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DNA- methylation changes in a human cell model of breast cancer progression

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Abstract

Epigenetic inactivation of genes by DNA hypermethylation plays an important role in carcinogenesis. An *in vitro* model of human breast epithelial cell transformation was used to study epigenetic changes induced by estradiol during the neoplastic process. Different stages of tumor initiation and progression are represented in this model being MCF-10F the normal stage; trMCF cells, the transformed stage; bsMCF cells, the invasive stage and, caMCF cells, the tumor stage. Global methylation studies by restriction landmark genomic scanning (RLGS) showed an increased DNA-methylation during the in the invasive and tumor stages. Expression studies showed that *NRG1* (neuregulin 1), *CSS3* (chondroitin sulfate synthase 3) and *SNIP* (SNAP-25-interacting protein) were downregulated in the invasive and tumor cells. The transformed cells showed low expression of *STXBP6* (amysin) compared to the parental cells MCF-10F. The treatment of these cells with the demethylating agent 5-aza-dC alone or in combination with the histone deacetylase inhibitor trichostatin increased the expression of *NRG1*, *STXBP6*, *CSS3* and *SNIP* confirming that DNA methylation plays an important role in the regulation of the expression of these genes. The *NRG1* exon 1 has a region located between −136 to +79 (considering +1, the translational initiation site) rich in CpG sites that was analyzed by methylation specific PCR (MSP). *NRG1* exon 1 showed progressive changes in the methylation pattern associated with the progression of the neoplastic process in this model; *NRG1* exon 1 was unmethylated in MCF-10F and trMCF cells, becoming hypermethylated in the invasive (bsMCF) and tumor (caMCF) stages. Studies of human breast tissue samples showed that *NRG1* exon 1 was partially methylated in 14 out of 17 (82.4%) invasive carcinomas although it was unmethylated in normal tissues (8 out of 10 normal breast tissue samples). Furthermore, *NRG1* exon 1 was partially methylated in 9 out of 14 (64.3%) morphologically normal tissue samples adjacent to invasive carcinomas.

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Keywords

Epigenetic changes; Breast cancer; DNA methylation; Neuregulin; Cell transformation

1. Introduction

Breast cancer (BC) is a hormone dependent malignancy whose incidence is steadily increasing in most western societies and industrialized countries. Each year 44,000 women die of breast cancer, making it the second leading cause of cancer deaths among American women, after lung cancer, and the leading cause of death among women aged 40 to 55 years. The elevated incidence of breast cancer has been associated with prolonged exposure to high levels of estrogen such as early onset of menstruation and late menopause [1,2]. The relationship between estrogen and breast cancer is further supported by epidemiological data that have demonstrated that women who receive estrogenic hormone replacement therapy (HRT) are more likely than never users to develop breast cancer [3–5].

We have developed an *in vitro* model of human breast epithelial cells transformation induced by estradiol (Fig. 1) [6]. In this model, the human breast epithelial cell line MCF-10F that is estrogen receptor α (ESRα) negative, was transformed by estradiol and different cell lines that represent different stages of breast cancer progression were isolated [6]. The MCF-10F progression model consists of four derived cell lines: a) the spontaneously immortalized cell line MCF-10F, which does not show any characteristic of invasiveness or tumor formation and therefore is considered to be a normal-like breast epithelial cell line; b) the transformed trMCF cells; c) the invasive bsMCF cell line and, d) the tumor cell lines, caMCFs, which shown all characteristics of a fully malignant breast cancer cell types [6] (Fig.1). The bsMCF cells induced tumors in SCID mice that were poorly differentiated adenocarcinomas that were ESRα, progesterone receptor (PR) and ERBB2 negatives. The highest number of deregulated genes was observed in caMCF, being slightly lower in bsMCF, and lowest in trMCF and this order was consistent with the extent of chromosome aberrations (caMCF > bsMCF ⋙ trMCF) [7].

Breast cancer is characterized by a variety of genetic lesions that include gene amplification and deletion, point mutations, loss of heterozygosity, chromosomal rearrangements, and overall aneuploidy [8]. Alternative mechanisms are represented by DNA methylation and covalent modifications of histone proteins, two epigenetic modifications important in transcriptional control [9]. DNA-methylation of cytosine residues at CpG dinucleotides that span the promoter and the first exon of some genes, occurs by the addition of a methyl group to the carbon-5 position of cytosine through the action of the *de novo* DNA methyltransferase (DNMT) enzymes [10]. Hypermethylation of CpG-rich sequences present in the promoters is associated with gene silencing. Histone modification such as acetylation, methylation, phosphorylation and ubiquitination is another epigenetic modification. Histone acetylation is controlled by histone acetyltransferases and histone deacetylases (HDACs) which control gene expression by remodeling the nucleosomes [11]. Highly acetylated histones are usually associated with transcriptional active sequences and hypoacetylated histones with silenced genes.

In contrast to mutations, epigenetic changes are reversible, raising the possibility of developing therapeutics based on restoring the normal epigenetic state to cancer-associated genes. The drugs 5-azacytidine (5-aza-C) and 5-aza-2'-deoxycytidine (5-aza-dC) are capable of reactivating gene expression. Subsequent to the incorporation into nucleic acid, they have the ability to directly target DNA methyltransferases (DNMTs) and in this way deplete cells from DNMT activities [12,13]. The covalent interaction between 5-aza-C and DNMT1 forces DNA

replication to proceed in the absence of DNA methylation, thereby causing genomic DNA hypomethylation, including the loss of methylation at the promoters of previously silenced genes [12]. Another chemical agent commonly used to modulate the expression of silenced genes in cancer cells is the histone deacetylase inhibitor trichostatin (TSA) [14,15].

We have demonstrated that treatment of the human breast epithelial cells MCF-10F with 17-β estradiol (E_2) induces the expression of phenotypic and genomic changes indicative of cell transformation [6]. In the present work, DNA methylation analysis of the different stages of the *in vitro* model revealed that epigenetic modifications of *NRG1, CSS3 and SNIP* were involved in the progression to the invasive and tumor stages. Results reported here are the first ones to demonstrate that *NRG1* exon 1 showed methylation changes during the progression of the neoplastic process. Studies of human breast tissue samples showed that *NRG1* exon 1 was partially methylated in invasive carcinomas although it was unmethylated in normal tissues. Furthermore, *NRG1* exon 1 was partially methylated in a high percentage of morphologically normal tissue samples adjacent to invasive carcinomas.

2. Materials and Methods

2.1. Cell lines and culture conditions

MCF-10F is a human breast epithelial cell line spontaneously immortalized estrogen receptor alpha (ERα)-negative and beta (ERβ)-positive, progesterone receptor (PR) and ERBB2 negatives. MCF-10F, trMCF, bsMCF and caMCF cells were cultured in Dulbecco's modified Eagle medium [DMEM/F-12, Gibco®; Formula 90–5212 EF: containing DMEM/F12 (1:1) with L-glutamine and phenol red, with 315 mg/L D-glucose, with 55 mg/L sodium pyruvate] with 5% horse serum, 2.43 g/l sodium bicarbonate, 20 μg/l epidermal growth factor (EGF), 100 μg/l Vibrio cholera toxin, 10 mg/l insulin, 0.5 mg/l hydrocortisone, 1.05 mM calcium, antibiotics and antimicotic (100 units/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin) [6]. The caMCF1, caMCF2, caMCF3 and caMCF4 cell lines were isolated from four single xenograft tumors grown in four animals; previous studies showed that these tumorigenic cells are very similar between them [6,7].

The human breast cancer cell lines T47D and MCF-7, both ERα positive and the ERα negative cell lines MDA-MB-231, MDA-MB-468, HS578T and BT549 were obtained from the Cell Culture Facility at Fox Chase Cancer Center and grown in RPMI 1640 media (Gibco-BRL, Gaithersburg, MD) with L-glutamine plus 10% fetal bovine serum (FBS) with antibiotics (100 units/ml penicillin, 100 μg/ml streptomycin).

2.2. Human Breast tissue samples

Breast tissue samples from 42 patients enrolled from Fox Chase Cancer Center was used in this study. The breast tissue samples included: 11 normal breast tissues (controls), 17 invasive carcinoma (IC) samples and, 14 morphologically normal tissue samples adjacent to IC. The study protocol was approved by the Institutional Review Board (IRB) and written informed consent was obtained from every participant. All patients were identified by coded numbers in place of names in the FCCC database; patient names and/or medical record numbers were known only by the treating physician and ancillary personnel. The samples from the control group were histological normal tissue adjacent to breast lesions with minimal pathological changes such as fibrosis, fibroadenomas, adenosis or papillomas. The histological normal breast tissue included in this study was taken from a distance of approximately 2 cm from the main gross palpable lesion and divided into two equal portions, one portion was fixed and embedded in paraffin for evaluation by a pathologist and a second half was frozen at −80°C. The samples were considered morphologically normal if they contained clusters of small ducts composed of a single myoepithelial basal layer and single cubical luminal layer with clear and

prominent lumen and, if the cells had uniform, small nuclei with diffusely distributed chromatin. The tumor samples (Group 2 in Table 3) and the morphologically normal tissues adjacent to IC (Group 3 in Table 3) were from different patients.

2.3. Real time RT-PCR

Total RNA was isolated from growing cells at 70%–80% confluence using Trizol (Invitrogen, Carlsbad, CA) according to manufacture's instructions. The RNAs were treated with DNase I (Invitrogen, Carlsbad, CA) and cleaned using RNeasy kit (Qiagen, Valencia, CA). The RNA concentration and quality were determinate using a spectrophotometer (NanoDrop Technologies, Wilmington, DE) and capillary gel electrophoresis (Agilent 2100 Bioanalyzer, Palo Alto, CA). The TaqMan One Step RT-PCR kit (Applied Biosystems, Foster City, CA) was used and the assays were run using Applied Biosystems 7900 HT instrument. The Ct (threshold cycle) was calculated using two different amounts of RNA for each sample (50 ng and 100 ng total RNA) to allow measurement of the PCR efficiency. The Taqman primers/probe sets used were Hs00247620_m1 for *NRG1*, Hs00365956_m1 for *HOXA9*, Hs00233470_m1 for *BMP6*, Hs00398096_m1 for *SPRY1*, Hs00194833_m1 for *TM4SF9*, Hs00986118_m1 for *SNIP*, Hs00274072_m1 for *STXBP6*, Hs00545664_m1 for *CSS3*, Hs00232429_m1 for *TBR1*, Hs00270117_s1 for *FOXD1* and Hs00427620_m1 for *TBP* (Applied Biosystems, Foster City, CA). 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 method calculates the relative gene expression using the following equation: Relative quantity= $2^{-\Delta\Delta Ct}$. For expression studies with 5-aza-dC and/or TSA, the treatments were performed in three independent experiments, RNA was isolated from the cells and the real time RT-PCR reactions were made in duplicate for each sample-primer/probe. *TBP* (TATA-box binding protein) was used as endogenous control and the expression levels of the different genes were referred to the expression in the parental cell line MCF-10F. The *NRG1* primer/probe detects mRNAs corresponding to HRGα, HRGβ1, HRGβ2, HRGβ3, HRGγ, GGF, GGF2 and NDF43, type I isoforms [16,17].

2.4. Treatments with 5-aza-dC and TSA

MCF-10F, trMCF, bsMCF and caMCF cells were treated with 2.5 μM 5-aza-2'-deoxycytidine (5-aza-dC) (Sigma Chemical Co, St. Louis, MO) for 96 hours or the histone deacetylase inhibitor trichostatin (TSA) at a concentration 100 ng/ml (Wako Pure Chemical Industries Ltd., Osaka, Japan) for 12 hours. For combination studies, 5-aza-dC was added to the culture medium for 96 hours and TSA was added for the last 12 hours. As the four caMCF cell lines showed similar characteristics [6,7,18], caMCF2 and caMCF4 were randomly chosen to be treated with 5-aza-dC and/or TSA. The treatments with 5-aza-dC and/or TSA were performed in three independent experiments and the RNAs were isolated from the cells for expression studies. For NRG1 protein studies, the cells were treated with 5-aza-dC in two independent experiments and protein extracts were prepared as it is indicated below.

2.5. Methylation specific PCR (MSP)

DNA was isolated from the cells and human breast tissue samples using DNeasy Blood and Tissue kit (Quiagen). For the sodium bisulfite modification, 1μg of DNA was denatured by adding 0.3M NaOH, incubated at 42°C for 30 minutes and, treated with 3.3M sodium bisulfite and 0.49 mM hydroquinone at 55°C for 16h, and purified using the Wizard DNA purification resin (Promega, Madison, WI) [19]. Approximately, 150 ng of sodium bisulfite-modified DNA was used as template in each PCR reaction. The PCR mixture contained 1x PCR buffer, 1.5 mM $MgCl_2$, 200 μM dNTPs, 0.2 μM each primers and 1 unit Platinum Taq Polymerase (Invitrogen). The PCR was performed as followed: an initial denaturalization step at 94°C during 5 min followed by 35 cycles at 94°C for 45 sec, annealing temperature for 45 sec and,

an extension step at 72°C for 45 sec; a final extension for 10 min at 72°C was included at the end. The primers were designed using Methyl Primer Express Software 1.0 from Applied Biosystems (Table 1). The DNA from MCF-10F cells treated with SssI methyltransferase (New England Biolabs Inc., Bervery, MA) was used as positive control for methylated alleles. PCR products were analyzed on 1.5% agarose gels and visualized under UV illumination. Three independent DNA extractions were done for each cell line and converted by bisulfite treatment and, the MSP reactions were performed at least two times for each DNA.

2.6. Western blot

Proteins were extracted from the cells using M_PER Mammalian Protein Extraction Reagent (Thermo Scientific) according to the manufacture's instructions and measured using Bradford assay. Approximately, 50 μg were loading in a 4–20% tris-glycine gel (Invitrogen). A polyclonal goat anti human neuregulin-1α (C19) (Santa Cruz, sc-1791) in a dilution 1:250 and a polyclonal goat anti human β-actin (Santa Cruz, sc-1616) at 1:500 dilution were used as primary antibodies. Donkey IgG anti goat with horse radish peroxidase (Santa Cruz, sc-2020) was used as secondary antibody (1:3,000 dilution). The ECL Western blotting detection Reagent kit (Amersham Biosciences) for a chemiluminescent detection was used to develop. Western blots were repeated at least 2 times for each protein extract. The bands from the films were scanned and their intensities were quantified using the gel image analysis software (Nonlinear Dynamics, Inc.). All bands of the NRG1 were normalized against the corresponding β-actin intensity and then compared to each other.

2.7. Statistical analysis

The mRNA expressions results are representative of three independent 5-aza-dC/TSA treatments. For NRG1 protein studies, two independent 5-aza-dC treatments were made for each cell line and at least two independent Western blots were made for each cell extract. The results are expressed as the means± standard errors. Statistical analysis of the data was performed using a Student's *t*-test. The statistically significant differences are indicated ($p \leq 0.05$).

3. Results

3.1. Epigenetic changes in a model of breast cancer progression

Global CpG island methylation was study at the different stages of the *in vitro* model of breast cancer progression by restriction landmark genomic scanning (RLGS) [20]. RLGS is based on the DNA digestion with the restriction enzyme NotI which is methylation-sensitive [21]. Thirty-eight genes were hypermethylated in the transformed (trMCF), invasive (bsMCF) and/or tumor (caMCF) stages compared to normal-like breast epithelial cells MCF-10F [20]. In order validate the RLGS results and identify genes that show progressive changes in their methylation pattern associated with the progression of the neoplastic process, expression studies were performed in these cells. As hypermethylation is associated with mRNA downregulation, the expression of 10 out of 38 genes was studied by real time RT-PCR. *FOXD1*, *TBR1*, *BMP6*, *HOXA9* and *TM4SF9* mRNA levels were similar at the different stages suggesting that methylation pattern of these genes was similar in the different cells of this model (data not shown). Although *NRG1*, *SPRY1*, *STXBP6*, *CSS3* and *SNIP* were downregulated at one or more stages compared to MCF-10F (Fig.2) suggesting that increased DNA-methylation of these genes could be involved in their downregulation. *NRG1* mRNA level was higher in MCF-10F compared to other stages and it was downregulated in trMCF (-1.4-fold change), bsMCF (-188-fold change) and absent in caMCF cells, although the downregulation in trMCF was not significant compared to the control MCF-10F (Fig.2). *SPRY1*, *CSS3* and *SNIP* showed downregulation in the invasive (bsMCF) and tumor (caMCF) cells and, *STXBP6* was downregulated only in the transformed trMCF cells (Fig. 2).

Furthermore, the transformed stage (trMCF) showed increased expressions of *SPRY1*, *CSS3* and *SNIP* and the tumor caMCF cells showed increased expression of *STXBP6* (Fig. 2).

3.2. Expression studies after treatment with the demethylating agent 5-aza-dC and the histone deacetylase inhibitor trichostatin (TSA)

The different cells of the model (MCF-10F, trMCF, bsMCF and caMCF2) were grown in regular media or media with 5-aza-dC and/or TSA and, the expression of *NRG1*, *STXBP6*, *CSS3*, *SNIP* and *SPRY1* were studied using real time RT-PCR (Table 2). The invasive bsMCF and tumor cells caMCF2 showed higher induction of *NRG1* expression than parental or transformed cells after treatment with 5-aza-dC. *NRG1* expression increased in the invasive (bsMCF) and tumor (caMCF2) cells at least 48-fold times after 5-aza-dC treatment indicating that this genes became hypermethylated in bsMCF and remain hypermethylated in caMCF2 (Table 2).

The expression of *STXBP6* increased in all the cells after treatment with 5-aza-dC in combination with TSA although there was a higher increase in trMCF. As it is showed in Table 2, the transformed cells (trMCF) showed a 27-fold times increase in *STXBP6* expression with 5-aza-dc, 24.8-fold increase expression with TSA and, 99.3-fold induction when TSA was used in combination with 5-aza-dC. Lower *STXBP6* increases were observed in bsMCF and caMCF2 after 5-aza-dC (7.6- and 3.9-fold time increases respectively), TSA (2.6- and 1.1- fold increases respectively) or combinational treatments (6.3- and 1.7-fold increases respectively). These results indicate that *STXBP6* downregulation in the transformed cells trMCF was probably due to increased methylation and the hypoacetylation of the histones bind to this region of the chromatin. *CSS3* showed higher expression with 5-aza-dC in all the cells from the model although, higher increases were observed in bsMCF and caMCF2 (at least 96.76-fold times induction) (Table 2). In the invasive bsMCF cells, *CSS3* expression showed higher increases after combinational treatment with 5-aza-dC and TSA although caMCF2 shown higher levels of induction with 5-aza-dC alone (Table 2).

For *SNIP*, 5-aza-dC alone or in combination with TSA also increased its expression in all the cells from the model although higher increases were observed in bsMCF and caMCF2 cells. *SNIP* expression showed higher increases when the cells were treated with TSA in combination with 5-aza-dC: 11.04-fold in MCF-10F, 8.4-fold in trMCF and, 66.5- and 18.4-fold increase in the invasive bsMCF and tumor cells caMCF2, respectively (Table 2). These results indicated that the low *SNIP* expression in the invasive and tumor cells was related probably to increased methylation of this gene and hypoacetylation of the histones that were binding to the chromatin in this region making *SNIP* less accessible for its transcription.

All the cells from the *in vitro* model showed low *SPRY1* increases after treatment with 5-aza-dC (2.32-fold in MCF-10F, 2.6- fold in trMCF, 4.9-fold in bsMCF and 1.5-fold in caMCF2) suggesting that methylation was not involved in the downregulation of this gene in bsMCF and caMCF cells (Table 2).

We observed that treatment with trichostatin (TSA) decreased the expression of some genes at certain stages but not in others (Table 2). The *SPRY1* mRNA level decreased in trMCF cells treated with TSA and the difference was significant compared to the expression in trMCF grown in regular media ($p \leq 0.05$). Also these cells (trMCF) showed a decreased *NRG1* expression after treatment with 5-aza-dC in combination with TSA but, the difference was not significant compared to the cells grown in regular media ($p=0.54$) (Table 2). A similar effect with TSA treatment was observed in bsMCF for *NRG1* and in caMCF2 for *CSS3* although the variations were not significant compared to their expressions in these cells grown in regular media ($p= 0.8$ and $p=0.07$, respectively) (Table 2).

3.3. Methylation studies by methylation specific PCR (MSP)

Methylation specific PCR (MSP) was performed to study the methylation status of *SPRY1*, *STXBP6*, *CSS3*, *SNIP* and *NRG1* at the different stages of the *in vitro* model. For these studies, genomic DNA was treated with bisulfite which converts unmethylated cytosine to uracil for generating thymidine during the subsequent PCR process [22]. For *SPRY1*, all the cell lines from the model showed a band only with the U-primers indicating that this gene was unmethylated (Fig. 3). For *STXBP6 and SNIP*, all the cell lines showed a band with the M-primers indicating that these genes were methylated in all the stages of the *in vitro* model (Fig. 3). Although *STXBP6* and *SNIP* showed significant differences in their expression in the different cells, these genes did not show to be unmethylated in some cells becoming methylated in others. A progressive methylation change was not observed for *STXBP6* and *SNIP* in this model of breast cancer progression in the regions that were studied by MSP (Fig. 3A). *CSS3* showed a band with both, the U- and M-primers in all the cells although even using different DNA extractions, MCF-10F and trMCF cells showed a stronger band with the U-primers and, bsMCF and caMCF cells with the M-primers (Fig. 3).

When *NRG1* exon 1 was studied by MSP, MCF-10F and trMCF cells showed a band only with the U-primers that recognize the unmethylated sequence and no bands were obtained with the M-primers (Fig. 4A). Conversely, PCR bands were obtained in bsMCF, caMCF1, caMCF2, caMCF3 and caMCF4 with the M-primers, but no band was obtained with the U-primers (Fig. 4A). These data indicated that *NRG1* exon 1 is unmethylated in MCF-10F and trMCF cells, becoming methylated in the invasive (bsMCF) and tumor (caMCF) stages. In this model of breast cancer progression, *NRG1* showed a progressive methylation change. Furthermore, *NRG1* was study by methylation specific PCR (MSP) in different breast cancer cell lines (Fig. 4B). T47D and MCF-7, both ERα positive and, the ERα negative cell lines MDA-MB-231, HS578T and BT549 showed a band with the U-primers indicating that this gene was unmethylated in these cells. The cell line MDA-MB-468 (ERα negative) showed a band with the M-primers and no band was obtained with the U-primers indicating that *NRG1* exon 1 was hypermethylated in MDA-MB-468 (Fig. 4B).

3.4. NRG1 in the model of breast cancer progression

The levels of the NRG1 protein was studied by Western blot (Fig. 5A). NRG-1α protein level was lower in the transformed (trMCF), invasive (bsMCF) and tumorigenic (caMCF4) stages and the differences were significant compared to the control MCF-10F ($p<0.05$) for all of them, including trMCF cells ($p=0.003$). The invasive bsMCF and tumor caMCF4 cells showed significant increases of NRG1 after treatment with 5-aza-dC at both mRNA and protein levels (Fig. 5). Although NRG1 mRNA expression was very low in bsMCF and caMCF cells, the NRG-1α protein was present in these cells although at lower levels compared to the parental MCF-10F cells.

3.5. NRG1 methylation in human breast invasive carcinomas

NRG1 exon 1 methylation was study in invasive carcinomas (IC) and morphologically normal tissue adjacent to invasive carcinomas by methylation specific PCR (MSP) (Table 3). As it is indicated, it was not possible to include tumor and adjacent tissue samples from the same patients. As control, breast tissue samples adjacent to benign lesions were used as it was described (Table 3). We found that *NRG1* exon 1 was partially methylated in 14 out of 17 (82.4%) invasive carcinomas although it was unmethylated in normal breast tissue (8 out of 10 normal breast samples). Furthermore, *NRG1* exon 1 was partially methylated in 9 out of 14 (64.3%) morphologically normal tissue samples adjacent to IC (Table 3).

4. Discussion

Cancer progression models have become an invaluable tool for the study of genetic aberrations that correlate with a shift from a normal to a disease phenotype. In the present work, we used a human breast cancer progression model to study DNA methylation changes that occur during the transformation of breast epithelial cells with the idea to find biomarkers for an early detection of breast cancer. Using this model, we found that NRG1 was expressed by the normal human breast epithelial cells MCF-10F and transformed cells (trMCF) although it was downregulated at the invasive and tumorigenic stages (bsMCF and caMCF, respectively). DNA methylation studies showed that *NRG1* was unmethylated in the normal and transformed cells becoming hypermethylated in the invasive and tumorigenic cells. Results presented here showed progressive methylation changes in *NRG1* exon 1 in this breast cancer progression model. Furthermore, *NRG1* exon 1 methylation was studied in normal human breast tissues and invasive breast carcinomas. We found that *NRG1* exon 1 was partially methylated in a high percentage of human invasive carcinomas (IC) and morphologically normal tissue adjacent to IC although it was unmethylated in normal breast tissues.

Neuregulins are ligands for the ERBB human epidermal growth factor receptor (HER) family and they binds to ERBB-3 and/or ERBB-4 [23–25]. Loss of chromosome 8p, where *NRG1* is located, is one of the most frequent genomic events reported in epithelial cancers including breast, colon, bladder and prostate. *NRG1* has been proposed as the principal candidate tumor suppressor gene in this region [26–28]. The *NRG1* gene is approximately 1.4 megabases long and as a consequence of alternative splicing and multiple promoters (possible nine) with the use of at least six transcription initiation sites [17,29] gives rise to the production of more than 15 protein variants [16,17,30]. The extraordinary diversity of the neuregulin signaling pathway is achieved by alternative splicing of this gene. *NRG1* has been proposed both as candidate oncogene and as a tumor suppressor gene [31] and although they can be mitogens, they can also be pro-apoptotic proteins [32]. Also, neuregulins stimulate mammary epithelial growth and differentiation and, branching morphogenesis [33].

In the *in vitro* model of breast cancer progression, *SNIP* (SNAP-25- interacting protein) and *CSS3* (chondroitin sulfate synthase 3 or CHSY2) expressions were lower at the invasive and tumor stages but progressive changes in the methylation status of their promoters were not detected by methylation PCR (MSP). Studies with the demethylated agent 5-aza-dC and the histone deacetylase inhibitor trichostatin, indicated that the lower *SNIP* expression in bsMCF and caMCF cells could be related to increase *SNIP* methylation and hypoacetylation of the bounded histones, making *SNIP* less accessible for its transcription. It has been showed that *SNIP* silencing increases cell spreading and migration in the normal breast MCF-10 cells (Di Stefano and Difilippi, unpublished data) [34]. *CSS3* was partially methylated in all the cells although *CSS3* promoter methylation seemed to increase in the invasive and tumorigenic stages. *CSS3* is a glycosyltransferase involve in the biosynthesis of chondroitin sulfates (CS)-proteoglycans, molecules distributed on cell surfaces and in the extracellular matrix playing crucial roles in cell adhesion, morphogenesis and cell division [35]. The decreased SNIP and CSS3 levels could be related to the fibroblastic-like shape, decreased adhesion and increased motility and invasion capacity of the bsMCF and caMCF cells. In the *in vitro* model of breast cancer progression, *STXBP6* (amysin) was downregulated in the transformed cells and increased its expression at the invasive and tumorigenic stages. Methylation studies showed that *STXBP6* promoter was hypermethylated in all the cells of the model. STXBP6 binds components of the SNARE complex that has a central role in vesicle targeting and fusion in eukaryotic cells; it has been shown that epimorphin, one of the SNARE proteins, directs epithelial morphogenesis [36].

NRG1 exon 1 has a region located between −136 to +79 (considering +1, the translational initiation site) rich in CpG sites that was analyzed by methylation specific PCR (MSP). This region showed to be unmethylated in the immortal human breast MCF-10F and transformed cells (trMCF) but, hypermethylated in the invasive and tumorigenic cells (bsMCF and caMCF). These data indicated that *NRG1* exon 1 is unmethylated in the parental MCF-10F and transformed trMCF cells, becoming hypermethylated in the invasive (bsMCF) and tumor (caMCF) stages. Furthermore, treatment with 5-aza-dC increased *NRG1* expression in the invasive and tumorigenic stages supporting that this gene was hypermethylated at these stages. Interesting, although *NRG1* was partially methylated and downregulated at the mRNA level, the protein was still present in bsMCF and caMCF cells although at lower level compared to MCF-10F.

The methylation status of *NRG1* was also study in different breast cancer cells lines such as MCF-7, T47D, MDA-MB-231, MDA-MB-468, HS578T and BT-549. *NRG1* was unmethylated in all the cells except in breast cancer cell line MDA-MB-468. The MDA-MB-468 cell line was isolated from a pleural effusion of a 51-year-old African American female patient with metastatic adenocarcinoma of the breast and this cell line produced tumors when it was inoculated in nude mice. Based on global gene expression analyses, breast cancer cell lines were classified into two major branches, luminal (MCF-7 and T47D) and basal [37,38]. Luminal cell lines display moderate to high expression of ERα and luminal cytokeratins [39]. The basal cell lines included the normal breast cell lines MCF-10A/F and the tumor cell lines MDA-MB-468, MDA-MB-231, HS578T and BT-549 [37]. The basal like carcinomas have a more aggressive clinical behavior [39].

NRG1 methylation was study in invasive carcinomas (IC) of the breast and morphologically normal tissue adjacent to IC. As control, breast tissue samples adjacent to benign breast lesions were used. We found that *NRG1* exon 1 was partially methylated in 14 out of 17 (82.4%) IC and it was unmethylated in normal breast tissues (80% normal breast samples). Recently, it was shown that *NRG1* was unmethylated in normal breast tissues and methylated in breast tumor samples [31] [40]. Although *NRG1* was hypermethylated in the invasive bsMCF and tumorigenic caMCF cells, NRG1 protein was detected in these cells although at lower levels compared to the normal cell line MCF-10F. Raj et al (2000) have showed using immunohistochemistry, that advanced breast adenocarcinomas with absent or low levels of NRG-1α were associated with poorer prognosis [41].

In women with breast cancer, DNA methylation of the normal tissue adjacent to the tumor may be a marker for an increased risk of local recurrence. Since the density of DNA methylation has been shown to increase progressively during neoplastic transformation from premalignant lesions to invasive cancers, frequent promoter hypermethylation of tumor suppressor loci in normal breast tissue is a harbinger for local recurrence. In order to determine if *NRG1* methylation was an early event manifested even before the morphological changes in the ductal cells occur, we have analyzed the normal breast tissue adjacent to invasive carcinomas. We found that *NRG1* exon 1 was partially methylated in a high percentage of morphologically normal breast tissue adjacent to invasive carcinomas (9 out of 14 samples; 64.3%).

Altogether our data indicate that *NRG1* exon 1 is methylated during the progressive stages of transformation in human breast epithelial cells. Importantly, the finding that the normal structures of the breast have *NRG1* exon 1 partially methylated although the absence of histological or cytological abnormality, points to the use of this gene as a marker of neoplasia.

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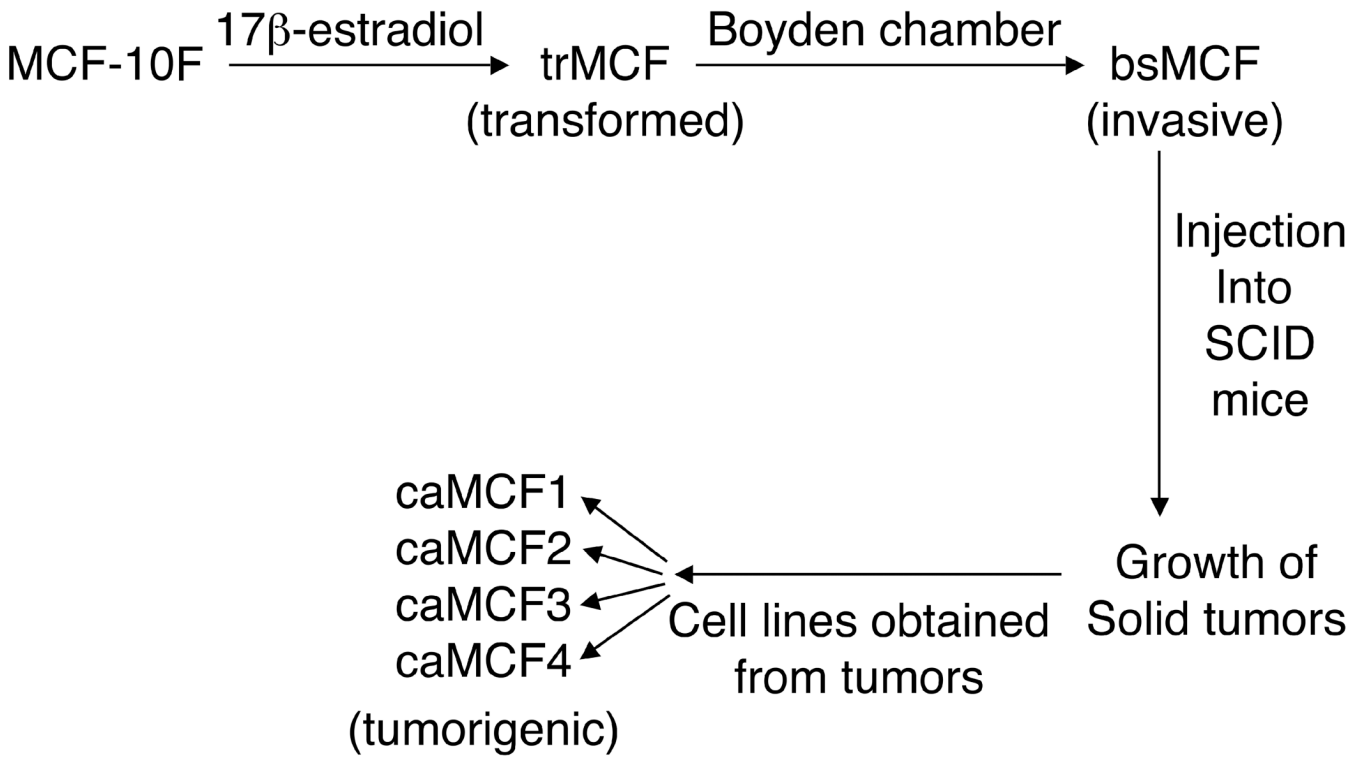


Figure 1. Different stages in *the in vitro* model of breast cancer progression

MCF-10F cells were treated with 70nM 17 β-estradiol for 24 hours, twice a week for two weeks. After the treatment, the trMCF cells (transformed stage) were maintained in culture and after several passages they were placed on matrigel Boyden chambers. The cells that passed through the membrane, named bsMCF cells (invasive cells) were collected, expanded and injected into the mammary fat pad of SCID mice. These invasive bsMCF cells formed tumors that were dissected originating the caMCF cell lines. The caMCF1, caMCF2, caMCF3 and caMCF4 cell lines were isolated from four single xenograft tumors and these cells showed similar characteristics according to previous studies [6,7,18].

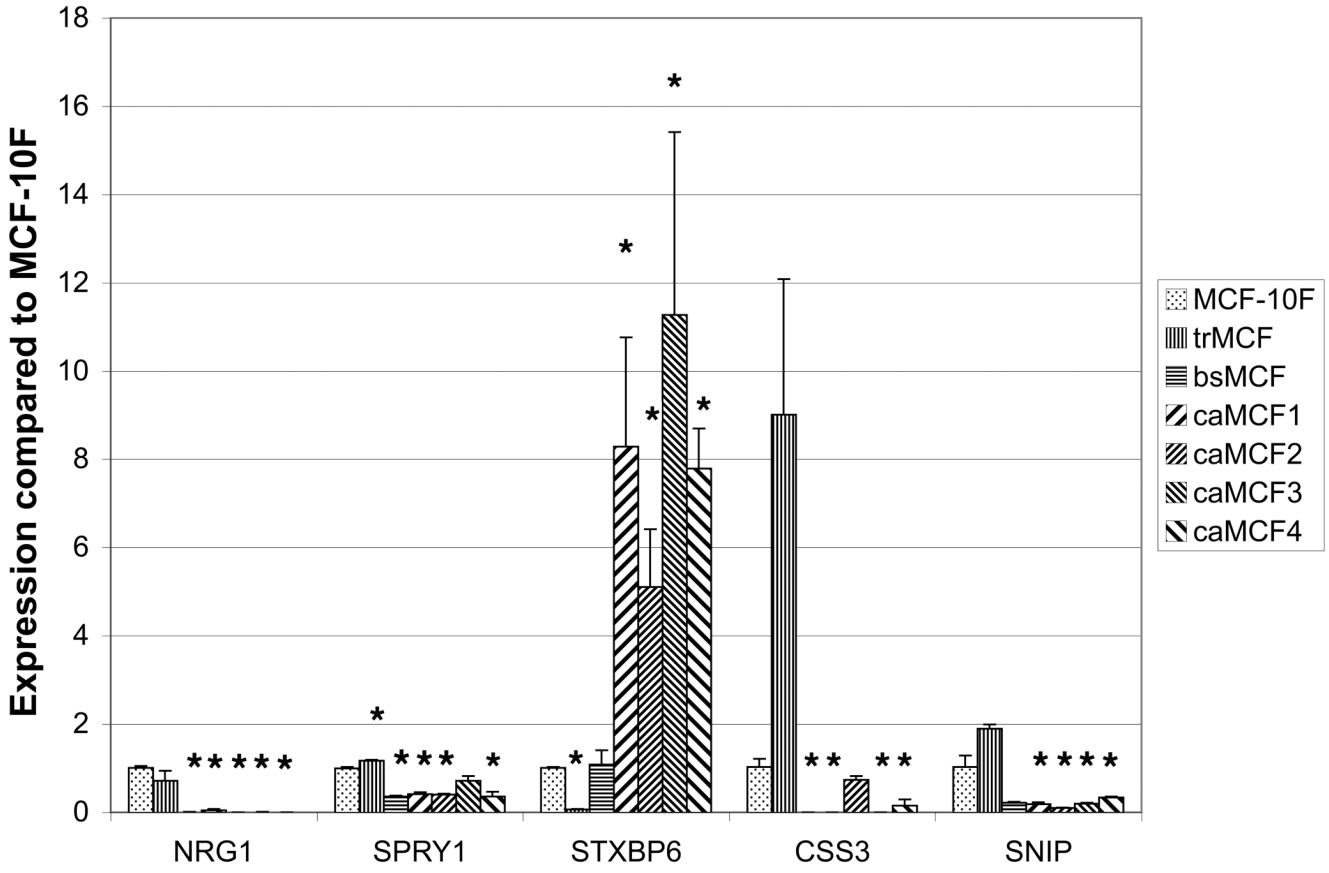


Figure 2. ***NRG1, SPRY1, STXBP6, CSS3*** **and** ***SNIP*** **were downregulated at different stages in the** ***in vitro*** **model of breast cancer progression**

RNA was isolated from: MCF-10F (normallike stage), trMCF (transformed stage), bsMCF (invasive stage) and the tumor caMCFs cells (caMCF1, caMCF2, caMCF3 and caMCF4). RNAs were isolated from each cell line in three independent experiments and the quantitative real time PCR was made in duplicated for each sample-primer-probe The results represent the means ± standard errors. The statistically significant variations compared with MCF-10F are indicated (*) ($p \leq 0.05$).

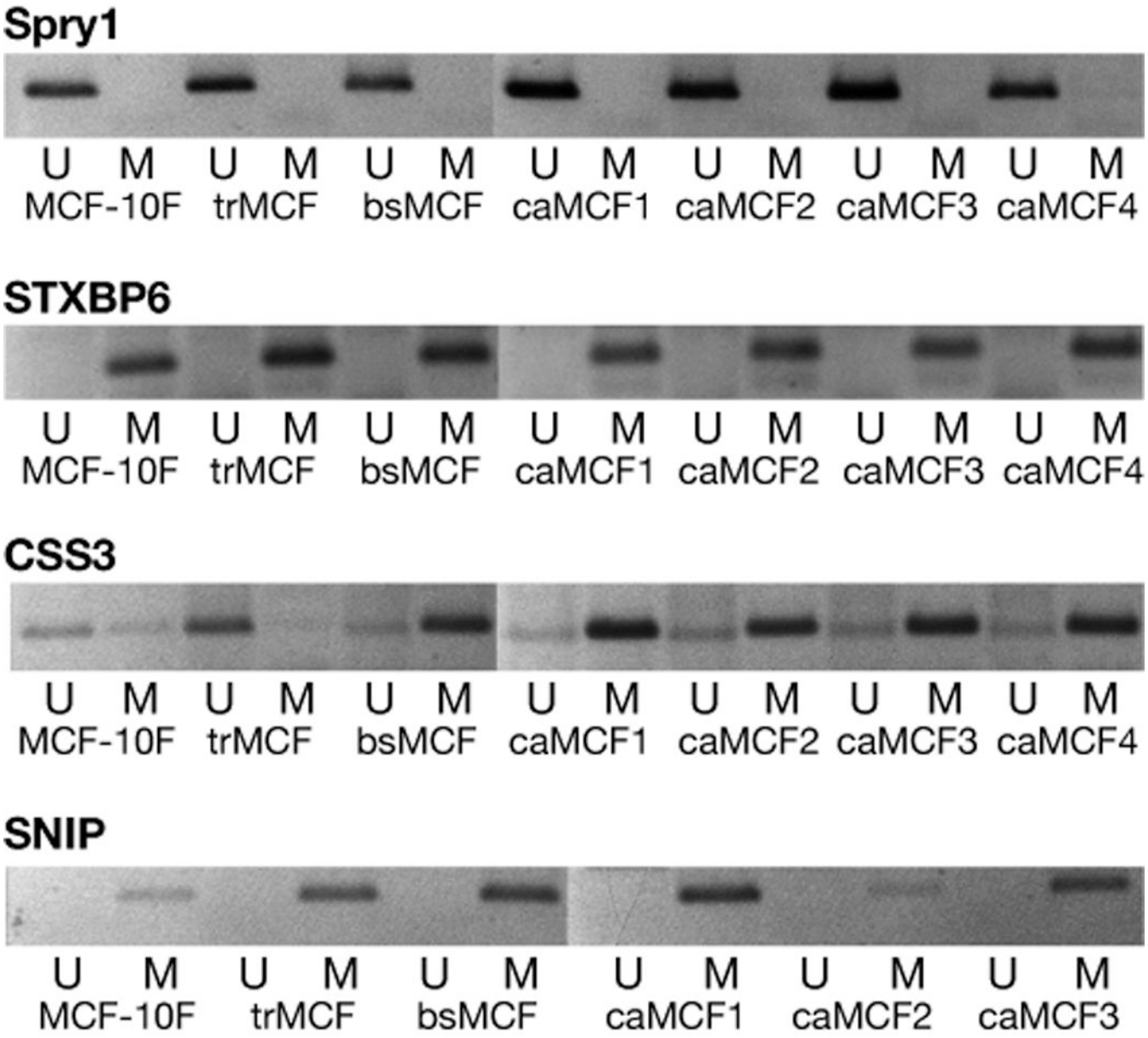


Figure 3. *STXBP6, SNIP* and *CSS3* methylation in the *in vitro* model

The methylation status of the promoter/exon 1 of *STXBP6, SNIP* and *CSS3* was studied in MCF-10F, trMCF, bsMCF and caMCF cells. A representative experiment from three independent ones is showed.

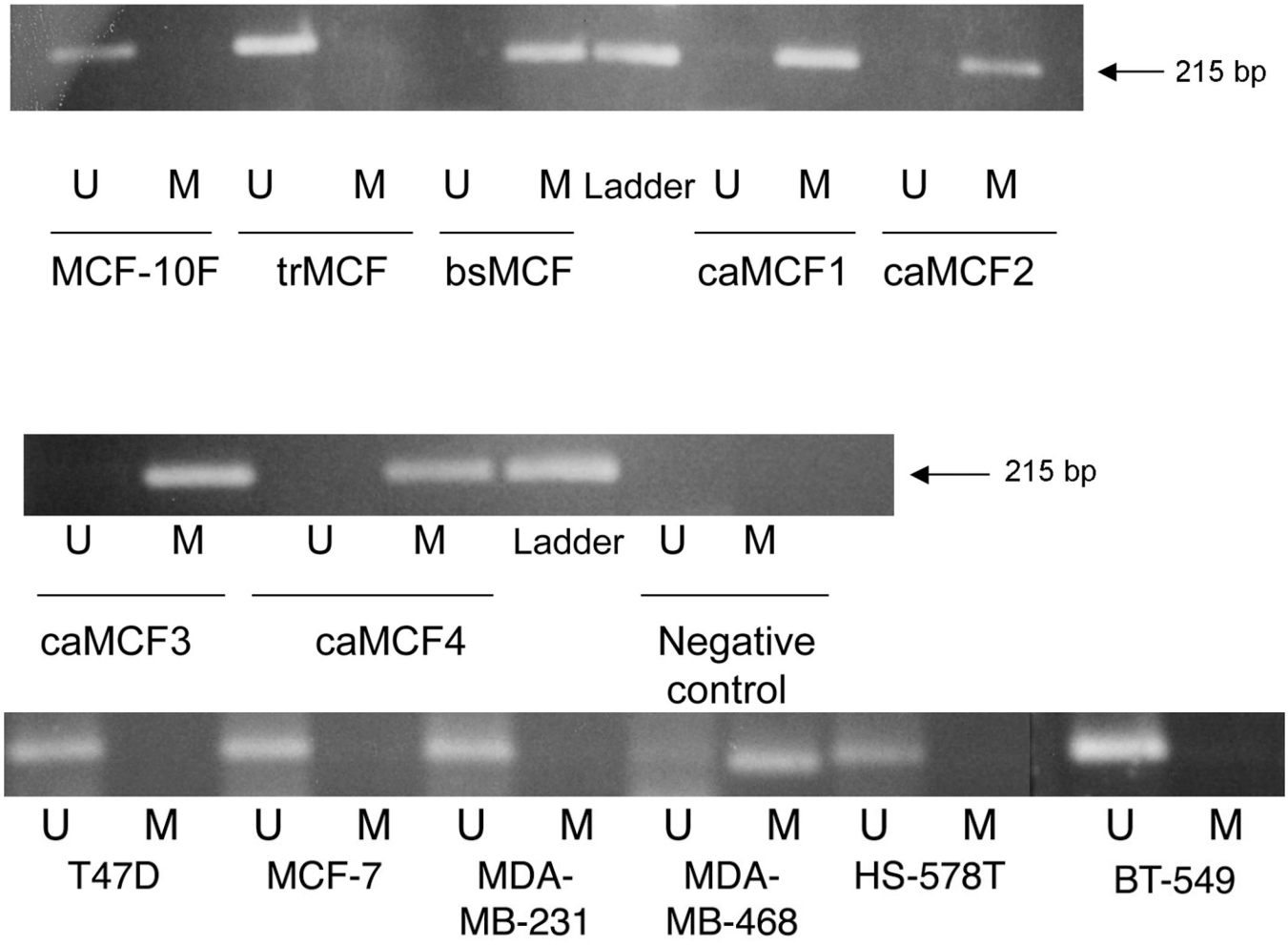
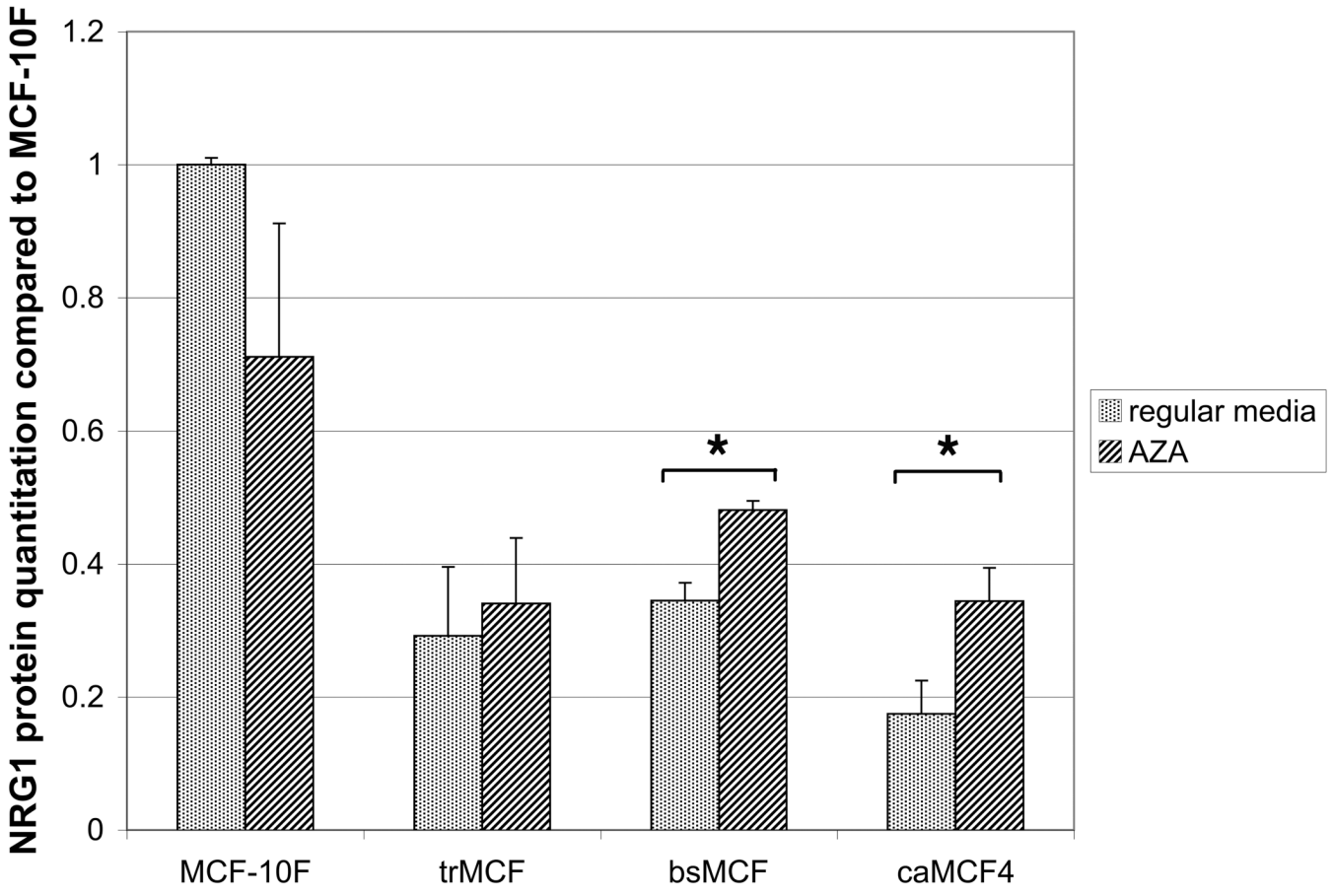


Figure 4. *NRG1* hypermethylation in the invasive and tumorigenic stages of the *in vitro* model and the estrogen receptor negative cell line MDA-MB-468

NRG1 methylation was studied by methylation specific PCR (MSP) in: A) the *in vitro* model of breast cancer progression and, B) in the human breast cancer cell lines T47D and MCF-7, both ERα positive and, the ERα negative cells MDA-MB-231, MDA-MB-468, HS578T and BT549. A band corresponding to *NRG1* exon 1 was identified with two sets of primers, one pair recognized a sequence in which CpG sites present in the primer region were unmethylated (bisulfite modified to UpG), and the other recognized a sequence in which CpG sites were methylated (unmodified by bisulfite treatment). A representative experiment from three independent ones is showed.



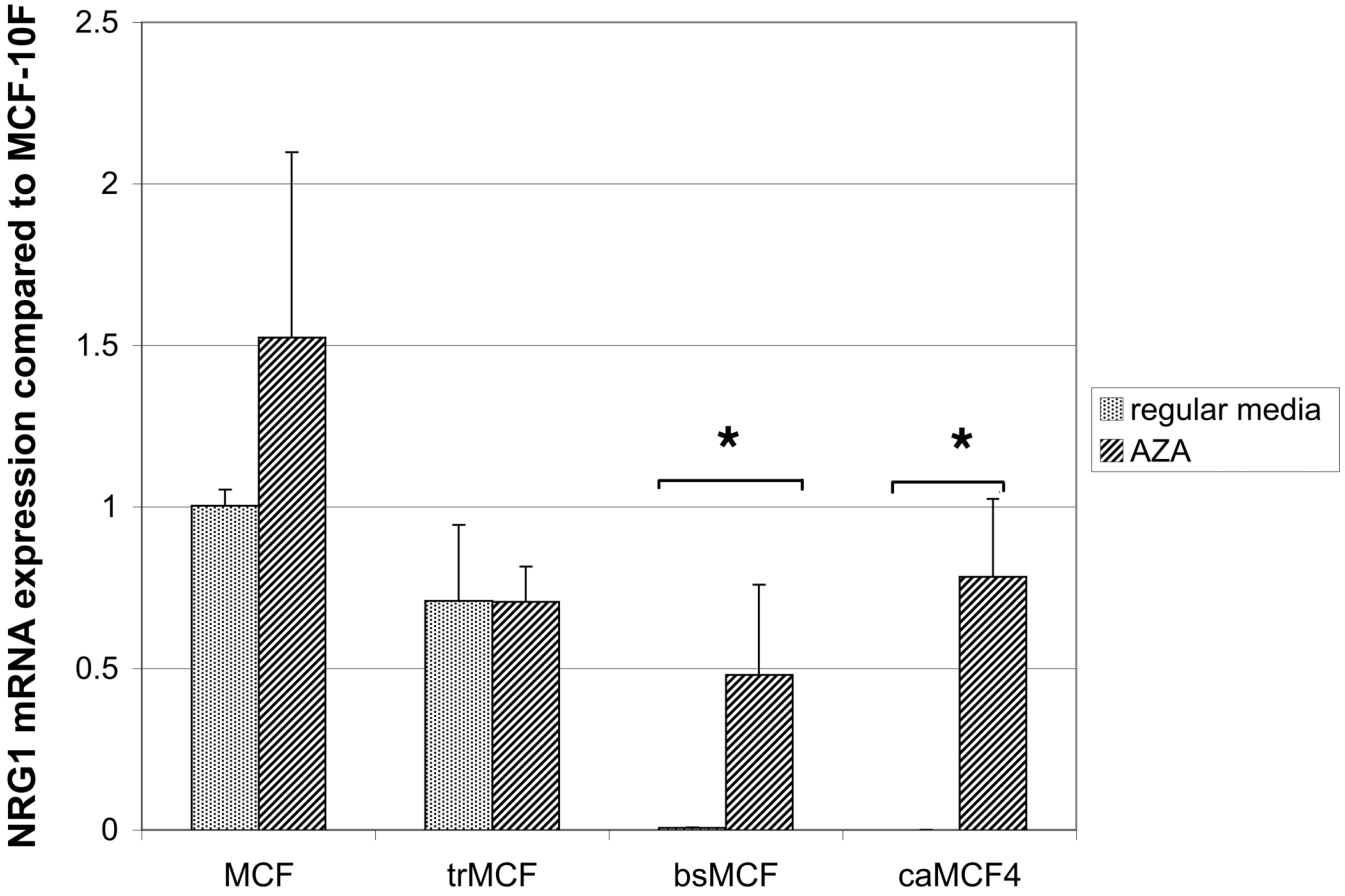


Figure 5. NRG1 expression *in vitro* model of breast cancer progression

The different cells of the model were treated with 2.5μM 5-aza-dC during 96h or grown in regular media. A) NRG1 protein (44-kDa) was present in all cell lines although it was downregulated in trMCF, bsMCF and caMCF4 cells compared to the expression in the parental cell line MCF-10F. B) *NRG1* mRNA expression: RNA was extracted from the cells and real time RT-PCR was performed and compared to the expression in MCF-10F. The averages ± standard errors are shown and the significant differences between cells grown without (regular media) or with 5-aza-dC (AZA) are indicated (*).

Table 1
Primers used for methylation specific PCR (MSP)

The U-primers and M- primers recognize the unmethylated and methylated sequences, respectively. The annealing temperatures and the size of the PCR product are indicated in each case.

Gene	Primers 5'→3'	Annealing	PCR (bp)
NRG1	Forw (U): GTAGTGTGAGTGTTTTAGTGTGGTTG Rev (U): CAAACTCCAACTTCTTACCA	56°C	215
	Forw (M): GTTTTAGCGCGGTCGTTC Rev (M): CGAACTCCGACTTCTTACCG	58°C	
SPRY1	Forw (U): GGTATATATAAATGAAAGAGGAAAT Rev (U): AACACACAATAATTTACAAAACA	52°C	175
	Forw (M): GGTATATAAACGAAAGAGGAAAC Rev (M): CACGCAATAATTTACAAAACG	52°C	
STXBP6	Forw (U): TGAGTATGTTTAGAGGTGGTT Rev (U): AACTTAACCAACCCAAATAC	52°C	209
	Forw (M): GAGTATGTTTAGAGGCGGTC Rev(M): ACTTAACCGACCCGAATAC	55°C	
CSS3	Forw (U): TTAGAGTTGAGTGGGAGTTT Rev (U): CAAACA CTCACTAAACTATCA	52°C	193
	Forw (M): TTAGAGTTGAGCGGGAGTTC Rev(M): CGAACGCTCGCTAAACTATCG	58°C	
SNIP	Forw (U): TTATTTTTTGTAGATTTGGAGT Rev (U): AAAAACATCACACTTACAATCAA	54°C	237
	Forw (M): TTTTTCGTAGATTCGGAGC Rev(M): AAAAACATCACGCTTACGAT	52°C	

Table 2

Expression studies after treatment with 5-aza-dC and/or the inhibitor of the histone deacetylase TSA (trichostatin)

MCF, trMCF, bsMCF and caMCF2 were treated with 2.5 μM 5-aza-dC during 96h or 100ng/ml TSA during 12 h. For combinational studies, 5-aza-dC was added for 96h, and TSA was added for the last 12 h. After treatments, the RNAs were isolated and real time RT-PCR assays were performed and the values compared to MCF-10F. The expression values are relative to the expression of each gene in MCF-10F grown in regular media. The cells were treated in three independent experiments and the real time RT-PCR assays were performed in duplicated for each sample/primer-probe. The means ± standard errors are shown.

	NRG1	STXBP6	CSS3	SNIP	SPRY1
MCF-10F	1±0.05	1±0.03	1.03±0.19	1.03±0.26	1±0.03
MCF-10F AZA	1.52±0.57	2.32±0.77	26.65±1.01	1.93±0.19	2.32±0.08
MCF-10F TSA	0.94±0.05	4.19±0.32	15.5±1.02	14.27±0.25	0.87±0.19
MCF-10F AZA+TSA	1.04±0.08	7.15±2.15	160.17±11.1	11.04±0.08	1.03±0.12
trMCF	0.71±0.24	0.06±0.01	9.01±3.07	1.89±0.098	1.17±0.02
trMCF AZA	0.71±0.11	1.62±0.07	93.18±15.85	2.81±0.34	3.07±0.08
trMCF TSA	0.55±0.06	1.49±0.07	44.89±13.87	8.76±0.33	0.41±0.12
trMCF AZA+TSA	0.498±0.12	5.96±0.42	164.20±55.9	15.68±1.95	0.93±0.04
bsMCF	0.01±0.001	1.08±0.32	0±0	0.22±0.02	0.35±0.04
bsMCF AZA	0.48±0.28	8.17±2.37	1.29±0.9	1.48±0.11	1.72±0.64
bsMCF TSA	0.005±0.003	2.84±0.47	0.07±0.07	3.79±0.63	0.49±0.07
bsMCF AZA+TSA	0.22±0.13	6.78±2.03	2.18±1.3	14.63±0.4	1.01±0.19
caMCF2	0±0	5.1±1.32	0.73±0.09	0.1±0.004	0.35±0.01
caMCF2 AZA	1.02±0.08	19.78±1.78	70.64±9.25	1.57±0.08	0.51±0.04
caMCF2 TSA	0±0	5.41±0.169	0.13±0.13	0.88±0.14	0.72±0.05
caMCF2 AZA+TSA	0.01±0.002	8.61±0.3	0±0	1.84±0.15	1.74±0.3

Table 3

Methylation of NRG1 exon 1 in invasive carcinomas (IC), morphologically normal tissue adjacent to IC and normal human breast tissues

As control, normal tissue adjacent to benign lesions of the breast was used (control group). The methylation status of *NRG1* is indicated as: U (unmethylated) or P (partially methylated: PCR products were obtained with both set of primers). M: methylated. ND: no determinate. The age of the patients at the moment that the sample was taken and type of lesion are indicated. IDC: invasive ductal carcinoma. ILC: invasive lobular carcinoma. DCIS: ductal carcinoma in situ. p

Patient number	Age	Diagnostic	NRG1
Group 1: Control Group (normal tissue adjacent to benign lesions)			
148115	60	Bening breast disease	**U**
135447 S8	77	Apocrine metaplasia	**U**
49101	56	Fibroadenoma	**ND**
136620	67	Fibrocystic changes	**P**
131665	64	Fibrocystic changes	**U**
85453	59	Epithelial hyperplasia	**U**
137340 S15	63	Epithelial hyperplasia	**P**
135990 S9	64	Adenosis	**U**
141007 S21	77	Adenosis	**U**
141300 S24	78	Adenosis	**U**
S14 136880	59	Papilloma	**U**
			P=2/10=20%
Group 2: Breast tumors			
1004172 (287767)	50	IDC, DCIS (GRADE III)	**P**
1003361 (285220)	68	IDC	**P**
1004363 (276283)	58	IDC (HIGH GRADE)	**P**
1004783 (214003)	73	IDC, DCIS(GRADE III)	**P**
1003356 (261845)	57	IDC (GRADE II)	**U**
1000851 (265718)	59	IDC, DCIS	**P**
1004041 (286233)	37	IDC (GRADE III)	**P**
1003341 (279135)	44	IDC, DCIS (HIGH GRADE)	**P**
1004582 (296190)	52	IDC (GRADE III)	**P**
1004092 (284610)	78	IDC (GRADE III)	**U**
1000826 (279038)	73	IDC (GRADE III)	**P**
1002833 (268295)	64	IDC (GRADE III)	**P**
1001581 (230160)	64	IDC (GRADE III)	**P**
1001380 (243278)	59	IDC (GRADE II)	**U**
1001168 (254401)	51	IDC (GRADE II)	**P**
1001484 (239086)	76	IDC (GRADE I)	**P**
1003731 (241220)	51	IDC (GRADE III)	**P**
			P= 14/17= 82.4%

Patient number	Age	Diagnostic	NRG1
Group 3: Morphologically normal tissue adjacent to IC			
140569 C26	67	IDC	**P**
145565	62	IDC, DCIS	**U**
145564	74	IDC	**P**
133360	57		**U**
141008 S22	55	IDC, DCIS	**U**
145813	53	IDC	**P**
147043	73	IDC, DCIS	**M**
145563	65	IDC	**P**
139128 S19	84	IDC	**P**
141299 S23	75	IDC, DCIS	**P**
154855	59	IDC	**U**
143061	73	IDC and ILC	**P**
157678	92	ILC	**P**
156105	73	ILC	**U**
			P or M=9/14 = 64.3%