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Entropy of Feulgen-stained 17-ß-estradiol-transformed human breast epithelial cells as assessed by restriction enzymes and image analysis

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Abstract. MCF-10F human breast epithelial cells when transformed with 17-ß-estradiol (E2) give rise to highly invasive C5 cells that generate adenocarcinomas in SCID mice. From these tumors, cell lines such as C5-A6-T6 and C5-A8-T8 have been derived. Variable patterns of chromatin supraorganization have been demonstrated for these cells during the transformation/tumorigenesis progress, when assessing chromatin entropy by image analysis in Feulgen-stained preparations. Since epigenetic dysregulation might contribute to the chromatin textural repatterning in transformed MCF-10F cells, the association of the variable chromatin packing states with global DNA methylation was investigated in these cells after their treatment with restriction enzymes followed by Feulgen staining and chromatin entropy evaluation by image analysis. The results indicate that although -C^{m}CGG- sequences may affect chromatin supraorganization in some of the analyzed cell types (perhaps due to localized hypermethylation), not all the chromatin condensation patterns in these cells with transformation and/or tumorigenesis are associated with DNA methylation (e.g. E2 cells). Chromatin supraorganization remodeling in C5-A6-T6 and C5-A8-T8 cells may be attained by different mechanisms, with C5-A6-T6 chromatin packing states perhaps being associated with local DNA hypermethylation or other epigenetic factors, and C5-A8-T8 likely being associated with global DNA hypomethylation, as reported in the literature for other cell types. Thus, we assume that a variable epigenetic modulation affecting the higher-order packing states of chromatin in the estrogen-transformed MCF-10F cell model could be evident with the chromatin entropy study by image analysis.

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Introduction

Treatment of MCF-10F immortalized human breast epithelial cells with 17-ß-estradiol (E-2) has been found to induce transformation and tumorigenesis (1). These phenomena are characterized by several phenotypic changes including those involving DNA amounts, nuclear sizes and chromatin supraorganization, as assessed by image analysis (2). The neoplastic transformation induced by E-2 in MCF-10F cells is independent of the presence of α-receptors for estrogen since the above-mentioned changes are not abrogated under concomitant treatment with E-2 and the estrogen antagonist, ICI182,780 (2,3).

E-2-transformed MCF-10F cells (E2) selected in Matrigel chambers give rise to highly invasive C5 cells, capable of generating adenocarcinomas, when injected in SCID mice (4). Cell lines derived from these tumors, among which C5-A6-T6 and C5-A8-T8 have been subsequently obtained (4).

Among the various image analysis textural parameters estimated in Feulgen-stained cells, inclusive in transformed MCF-10F cells, entropy has been shown to be relevant for discrimination of changes in chromatin supraorganization (2,5-7). Nuclear entropy as studied by video image analysis has been defined as the number of bits necessary to store the densitometric values per nucleus image or the amount of gray value variability contained in a nucleus (5,7,9). This parameter measures the complexity of an image (10), such that a high entropy value for Feulgen-stained cells is found when there is a high contrast between condensed and non-condensed chromatin, whereas a low entropy value represents a homogeneously packed chromatin state (fairly constant gray levels) (7,8) (Fig. 1). The entropy studied in Feulgen-stained preparations of the transformed MCF-10F cells has revealed a variable heterogeneity in chromatin packing states, attaining its highest and lowest expression in E2 and C5-A8-T8 cells, respectively (7) (Table I). Expressive chromatin remodeling with advancing cell transformation and tumorigenesis has thus been suggested for this cell system (7).

Since it has been considered that higher order chromatin packing states and nuclear organization in animal cells be affected by epigenetic mechanisms (7,11), epigenetic dysregulation might contribute to the chromatin textural

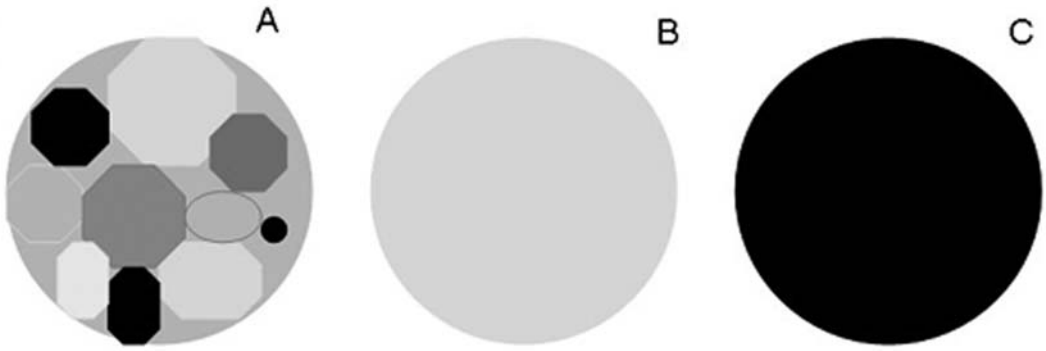

Figure 1. Images containing highly contrasted absorbing compartments (high entropy values) (A), and one homogeneously distributed compartment exhibiting low (B) or high (C) absorbing components (low entropy values).

Table I. Entropy of Feulgen-stained 17-ß-estradiol-transformed MCF-10F cells (7).

	Entropy	
Cells	X	S
MCF-10F (control)	4.99a	0.35
E2	5.50b	0.35
C5	4.96a	0.38
C5-A6-T6	4.94a	0.36
C5-A8-T8	4.82c	0.34

n, 200; E2, 17-ß-estradiol-treated MCF-10F cells; C5, cells expanded from E2; C5-A6-T6 and C5-A8-T8, tumor cells; S, standard deviation; X, arithmetic mean. Different letters in the X column denote differences significant at P=0.01 (ANOVA).

repatterning in transformed MCF-10F cells. Indeed, global genome hypomethylation and regional site hypermethylation have been reported in human breast cancer cells (Fernandez *et al*, Proc AACR Annual Meeting 48: abs. 2850, 2007; 12,13), with a prominent loss of DNA methylation accompanied by other epigenetic alterations being associated with the increased malignant properties of these cells (12). In the human breast cancer cell lines studied by Shann and co-workers (13), extensively hypomethylated genes are all silenced, as shown by microarray analysis. On the other hand, in E-2-transformed MCF-10F cells, expression silence specifically associated with DNA hypermethylation has been pointed out for a few genes (SPRY1 and NRG1), although the expression of other genes (STXBP6, BMP6, CSS3, TM4SF9 and SNIP) has been hypothesized to also be potentially capable of being affected by DNA methylation.

CpG methylation affecting extensive global genome sites can be investigated in chromosomes and in interphase chromatin *in situ* after their treatment with the restriction enzymes *Msp*I and *Hpa*II (14-18). Both enzymes cleave the sequence -CCGG-, but *Hpa*II does not cleave it if the central cytosine of this sequence is methylated (19). Image analysis of Feulgen-stained cells previously treated with *Msp*I and *Hpa*II has permitted investigation into whether extensive CpG methylation participates in chromatin higher order structures (17,18). This approach could be extended to evaluate the participation of global DNA methylation in nuclear phenotypes that have various chromatin higher order packing states as transformation and tumorigenesis progress.

Considering that variable patterns of chromatin supraorganization have been observed in E2-transformed MCF-10F cell lines (7), the association of heterogeneous chromatin packing states (as estimated by chromatin entropy) with extensive DNA methylation was investigated here by image analysis after cell treatment with the restriction enzymes *Msp*I and *Hpa*II and Feulgen staining.

Materials and methods

Cells. The spontaneously immortalized human breast epithelial cell line MCF-10F, 17-ß-estradiol-transformed MCF-10F cells (E2), highly invasive E2 cells (C5), and cells derived from tumors generated by injection of C5 cells in SCID mice (C5-A6-T6 and C5-A8-T8) (4) were used.

MCF-10F cells were cultured in DMEM:F-12 medium containing 1.05 mM calcium, antibiotics, antimycotics, hormones, growth factors and equine serum as reported previously (20). E2 cells resulted from MCF-10F cells in their 123rd passage, treated with 70 nM 17-ß-estradiol as described previously (4). The E2 cells used were at their 10th passage. C5 cells were obtained by expansion from the 10th passage of E2 cells; injection of these has been previously reported to elicit development of poorly differentiated adenocarcinomas in 9 out of 10 SCID mice (4). C5-A6-T6 and C5-A8-T8 tumoral cell lines were used at passage three, for which fingerprint and tumorigenic assay analyses have been described (4).

The cells were cultured for 96 h (MCF-10F cells) and 48 h (the other cell types) at 70-80% confluence on 9.4 cm^2 plastic chamber slides (21), fixed in an absolute ethanol-glacial acetic acid mixture (3:1, v/v) for 1 min, rinsed in 70% ethanol for 5 min, and air dried.

Digestion with restriction enzymes. The cells were initially treated with 1% Triton X-100 in the presence of 4 M glycerol

Entropy of Feulgen-stained 17-ß-estradiol-trans-MCF-10F cells after treatment with restriction enzymes.

Cells	Tests	X	S
MCF-10F	Buffer 1	5.09a	0.38
	*Msp*I	4.58b	0.40
	Buffer 2	5.09a1	0.42
	*Hpa*II	4.53b1	0.40
E2	Buffer 1	5.42a2	0.47
	*Msp*I	5.31b2	0.45
	Buffer 2	5.44a3	0.36
	*Hpa*II	4.56b3	0.51
C5	Buffer 1	5.12a4	0.30
	*Msp*I	4.86b4	0.37
	Buffer 2	4.97a5	0.38
	*Hpa*II	4.10b5	0.33
C5-A6-T6	Buffer 1	5.14a6	0.40
	*Msp*I	4.79b6	0.45
	Buffer 2	4.77a7	0.37
	*Hpa*II	4.82a7	0.59
C5-A8-T8	Buffer 1	4.96a8	0.34
	*Msp*I	4.60b8	0.38
	Buffer 2	4.69a9	0.35
	*Hpa*II	4.95b9	0.39

n, 200; E2, 17-ß-estradiol-treated MCF-10F cells; C5, cells expanded from E2; C5-A6-T6 and C5-A8-T8, tumor cells; S, standard deviation; X, arithmetic mean. Different letters and numbers in the X column for comparison of enzyme vs respective buffer results denote differences significant at P=0.01 (ANOVA).

for 2 min to make the nuclear envelope more permeable to the enzyme digestions (22,23). Then, the preparations were incubated with 1.0 U/μl *Msp*I and *Hpa*II (New England Biolabs, Ipswich, MA, USA) restriction enzymes in appropriate buffers and then covered with coverslips (15,18,24). The incubations were accomplished in a moist chamber at 37˚C for 10 h, after which the slides were rinsed in distilled water and air dried (18,24). As controls, preparations were treated with Triton X-100 and the assay buffers recommended for each of the enzymes used (18).

Cytochemistry. The enzyme-treated and control preparations were simultaneously processed for the Feulgen reaction (4 M HCl at 24˚C, 75 min), cleared in xylene, and mounted in Cargille oil (Cedar Grove, NJ, USA) (n_D = 1.54). Three slides were used for each experimental condition.

Video image analysis. Two hundred interphase nuclei chosen at random for each experimental condition were measured using Carl Zeiss/Kontron equipment and Kontron KS400 version 3.0 software (Oberkochen, Munich, Germany). The microscopic images were obtained with a Zeiss Axiophot 2 microscope equipped with a Neofluar 40/0.75 objective, optovar factor 2, 0.90 condenser, and light of wavelength equal to 546 nm, provided by a Schott interference filter. A 100-W halogen illuminator, a voltage regulator for light intensity maintained constant at point 4, filter wheels 1 and 2 rotated into position 100 (open position), and a luminous-filter diaphragm (transmitted light) at its maximal opening were used. These illumination conditions were maintained for all nuclei investigated. The images to be processed were fed from the microscope into a Pentium computer through a Sony CCD IRIS/RGB Hyper HAD color video camera. Two threshold values determined which gray value range of the image Input is retained or deleted in the image Output. Low (L) and high (H) threshold values (gray values) were obtained by moving the borders in the gray value histogram such that the nuclear images appeared well segmented from each other and from the background. Since 'green' was selected as the parameter 'color', the pixels inside the gray value range (L, H) were displayed in this color. Under these conditions, 1 μm corresponds to 7.23 pixels. The minimum area possible to be measured with this apparatus corresponded to four pixels. Kontron KS400 software provided quantitative information on chromatin entropy (9).

Statistical analysis. Calculations and the ANOVA test were performed using Minitab 12™ software (State College, PA, USA).

Results and Discussion

The entropy values of the Feulgen-stained control and 17-ß-estradiol-transformed MCF-10F cells after treatment with *Msp* I decreased significantly (Table II), which indicates that -CCGG- sequences are involved in the previously reported chromatin packing states of these cells (Table I) (7). After the different cell types were treated with *Hpa*II, the entropy values significantly decreased for MCF-10F, E2, and C5 cells, but they did not change for the C5-A6-T6 cells and even increased for the C5-A8-T8 cells (Table II). A conspicuous entropy decrease with *Hpa*II digestion occurred mostly in the E2 cells (Table II).

The findings obtained after treatment with *Hpa*II indicate that the high heterogeneity in chromatin packing states that was originally evident in E2 cells (7) is not directly associated with DNA methylation, because E2 cells showed the largest decrease in entropy when DNA sequences containing non-methylated cytosines were removed. This occurred in spite of the presence of a locally hypermethylated and silent DNA, which was expressed following 5-aza-dC, but not following trichostatin A treatments. The former has been reported to affect the SPRY1 and NRG1 genes in E2. It is possible that, in this case, epigenetic factors other than extensive cytosine methylation are responsible for the condensation levels attained with the 17-ß-estradiol transformation, the organization of which is changed when -CCGG- sequences are excised [possibly a result of histone modifications and linker histone binding (25)]. In mutant

mouse ES cells, the absence of DNA methylation has been observed to change nuclear organization with an increase in the level of chromocenter clustering (25).

In C5-A6-T6 cells, one of the tumor cell lines derived from the adenocarcinomas induced by injection of C5 cells in SCID mice (4), the contrast between condensed and non-condensed chromatin was originally lower than in E2 cells (Table I) (7). Since, in this case, chromatin entropy remained unchanged after *Hpa*II treatment, although it decreased following *Msp*I digestion, the heterogeneity levels originally attained by chromatin supraorganization may be contributed to by methylated C-containing DNA sequences. According to Fernandez and co-workers, molecular assays have revealed the presence of DNA hypermethylation in some tumor cells generated from C5 cells, although specifically on NRG1 and CSS3 genes.

In C5-A8-T8, another tumor cell line derived from an adenocarcinoma generated in SCID mice after C5 cell injection (4), chromatin organization was originally characterized by extensive homogeneity and low condensation state (Table I) (7). These characteristics changed after *Hpa*II treatment (entropy increase), indicating that heterogeneity in chromatin packing states was elicited (Table II). This was possibly due to structural chromatin remodeling following excision of non-methylated C-containing DNA sequences rather than being associated with some local DNA hypermethylation. Indeed, the chromatin packing state pattern of this tumor cell line could be better explained by global cytosine hypomethylation referred to occur extensively in breast cancer cells by Shann and co-workers (13).

Present findings indicate that -C^{m}CGG- sequences may affect chromatin supraorganization in 17-ß-estrogen-transformed MCF-10F cells. However, not all chromatin condensation patterns set up in these cells with transformation and/or tumorigenesis are associated with DNA methylation (e.g. E2 cells). In the tumor cell lines C5-A6-T6 and C5-A8-T8, generated from the same highly invasive estrogen-transformed MCF-10 cells (C5) (4), chromatin supraorganization remodeling may be attained by different mechanisms. While one of these cell lines (C5-A8-T8) likely had its chromatin packing states associated with DNA hypomethylation, as reported in the literature for other cell types (13), the other one (C5-A6-T6), which showed its pattern of chromatin supraorganization not affected by *Hpa*II treatment, is suggested to have packing states mostly associated with local DNA hypermethylation or other epigenetic factors. A variable epigenetic modulation affecting the higher-order packing states of chromatin in the estrogen-transformed MCF-10F cell model, evident with the chromatin entropy investigation by image analysis, is thus assumed.

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