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Image analysis of the AgNOR response in *ras*-transformed human breast epithelial cells

Maria Luiza S. Mello^{a,b}, Benedicto C. Vidal^{a,b}, Jose Russo^c, Wolfgang Planding^b, and Ulrich Schenck^b

^aDepartment of Cell Biology, Institute of Biology, State University of Campinas (UNICAMP), 13083-863 Campinas, Brazil

^bLabor für klinische Zytologie, Institut für Pathologie der Technischen Universität München, 81675 Munich, Germany

^cBreast Cancer Research Laboratory, Fox Chase Cancer Center, 19111 Philadelphia, USA

Summary

The argyrophylic staining of the nucleolar organizer regions (AgNOR positive response) in interphase nuclei is often related directly to the cellular demand for ribosome biogenesis and is considered of relevance in studies of tumor pathology. Transformation of human breast epithelial MCF-10A cells by the *c-Ha-ras* oncogene results in altered growth, invasiveness and tumorigenicity in nude mice. Since *ras* transformation may be associated with a more intense nucleolar activity, we examined the influence of transfection by the *Ha-ras* oncogene on AgNOR staining response in MCF-10A cells. Following assessment of the AgNOR response with video image analysis, the AgNOR-positive areas and the AgNOR area/nuclear area ratio, but not the number of AgNOR aggregates or dots per nucleus, were found to be much higher after *ras* transformation. A role of the *Ha-ras* transformation on the nucleolar activity of the MCF-10A is thus suggested as assessed by the AgNOR staining. Based on data of the literature, it is also hypothesized that a decreased wild-type p53 level, possibly promoted by the *ras* transformation, may be associated with the increased AgNOR response.

Keywords

AgNOR; *Ha-ras* oncogene; Human breast epithelial cells; *in vitro*; p53; Image analysis

Introduction

In human cells, the abundance of argyrophylic interphase nucleolar organizer regions (AgNOR) is, in many cases, considered to be of diagnostic and prognostic significance in tumor pathology, because of its direct relationship to the rate of cell proliferation and other demands for ribosome biogenesis (Derenzini and Trere, 1991). The AgNOR technique reveals the presence of nuclear proteins such as phosphoprotein C3 (nucleolin), B23 (numatrin), and factors necessary for rDNA transcription and early rRNA processing (Mehta, 1995). The close functional association between the AgNOR response and the cell cycle, cell proliferation and

Correspondence to: Maria Luiza S. Mello, mlsmello@unicamp.br.

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the rate of rRNA transcription and synthesis means that the number and size of the AgNOR regions may vary with alterations in any of these parameters (Mehta, 1995).

The spontaneously immortalized, non-transformed human breast epithelial cell line, MCF-10A, derived from a human breast epithelial cell strain designated MCF-10M (Soule et al., 1990), has been used as an important model for studies on damage to DNA and on human breast cell transformation *in vitro* (Ciardiello et al., 1990; Basolo et al., 1991; Bianco et al., 1994; Mello et al., 1994; Yoon et al., 2002; Starcevic et al., 2003; Cuendet et al., 2004; Burdick et al., 2006; Cuendet and Bolton, 2006). These cells are susceptible to *in vitro* transformation under overexpression of several genes, including the c-Ha-*ras* oncogene (Ciardiello et al., 1990; Basolo et al., 1991; Mello et al., 1994; Starcevic et al., 2003).

The transformation of MCF-10A cells by transfection with the c-Ha-*ras* oncogene results in altered growth, invasiveness and tumorigenicity in nude mice (Basolo et al., 1991), and remodeling in chromatin supraorganization (Mello et al., 1994). However, no investigation has yet been performed on intensity of nucleolar activity, as demonstrated with the AgNOR staining, in these cells under *ras* transformation, when an increased demand for ribosome biogenesis is supposed to occur. Indeed, in a different cell line derived from MCF-10M cells (MCF-10F), the transformation by benzo[a]pyrene followed by transfection with the c-Ha-*ras* oncogene resulted in increased nucleolar areas and nucleolar/nuclear area ratio, as estimated in toluidine blue-stained preparations (Barbisan et al., 1998).

To test the hypothesis of *ras* transformation being associated with a more intense nucleolar activity in human breast epithelial cells *in vitro*, we examined the AgNOR staining profile (Rueschoff et al., 1990; Derenzini and Ploton, 1991; Vidal et al., 1994) of MCF-10A cells transfected with the c-Ha-*ras* oncogene in comparison with that of nontransformed MCF-10A cells.

Materials and methods

Cell lines and culture conditions

MCF-10A cells in passage 98 and MCF-10A cells transfected with the plasmid Homer 6 alone, containing the neomycin-resistance gene (MCF-10Aneo cell line), or with this vector plus a construct containing the c-Ha-*ras* oncogene (MCF-10AneoT cell line), as described previously (Basolo et al., 1991), were used. The cells were grown in Dulbecco's minimal essential medium/F-12 medium (1:1) (Grand Island Biological Co.(GIBCO), Long Island, New York, USA) supplemented with 5% equine serum (GIBCO), 0.1 µg/mL cholera-toxin (ICN Biomedicals, Cleveland, OH, USA), 10 µg/mL insulin (GIBCO), 100 U/mL penicillin (GIBCO), 100 µg/mL streptomycin (GIBCO), 2.5 µg/mL amphotericin B (GIBCO), 0.5 µg/mL hydrocortisone (Sigma Chemical Co., St. Louis, MO, USA), and 0.02 µg/mL epidermal growth factor (Collaborative Research Inc., Palo Alto, USA), as reported previously (Soule et al., 1990; Basolo et al., 1991). The transfected cells were selected by their ability to grow and form colonies after three weeks at 37°C in a 5% CO₂ atmosphere in the medium described above but containing 750 µg/mL geneticin (GIBCO)(Basolo et al., 1991).

Cell preparation and staining

Cells grown on coverslips were fixed in an absolute ethanol-acetic acid (3:1, v/v) mixture for 1 min, rinsed in 70% ethanol for 3–5 min, and air-dried. Three coverslips of each cell line were used. AgNOR staining was done as described by Rueschoff et al (1990) and Derenzini and Ploton (1991), preceded by treatment with a 1% Triton X-100 solution in the presence of 4 M glycerol for 15 min at 37°C (Vidal and Mello, 1995). Briefly, the cells were treated with a solution containing 2 volumes 50% aqueous silver nitrate (Merck, USA) and 1 volume 2%

gelatin in 1% aqueous formic acid (v/v). The aqueous solutions were prepared with deionized water. The optimal staining time was found to be 10 min at 37°C. Immediately after the silver impregnation, the material was rinsed in deionized water, air dried, cleared in xylene and mounted in Canada balsam.

Image analysis

Zeiss-Kontron equipment (Oberkochen/Eching-Munich, Germany) was used for image acquisition, segmentation and featuring using a Kontron-IPS system and appropriate programs, according to the Kontron-IBAS 2000 instruction manual. The preparations were observed with a Zeiss Universal N microscope equipped with a Neofluar 100/1.30 objective, optovar 1.25, 1.3 condenser, and $\lambda = 546$ nm obtained with a Schott monochromator filter ruler. The images to be processed were fed from the microscope into a computer via a Hamamatsu C3077 (Hamamatsu Photonics, Japan) monochrome CCD video camera. In this investigation, $1 \mu\text{m} = 11.3$ pixels. Different grey levels were converted to pseudocolored images to facilitate the identification and discrimination of different areas of the image.

A macro developed by one of us (WP) provided quantitative morphological information on nuclear size and on the number and area of AgNOR-positive aggregates or dots. Since densitometric features were derived from whole nuclei as well as from distinct AgNOR-positive regions, two segmentation levels were used. The threshold levels for whole nuclear images and for AgNOR-positive areas were established based on previously determined grey level histograms of all image pixels of the MCF-10A cell nuclei. The same threshold levels were automatically used to analyze all the nuclei. Details of the image analysis procedure have been described elsewhere (Mello et al., 1994; Vidal et al., 1994). Although interactive image editing was possible while running the program, it was rarely required for the nuclei analyzed here.

Statistical analysis

All calculations and statistical analyses were done using the Minitab 12™ software (State College, PA, USA). For comparisons in which Anova was not applicable, nonparametric tests (Kruskal-Wallis, Mann-Whitney) for medians were used.

Results

Well-defined nuclear contours and conspicuous AgNOR-stained structures were detected in all of the cell nuclei. The nuclei stained pale yellow whereas the AgNOR dots or aggregates stained dark brown, as illustrated in Fig. 1a–c; 2a. Examples of nuclei that showed some of the steps of the Kontron-IPS system segmentation process were photographed from the color monitor, illustrated in Fig. 2b–g. Some AgNOR responsive dots outside the nucleoli and probably corresponding to Cajal bodies (Bilinski and Kloc, 2002; Niedojadlo and Gorska-Bryllass, 2003) were occasionally found and also considered as positive regions. These are shown in Fig. 2c,f.

Nuclei of different sizes were observed. Details are given in Table 1. In control MCF-10A cells, the smaller nuclei generally showed smaller AgNOR areas whereas larger nuclei presented larger AgNOR aggregates. In the other cells no correlation between the nuclear and AgNOR areas was detected. The abundance of AgNOR aggregates or dots was not correlated with the nuclear size or the AgNOR total area in any of the cells analyzed here. It is worth mentioning that the number of points considered as AgNOR dots or aggregates do not coincide with the number of nucleoli detected visually.

The nuclear areas of cells transfected with the plasmid alone or with the plasmid plus the *Ha-ras* oncogene did not differ significantly from each other, but were larger than the nuclear areas of the nontransformed controls, detailed in Table 2. The AgNOR-positive areas and the AgNOR area/nuclear area ratio of MCF-10AneoT cells were significantly greater than those of the controls and of cells transfected with the plasmid alone. The frequency of AgNOR aggregates or dots counted in *Ha-ras*-transformed cells did not differ from that of control cells, but was smaller than in cells transfected with the plasmid alone.

Discussion

The results of this study indicate that *Ha-ras* transformation induces enhancement in the nucleolar activity of human breast epithelial MCF-10A cells, as measured by increased AgNOR-stained areas and AgNOR area/nuclear area ratios. The enhanced metabolic activity involved is certainly related to an increased demand for a rapid supply of ribosomes. The present findings also agree with the increase in nucleolar areas reported for toluidine blue-stained preparations of MCF-10F cells transformed by benzo[a]pyrene and transfected with the *Ha-ras* oncogene (Barbisan et al., 1998), and in nucleolar function as expressed in breast neoplasms reported in several studies (Helpap, 1989; van Diest et al., 1989, 1990; Derenzini and Trere, 1991; Belotti et al., 1997; Kruger et al., 2000; Kanna et al., 2001).

The image analysis parameters were more useful for discriminating MCF-10AneoT cells from MCF-10neo or MCF-10A cells than was the number of AgNOR aggregates or dots per nucleus. Indeed, there was considerable variability in the AgNOR areas relative to the nuclear size in MCF-10AneoT and MCF-10Aneo cells, in contrast to the significant correlation between AgNOR and nuclear areas in MCF-10A cells (control). The larger variability in the AgNOR areas of MCF-10AneoT cells may be related to that in the Feulgen-DNA content induced by the *c-Ha-ras* oncogene in these cells (Mello et al., 1994), probably as an effect of the genomic instability created by transfection with the mentioned oncogene. Indeed, nuclear polymorphism has been observed during malignant progression of primary human breast cancer (van Diest et al., 1989; Tajima et al., 1991).

The AgNOR response in human breast cancer has been suggested to be affected by the status of the oncosuppressor proteins p53 and pRb (Derenzini et al., 2004; Trere et al., 2004). In 71 human primary breast carcinomas with a mutated p53, as studied by Trere and co-workers (2004), the mean AgNOR area was greater than in 272 tumors with a normal (wild-type) p53. Presence of a wild-type p53 has been reported for nontransformed MCF-10A cells cultivated in several other laboratories (Bianco et al., 1994; Merlo et al., 1995; Cuendet et al., 2006). However, with progressively elevated Ras protein and increased cellular resistance to experimental oxidative DNA damage, a tumorigenic MCF-10A cell line (MCF10ATG3B) has shown a marked decrease (58%) in wild-type p53 protein levels (Starcevic et al., 2003). Although the p53 status for the MCF-10A cells used in this study has not been estimated, based on Starcevic and co-workers' (2003) report, it is suggested that a decrease in the wild-type p53 levels may also have occurred under the *Ha-ras* oncogene transformation analyzed here. In addition, present results on increased AgNOR areas and AgNOR area/nuclear area ratios in *Ha-ras*-transformed MCF-10A cells would thus not be in disagreement with Trere et al (2004) consideration of an increase in AgNOR area response related to increase in levels of mutated p53 in human breast tumors.

The inclusion of the AgNOR-positive dots assumed to represent Cajal bodies in the evaluation of the overall nuclear silver-staining response in a few nuclei of control and transformed cells does not overlook the association of the AgNOR response and RNA metabolism, since these bodies have been considered as sites for the concentration of RNA-processing factors and

elements of the splicing system (Bilinski and Kloc, 2002; Niedojadlo and Gorska-Bryllass, 2003).

The transfection of the MCF-10A cells with the *c-Ha-ras* oncogene or the Homer 6 plasmid alone increased the nuclear area. A similar finding has been reported for these cells after the Feulgen reaction, and associated with a certain chromatin unraveling in response to transfection of the plasmid, an event which was more prominent in the MCF-10Aneo cells (Mello et al., 1994). It is thus possible that with the induced chromatin loosening in the MCF-10Aneo cell nuclei, their nucleolar organizer regions would become more separated from each other in comparison to those of the MCF-10A cells. Consequently, the accumulation of the proteins reactive to the silver staining in the NOR regions of the MCF-10Aneo cells, as discriminated by the image analysis software, would reveal a number of AgNOR-stained dots larger than that in MCF-10A cells. The same may have occurred in MCF-10AneoT cells. However, in this case there was an increase in AgNOR-reactive proteins resulting in a larger area being occupied and in the AgNOR-positive sites being discriminated individually as aggregates (Fig. 2 – different pseudocolors) and not as dots, on account of a more intense nucleolar activity. The usefulness of counting the number of AgNOR dots for this model should thus be disregarded.

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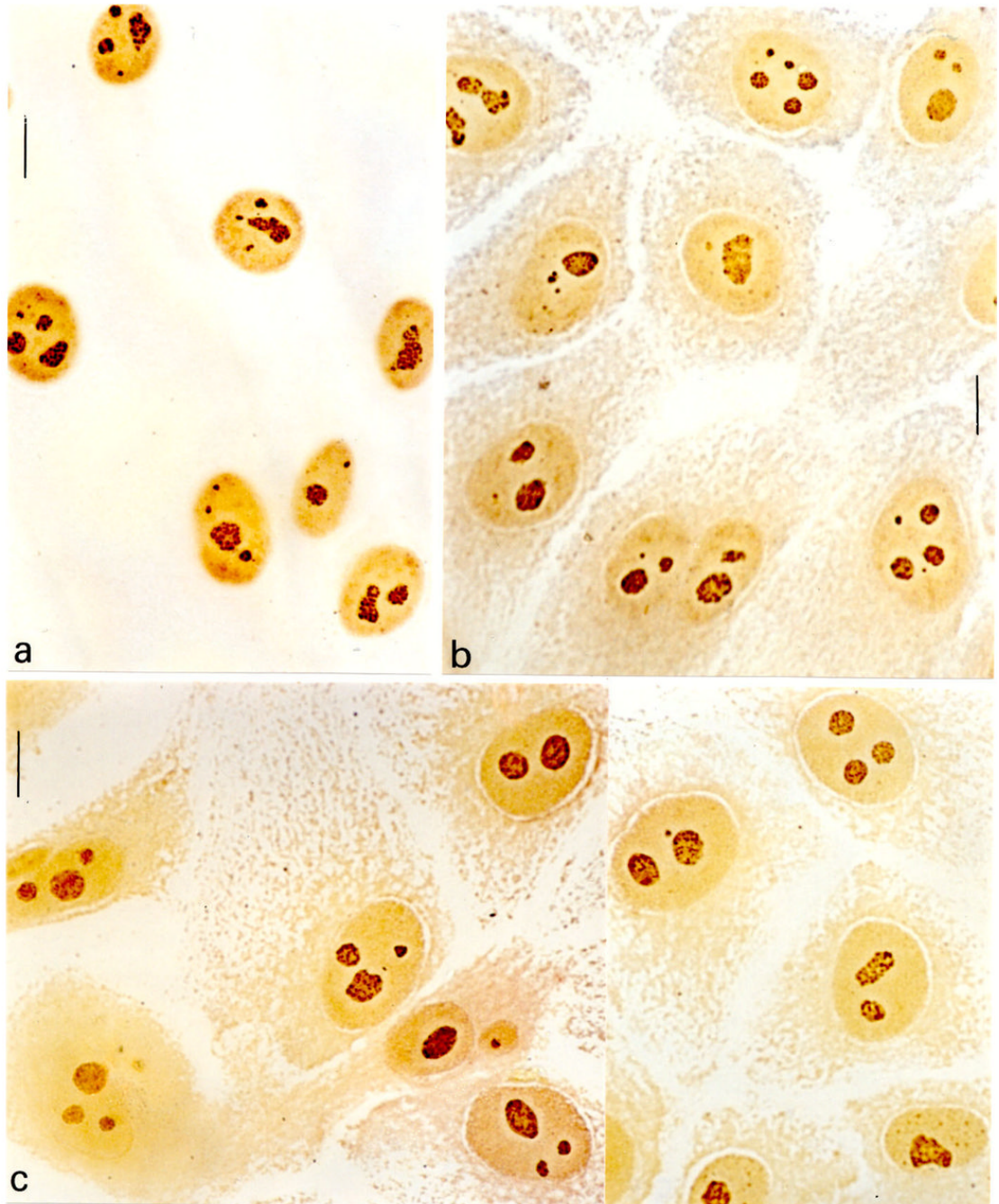


Figure 1. AgNOR staining in nucleoli (nu) of MCF-10A (a), MCF-10Aneo (b) and MCF-10AneoT (c) cells. Bar, 10 μm.

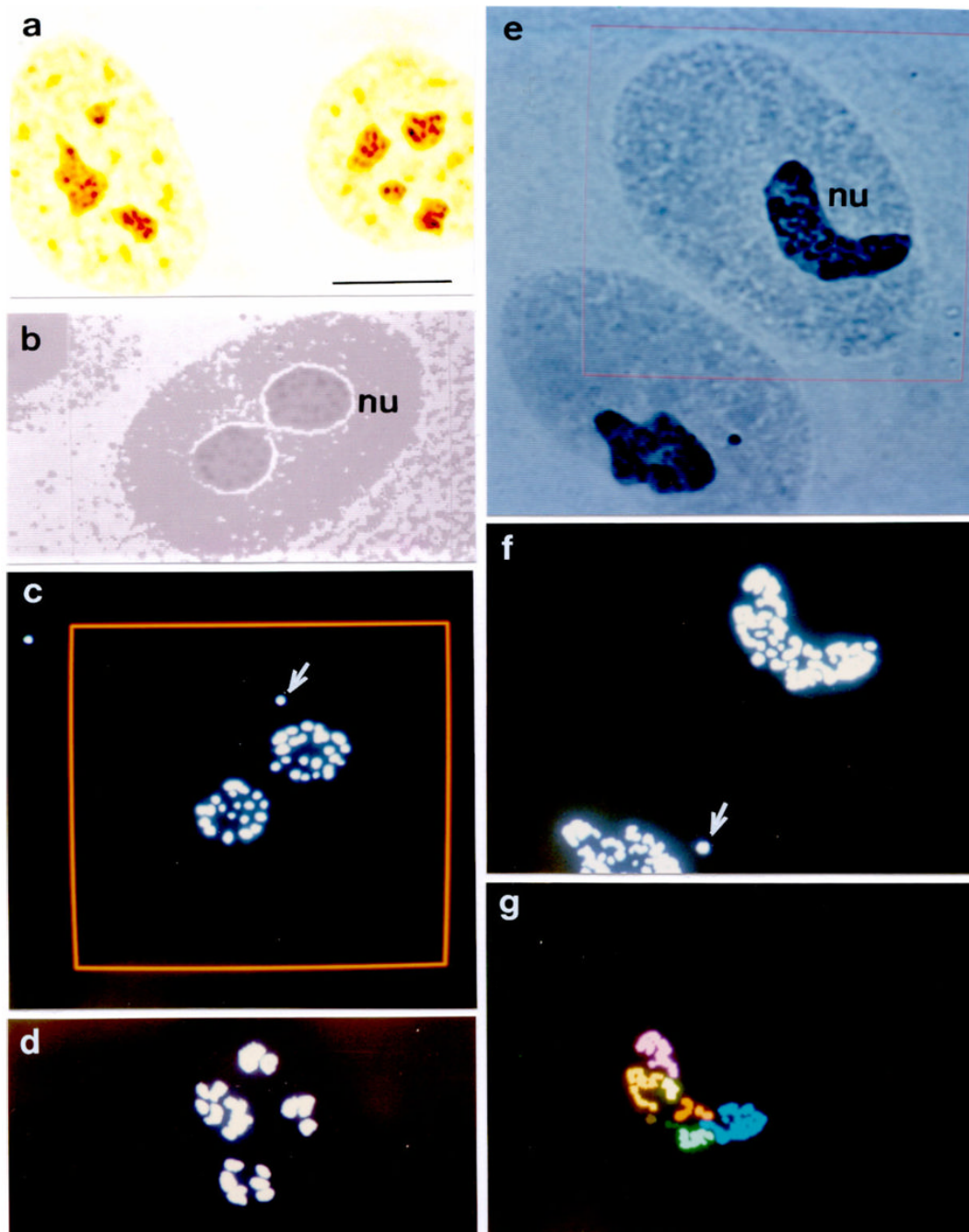


Figure 2.

AgNOR staining in nucleoli (**nu**) of nontransformed and transformed MCF-10A cells. **a.** AgNOR response (black dots) in control MCF-10A cells: photomicrograph obtained directly from the microscope. Bar, 10 μ m. **b–g.** Examples of some steps of the segmentation procedure and feature evaluation of the image analysis by Kontron-IPS system of AgNOR-stained particles or aggregates as photographed from the color monitor. **b.** Original image (TV on) of one MCF-10A cell nucleus. **c.** Grey level segmentation of the AgNOR-stained dots of the nucleus shown in **b**. The arrow indicates a positive dot in a putative Cajal body. The area under analysis is delimited by the red frame. **d.** Grey level segmentation of the AgNOR-stained dots of an MCF-10Aneo cell nucleus. **e.** Original image (TV on) of two MCF-10AneoT cell nuclei.

The nucleus to be analyzed is inside the area delimited by the red frame. **f.** Grey level segmentation of the AgNOR-stained particles of the image shown in **e.** The arrow indicates a positive dot in a putative Cajal body. **g.** Identification of the AgNOR-stained image of the nucleus shown inside the frame area in **e.** Different pseudocolors identify aggregates of dots not in contact with each other.

Table 1

Correlation coefficient **r** for the total area (A) and number (B) of AgNOR-stained aggregates or dots in the nuclear area (C), and for the number of AgNOR-stained aggregates or dots (B) in the total AgNOR area (A) in the MCF-10A cell lines.

Cells	n	Variable series	rand z(r)
MCF-10A	111	A × C	0.24*
		B × C	0.06
		B × A	0.02
MCF-10Aneo	91	A × C	0.16
		B × C	0.13
		B × A	0.00
MCF-10neoT	96	A × C	0.10
		B × C	0.11
		B × A	0.00

* P < 0.05. In all cases, a significance at the P_{0,05} level required **r** values > 0.19.

