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ORIGINAL ARTICLE

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# In vitro investigations of interphase and metaphase argyrophilic nucleolar organizer regions and cellular proliferation in the human urothelial cancer cell line HOK-1

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**Abstract** Detailed investigation of cell growth and nucleolar organizer region associated argyrophilic proteins (Ag-NORs) is necessary to asses a possible impact of Ag-NOR quantification on the diagnosis and prognosis of tumours. In this study, cellular proliferation of the transitional-cell carcinoma cell line HOK-1 was modulated over a period of 11 days by starvation and subsequent medium addition. Proliferation was determined daily by DNA flow cytometric estimation of S-phase fraction (SPF) and mitotic index (MI) calculation. The number and area of interphase Ag-NORs were quantified by automated image analysis daily and the number of Ag-NOR bearing chromosomes in metaphase was counted. In interphase nuclei, Ag-NOR area showed a highly significant correlation with SPF ($p < 0.0001$) whereas interphase Ag-NOR number showed significant correlation with MI ($p < 0.05$). A positive relationship between the number of Ag-NOR bearing chromosomes in metaphases and cellular proliferation was also observed. There is variability in Ag-NOR quantity during interphase and metaphase depending on growth conditions in vitro. Correlations of the number of interphase Ag-NORs with the MI on one hand and Ag-NOR area with SPF on the other provide further evidence that distribution and quantity of Ag-NORs are strongly influenced by the cell cycle phase within the structural-functional unit of the nucleolus.

**Key words** Nucleolar organizer regions · Silver staining
Image analysis · Cell cycle · Proliferation

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## Introduction

In man, nucleolar organizer regions (NORs) contain the ribosomal genes localized on the chromosomes 13, 14, 15, 21, and 22 (Goessens and Lepoint 1974; Goodpasture and Bloom 1975; Howell 1982). NORs are characterized by a unique feature; the presence of acidic proteins which can be selectively demonstrated by a histochemical one-step silver stain (Ploton et al. 1986). These acidic silver-staining NOR (Ag-NOR) proteins have been implicated in a variety of functions during ribosomal gene transcription (Lischwe et al. 1979; Herrera and Olsen 1986). During interphase, both ribosomal genes and their respective silver stained proteins are localized within the nucleolus (Wachtler et al. 1986). There is convincing evidence that the fibrillary components of the nucleolus represent the interphase counterpart of metaphase NORs (Hernandez-Verdun 1983, 1986; Goessens 1984).

In recent years staining for interphase Ag-NORs has attracted the interest of histo- and cytopathologists as a new technique in tumour diagnosis and prognosis (Öfner et al. 1990; Derenzini and Trere 1991; Egan and Crocker 1992). Several histopathological studies performed on routinely processed tissues from different malignant tumours have reported correlations between tumour grading and prognosis with number and area of Ag-NORs (for review see Egan and Crocker 1992). Moreover, statistically significant relationships between cellular proliferation, determined by Ki-67 immunoreactivity (Dervan et al. 1989; Shibuya et al. 1993) or S-phase fraction (SPF) calculation (Crocker et al. 1988), and Ag-NOR quantity have been demonstrated in a variety of human neoplasms.

In vitro studies of different malignant human cells revealed that the distribution pattern and total quantity of Ag-NORs are strongly influenced by cell proliferation and cell cycle phases. In neuroblastoma cells and in breast cancer cells a linear relationship between the population doubling time and the tritiated-thymidine uptake with the amount of Ag-NOR proteins has been

reported (Trere et al. 1989; Derenzini et al. 1990; Öfner et al. 1992). Derenzini et al. (1989) showed that the interphasic Ag-NOR area in the neuroblastoma cell line CHP 212 changes in parallel with DNA synthesis after culture medium deprivation and subsequent medium addition whereas no changes could be observed in metaphase Ag-NOR numbers. A recent study of our group (Öfner et al. 1993) demonstrated different behaviour of NOR-proteins in breast cancer cell lines treated with interferon-gamma and 4-hydroxy-tamoxifen indicating that Ag-NOR patterns may also reflect different mechanisms of growth suppression.

Estimation of the silver stained structures within cell nuclei by automated image analysis has been established as a reliable method of quantification (Rüschoff et al. 1990). However, the quantification of the complex and changeable appearence of Ag-NORs within interphase nuclei is still a matter of dispute (Hittmair and Öfner 1991). In general, both Ag-NOR number as well as Ag-NOR area have been shown to be correlated to cellular proliferation.

The aim of our present in-vitro study was to investigate the changes of Ag-NOR quantity (number and area) throughout the whole cell cycle in dependance of growth conditions modulated by starvation over a period of 11 days. We compared Ag-NOR quantity with cellular proliferation determined as SPF and mitotic index (MI), both methods used in routine diagnosis of solid tissues.

## Materials and methods

HOK-1 cells were grown in 25 $cm^2$ tissue culture flasks (Falcon, Becton Dickinson, Heidelberg, FRG) in a 1:1 mixture of Dulbecco's modified Eagle's Medium (DMEM, Seromed, Berlin, FRG) and MCDB-151 (Sigma, St. Louis, Mo. USA) as described previously (Offner et al. 1991). For experiments 500 000 cells were seeded in 12cm-petri dishes in a volume of 10 ml fresh medium and allowed to grow for 7 days. Thereafter the culture medium was discarded and starving cells were re-fed by the addition of fresh medium to initiate further cell growth. Ag-NORs and proliferative activity were assessed daily during the course of the experiment (1–11days). Briefly, cells were harvested using a mixture of 0.05% trypsin in 0.02% versene (Seromed) and divided in two fractions. One for the silver staining of NOR-associated proteins and MI estimation and the second for DNA flow cytometric measurement of the SPF.

After pretreatment with 0.075M potassium chloride the cells were fixed in three changes of a 3:1 (v/v) solution of methanol and acidic acid and subsequently dropped on cooled plain glass slides. No treatment with colchicine was performed. After rehydration in distilled water (10 min) silver staining was performed as described previously (Ploton et al. 1986) using a solution containing 1 volume of 2% gelatine in 1% aqueous formic acid and 2 volumes of 50% silver nitrate.

The freshly prepared solution was applied for 10 min at 37° C. Subsequently the cell preparations were thorougly rinsed with distilled water (3 × 5 min) to wash off silver precipitates. Finally the slides were dehydrated in a series of alcohols and permanently mounted with Entellan (Merck, Mannheim, FRG).

Randomly selected fields of the cell preparations were captured into digital memory using an Olympus BH-2 microscope (1000× magnification; oil immersion) connected with a video camera module (Sony CCM) and a Macintosh IIic computer equipped with a digitizer board. Image analysis was performed using Image Analyst (Automatix Inc.; Middlesex Turnpike Billerica, Mass. USA). The contrast of the image was enhanced by means of the histogram equalization method (Image Analyst). The threshold level was adjusted on metaphase Ag-NORs to get a clear cut differentiation between nuclear background and silver stained areas. One hundred cells were measured singulary in each cell preparation investigated.

Using the same slides as for automated image analysis the mean number of Ag-NOR bearing group D and group G chromosomes was counted in 20 metaphases. Chromosomes were identified by their size and number.

For DNA flow cytometry, after washing with phosphate buffered saline (PBS, pH 7.2, 0.01M) and fixation with freshly prepared chilled 70% ethanol DNA was stained with a solution consisting of propidium iodide (100 µg/ml) and ribonuclease A (1 mg/ml) for 30 min at 37° C in the dark. Cytometry was performed on a FACScan flow cytophotometer (Becton Dickinson, San Jose, Calif., USA) equipped with a 488nm argon laser to induce fluorescence. After doublet discrimination the SPF was calculated using the FACScan software.

Mitotic figures were counted within 1000 cells and the MI was calculated.

Statistical analysis was performed using the Spearman correlation test on a Systat computer program (Ill. USA) to compare SPF and mitotic index with Ag-NOR area and number, respectively.

## Results

Determination of SPF by DNA flow cytometry (Fig. 1) and calculation of MI (Fig. 2) revealed high cellular proliferation on the first three days of the experiment. Complete data on cell cycle kinetics are given in Table 1. SPF showed 31% on day 1 followed by a slight decrease to 25% on day 3. In contrast, the MI values increased from 0.3% up to 2.5% on day 3. Thereafter, due to the consumption of the culture medium the SPF and MI showed a continous decline reaching levels of 7% and 0%, respectively on day 7. Exchange of the culture medium on the 7th day immediately stimulated cellular proliferation with a fast rise of SPF up to 33% and MI values to 1.4% on day 9. Prolonged culture periods again led to a decrease of both parameters within 3 to 4 days.

Ag-NOR number (Fig. 3) as well as Ag-NOR area (Fig. 4) were influenced by the differing growth condi-

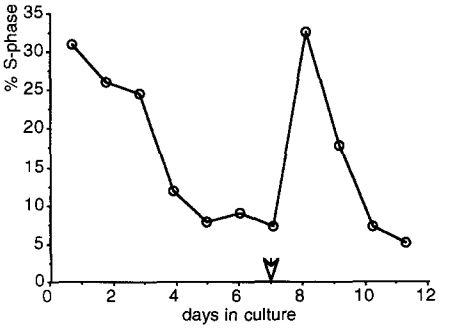


**Fig. 1** Percentage of HOK-1 urothelial cancer cells in S-phase fraction (SPF) determined by DNA flow cytometry over the period of 11 days. *Arrow* indicates culture medium exchange on day 7

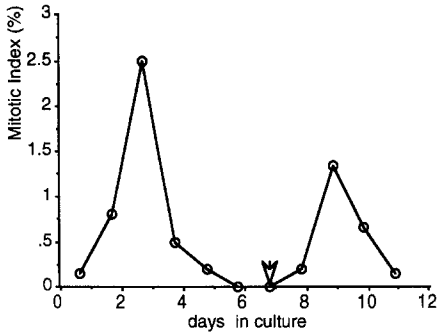


**Fig. 2** Mitotic indices over the period of 11 days. Arrow indicates culture medium exchange on day 7

**Table 1** Percentage of cells in cell cycle phase determined by DNA flow cytometry

| Days in culture | G0/G1 | S | G2/M |
|---|---|---|---|
| 1 | 57 | 31 | 12 |
| 2 | 59 | 26 | 15 |
| 3 | 60 | 25 | 15 |
| 4 | 72 | 12 | 16 |
| 5 | 85 | 8 | 7 |
| 6 | 82 | 9 | 9 |
| 7 | 83 | 7 | 10 |
| 8 | 52 | 33 | 15 |
| 9 | 55 | 18 | 27 |
| 10 | 72 | 7 | 21 |
| 11 | 81 | 5 | 14 |

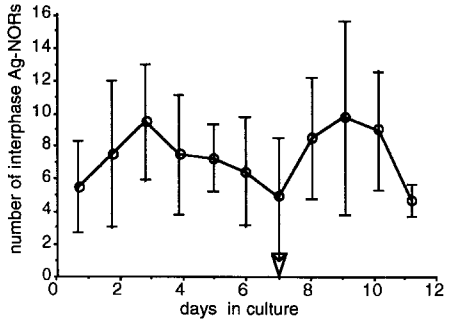


**Fig. 3** Mean Ag-NOR number with respective standard deviations over the period of 11 days. *Arrow* indicates culture medium exchange on day 7

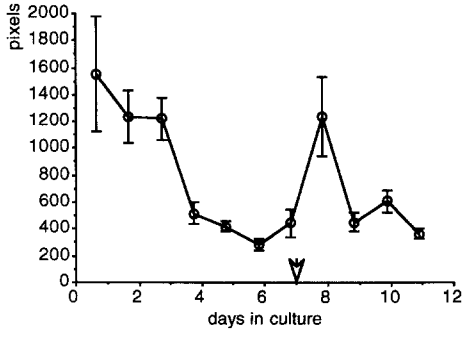


**Fig. 4** Mean Ag-NOR area with respective standard deviations over the period of 11 days. *Arrow* indicates culture medium exchange on day 7

**Table 2** Statistical correlation of S-phase fraction (SPF) and mitotic index (MI) with Ag-NOR number and area, using Pearson correlation matrix and Bonferroni-adjusted probabilities

| | SPF | Number | Area | MI |
|---|---|---|---|---|
| SPF | 1 | | | |
| Number | 0.31 | 1 | | |
| Area | 0.912** | 0.17 | 1 | |
| MI | 0.31 | 0.72* | 0.29 | 1 |

* $P<0.05$
** $P<0.0001$

tions. On the first 3 days of the experiment Ag-NORs were mainly seen clustered within a few big nucleoli (Fig. 5a) resulting in high values of the Ag-NOR area. Although an exact enumeration of Ag-NORs by image analysis was hampered by a considerable confluence of single black dots, an increase of Ag-NOR numbers was measured up to day 3 (Fig. 5b). During the following starvation period the Ag-NOR area decreased rapidly. In maximum starvation, few black dots were seen clustered in a few small nucleoli (Fig. 5c) giving minimal values for both, Ag-NOR area and number. Medium addition on day 7 resulted in a dramatic increase of the area of the Ag-NORs on day 8. As on day 1 numerous Ag-NORs were packed within few big nucleoli. In contrast, the Ag-NOR number did not reach its peak value before day 9, showing an enormous number of black dots of different sizes scattered all over the nucleolus (Fig. 5d). Starvation reduced size and number of Ag-NORs until the end of the experiment on day 11.

Statistical analysis comparing the Ag-NOR number and Ag-NOR area with MI and SPF revealed a highly significant correlation of Ag-NOR area with the SPF ($p<0.0001$) whereas Ag-NOR number correlated significantly with MI ($p<0.05$). No significant correlation could be observed neither between Ag-NOR number and SPF nor between Ag-NOR area and MI (Table 2).

Enumeration of Ag-NOR bearing chromosomes in metaphases (Figs. 5e and 6) showed more than 6 positive chromosomes on day 1 to 4 and 9 to 11. Differentiation of group D and G chromosomes revealed a synchronous increase and decrease of both groups. No metaphases could be demonstrated on day 6 and 7.

## Discussion

Most histo- and cytopathological studies have revealed a positive relationship between cell growth and Ag-NOR quantity indicating that this technique could be useful to determine proliferative activity on routinely processed tissues (Carbajo et al. 1993). In vitro studies comparing image analytical determined Ag-NOR quantity in different tumour cell lines with their population doubling times demonstrated significant correlations (Derenzini et al. 1990; Öfner et al. 1992). Derenzini et al. (1989) modulated cell growth in the established neurob-

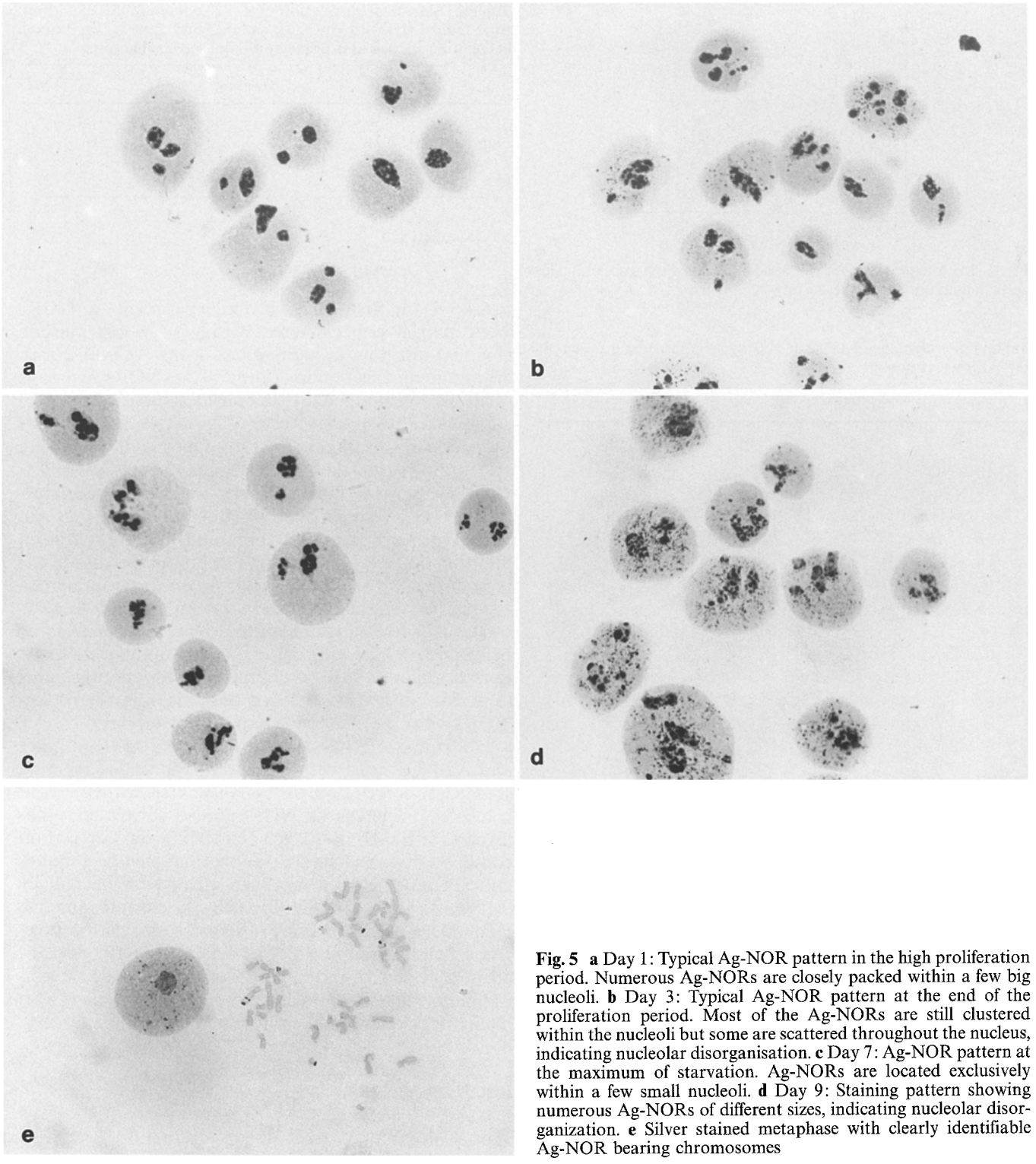


**Fig. 5** **a** Day 1: Typical Ag-NOR pattern in the high proliferation period. Numerous Ag-NORs are closely packed within a few big nucleoli. **b** Day 3: Typical Ag-NOR pattern at the end of the proliferation period. Most of the Ag-NORs are still clustered within the nucleoli but some are scattered throughout the nucleus, indicating nucleolar disorganisation. **c** Day 7: Ag-NOR pattern at the maximum of starvation. Ag-NORs are located exclusively within a few small nucleoli. **d** Day 9: Staining pattern showing numerous Ag-NORs of different sizes, indicating nucleolar disorganization. **e** Silver stained metaphase with clearly identifiable Ag-NOR bearing chromosomes

lastoma cell line CHP 212 by serum deprivation and subsequent medium addition and found a correlation between tritiated-thymidine uptake and the silver stained nuclear area. Quantitative data on the changes of the Ag-NOR number, a very commonly used variable in Ag-NOR quantification, were not given for the deprivation experiment in this study. The addition of colchicine for chromosome analysis to the cells for 30 min before harvesting probably strongly influences cell cycle kinetics and the number of Ag-NOR bearing chromosomes.

To get more detailed information on the relationships of both variables used in Ag-NOR quantification, number and area, with cellular proliferation we investi-

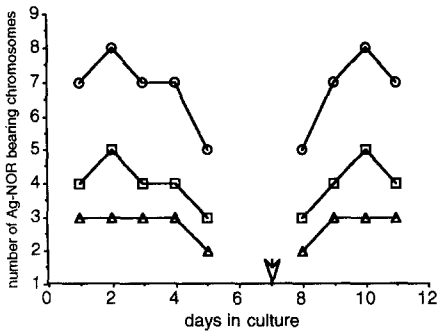


**Fig. 6** Number of Ag-NOR bearing chromosomes over the period of 11 days. *Arrow* indicates culture medium exchange on day 7. (*Circles* = total number of Ag-NOR bearing chromosomes; *squares* = group D chromosomes; *triangles* = group G chromosomes)

gated the diploid human urothelial cancer cell line HOK-1. DNA flow cytometry and MI calculation, both methods potentially usable in routine diagnosis were selected to determine proliferative activity. Our experimental design with continuous medium consumption by the tumour cell population followed by a single medium exchange on day 7 allowed a differentiated evaluation of Ag-NORs and growth parameters and resulted in statistically significant correlations between Ag-NOR number and MI on one hand and Ag-NOR area and SPF on the other.

These correlations might be explained by structural and functional changes due to cell cycle phases within the nucleolus. During phases of rapid cell growth (SPF > 20%) Ag-NORs are mostly seen clustered within a few big nucleoli. Quantitatively, this was reflected by high values of Ag-NOR area and increasing Ag-NOR mean numbers. Though cells were properly fixed and stained exact enumeration of Ag-NORs by image analysis was hampered by considerable confluency of single silver deposits during this growth period. The spatially close relationship due to nucleolar organization in G1 with overlapping single Ag-NORs, probably affects their enumeration in cytological specimens. Interestingly, the increase in Ag-NOR number was always paralleled by an increase of the MI indicating striking changes in the organization of the Ag-NORs due to the cell cycle phase. This finding is in accordance with data from HeLa cells (Hubbel et al. 1980) where Ag-NORs show comparable distribution patterns in G2 phase close to cell division whereas in phytohaemagglutinin-stimulated lymphocytes the distribution of interphase Ag-NORs seems to be more dependant on the number of generations after stimulation (Field et al. 1984).

Although we do not have information on the proliferative activity of single cells it seems to be likely that the correlation of Ag-NOR number and MI is due to nucleolar disorganization before forthcomig mitosis. The characteristic pattern of a high number of dispersed and distributed Ag-NORs might be caused by the onset of chromatin condensation in late cell cycle phases. Possibly, the very small, silver stained granules scattered throughout the whole nucleus represent argyrophilic proteins which have, due to chromatin condensation, already lost their contact with the NOR regions. This hypothesis is in accordance with the findings that in some interphase nuclei we observed paired arranged Ag-NORs comparable to metaphase Ag-NORs and that the amount of silver stainable proteins associated to the NOR region on metaphase chromosomes is strikingly less than that in interphase nuclei. Quantification of Ag-NOR bearing chromosomes without pretreatment of the cells by colchicine renders analysis more difficult but allows a realistic evaluation of this cell cycle phase. Although only 20 mitotic figures could be estimated for each day a clear correlation with proliferation and interphase Ag-NOR quantity was obtained.

During the starvation period (SPF < 10%), Ag-NORs were seen as clustered black dots within a few nucleoli. The significant difference from the high proliferation period was the size of the Ag-NOR aggregates. The Ag-NOR number as a possible determinant of nucleolar organization showed only a slight decrease because probably most of the cells stay in early G1. The increase of MI after culture medium exchange reflects the population doubling time of 29.6 h determined for HOK-1 cancer cells whereas the earlier decrease of the MI after the second medium addition when compared with the first medium addition is probably due to enhanced medium consumption.

In summary our in vitro model gives new detailed data on the correlations of the number and area of interphase Ag-NORs with cell growth in a human cancer cell line. Changes in the number of Ag-NOR bearing chromosomes are also dependent on cellular proliferation.

## References

Carbajo S, Orfao A, Vicente-Villardon JL, Carbajo-Perez E (1993) Expression of silver stained nucleolar organizer regions is coupled to cell cycle in rat thymic cells. Cytometry 14:46–52

Crocker J, Macartney JC, Smith PJ (1988) Correlation between DNA flow cytometric and nucleolar organizer region data in non-Hodgkin's lymphomas. J Pathol 154:151–156

Derenzini M, Trere D (1991) Importance of interphase nucleolar organizer regions in tumor pathology. Virchows Arch [B] 61:1–8

Derenzini M, Pession A, Farabegoli F, Trere D, Badiali M, Dehan P (1989) Relationship between interphasic nucleolar organizer regions and growth rate in two neuroblastoma cell lines. Am J Pathol 134:925–932

Derenzini M, Pession A, Trere D (1990) Quantity of nucleolar silver-stained proteins is related to proliferative activity in cancer cells. Lab Invest 63:137–140

Dervan PA, Gilmartin LG, Loftus BM, Carney DN (1989) Breast carcinoma kinetics. Argyrophilic nucleolar organizer region counts correlate with Ki67 scores. Am J Clin Pathol 92:401–407

Egan MJ, Crocker J (1992) Nucleolar organiser regions in pathology. Br J Cancer 65:1–7

Field DH, Fitzgerald PH, Sin FYT (1984) Nucleolar silver-staining patterns related to cell cycle phase and cell generation of PHA-stimulated human lymphocytes. Cytobios 41:23–33

Goodpasture C, Bloom SE (1975) Visualization of nucleolar organizer regions in mammalian chromosomes using silver staining. Chromosoma 53:37–50

Goessens G, Lepoint A (1974) The fine structure of the nucleolus during interphase and mitosis in Ehrlich tumour cells cultivated in vitro. Exp Cell Res 87:63–72

Goessens G (1984) Nucleolar structure. Int Rev Cytol 87:107–158

Hernandez-Verdun D (1983) The nucleolar organizer regions. Biol Cell 49:191–202

Hernandez-Verdun D (1986) Structural organization of the nucleolus in mammalian cells. Methods Achiev Exp Pathol 12:26–62

Herrera AM, Olson MOJ (1986) Association of protein C23 with rapidly labeled nucleolar RNA. Biochemisty 25:6258–6264

Hittmair A, Öfner D (1991) Silver stained nucleolar organizer regions in cancer cells (letter). Lab Invest 64:718–719

Howell WM (1982) In: Busch H, Rothblum L (eds) The cell nucleus. Academic Press, New York, PP 89–142

Hubbel HR, Lau YF, Brown RL, Hsu TC (1980) Cell cycle analysis and drug inhibition studies of silver staining in synchronous HeLa cells. Exp Cell Res 129:139–147

Lischwe MA, Smetana K, Olson MOJ, Busch H (1979) Proteins C23 and B23 are the major nucleolar silver staining proteins. Life Sci 25:701–708

Offner FA, Ott G, Povey S, Knuechel R, Preisler V, Fuezesi L, et al (1991) Characterization of the new bladder cancer cell line HOK-1: Expression of transitional, squamous and glandular differentiation patterns. Int J Cancer 49:122–128

Öfner D, Tötsch M, Sandbichler P, Hallbrucker CHR, Margreiter R, Mikuz G, Schmid KW (1990) Silver stained nucleolar organizer regions (AgNORs) as a predictor of prognosis in colonic cancer. J Pathol 162:43–49

Öfner D, Hittmair A, Marth C, Öfner C, Tötsch M, Daxenbichler G, Mikuz G, Margreiter R, Schmid KW (1992) Relationship between quantity of silver stained nucleolar organizer regions associated proteins (Ag-NORs) and population doubling time in ten breast cancer cell lines. Pathol Res Pract 188:742–746

Öfner D, Hittmair A, Marth C, Tötsch M, Daxenbichler G, Margreiter R, Böcker W, Schmid KW (1993) Relationship between quantity of silver stained nucleolar organizer region associated proteins (Ag-NORs) and growth rate suppression of breast cancer cell lines after interferon-gamma and 4-hydroxy-tamoxifen treatment. Virchows Arch [B] 63:359–363

Ploton D, Menager M, Jeannesson P, Himber G, Pigeon F, Adnet JJ (1986) Improvement in the staining and in the visualization of the argyrophilic proteins of the nucleolar organizer regions at the optical level. Histochem J 18:5–14

Rüschoff J, Plate KH, Contractor H, Kern S, Zimmermann R, Thomas C (1990) Evaluation of nucleolus organizer regions (NORs) by automatic image analysis: A contribution to standardization. J Pathol 161:113–118

Shibuya M, Ito S, Miwa T, Davis RL, Wilson CB, Hoshino T (1993) Proliferative potential of brain tumors. Cancer 71:199–206

Trere D, Pession A, Derenzini M (1989) The silver-stained proteins of interphasic nucleolar organizer regions as a parameter of cell duplication rate. Exp Cell Res 184:131–137

Wachtler F, Hopman AHN, Wiegant J, Schwarzacher HG (1986) On the position of the nucleolus organizer regions (NORs) in interphase nuclei. Studies with a new, non-autoradiographic in situ hybridization method. Exp Cell Res 167:227