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CENTROSOME ABERRATIONS AND TUMOR DEVELOPMENT

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STOCKHOLM 2003

The centrosome study started in this building, Z-01 huset.

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To My Dearest Family

CONTENTS

Abstract

The transformation of a normal cell to a tumor cell is a result of a failure somewhere in a complicated system of coordination, checkpoints and control mechanisms of inhibitors and activators working as triggers and transactivators of genes of importance for cell cycle progression. In the present thesis, the focus is on an important function that controls the segregation of the chromosomes during cell division to the next generation of two daughter cells. The conductor of this nice orchestration is the centrosome, which picks up the exact number chromosomes assembled in metaphase plate and brings them to each of the two creating nuclei. Since transformation to tumor cells includes rearrangements of the genetic material, the centrosome plays a crucial role and is therefore of interest to investigate.

We have used both in vivo and in vitro systems to elucidate the role of centrosomes in tumor development. Studying the progression of cancer in the human cervical epithelia, we analyzed condyloma, intraepithelial dysplasia and neoplasia (CIN I-III) and cervical cancer, and found an increased number of aberrant centrosomes first detected in the mild CIN I lesions. Thereafter in moderate CIN II and severe CIN III to invasive cancer a steadily increasing number of centrosomes above the normal number of 2 were registered in immunohistochemical staining with γ-tubulin. The results stress attention to the question whether the disturbances in centrosome function precedes malignification or not. We also found a transient appearance of tetraploid cells in the intraepithelial lesions. Tetraploidization results in an increased number of centrosomes for the cell to deal with when the tetraploid cell enters mitosis enhancing the risk for missegregation of chromosomes. All invasive cervical cancers investigated were aneuploid. The literature reveals a low frequency of p53 mutation in these cancers. But a high frequency of high-risk HPV revealed p53 function disturbance.

In adrenocortical tumors, both adenomas and carcinomas were analyzed with regard to aberrant centrosome numbers. Among fibroblasts, either one or two signals in γ-tubulin immunohistochemical staining was found in 99.7%. However in the benign adenomas, a mean of 4% of the cells showed more than two signals indicating amplification. In adrenocortical carcinomas, a mean of 10% of the cells had more than two signals. Again the question of centrosome aberrations to occur early in carcinogenesis is raised. The literature reveals a low frequency of p53 mutation in these cancer but most tumors are aneuploid. The in vitro system used was human breast cancer cell lines. In four selected lines, one was diploid-tetraploid (Hs578T), one tetraploid (MCF7) and one aneuploid (MDA-MB-231). Hs578T and MDA-MB-231 were p53 mutated. We also added a non-transformed fibroblast line (HDF). The two p53 mutated lines revealed the highest frequency of aberrant centrosomes. By

perturbing the mitosis with griseofulvin, we pushed in various frequencies all four lines to a higher ploidy level and found the p53 mutated lines to be more vulnerable according to centrosome function. By adding 13-*cis*-retinoic acid (13cRA) to MDA-MB-231, a reduction in aberrant centrosomes was obtained. However, no effect on cyclin A, D and E could be seen and thereby no restoration of G1 checkpoint function occurred related to the cyclins investigated. We conclude 13cRA to exert its effect upon structures as cytoskeleton and mitotic spindle formation and also push cells into apoptosis. Centrosomes have come into focus in the cancer literature the last decade. The present thesis shows that they must be studied further and maybe also be used in cancer diagnosis.

Keywords: centrosome, cervical carcinoma, adrenocortical tumor, tetraploidization, p53, griseofulvin, 13-*cis*-retinoic acid.

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ABBREVIATIONS

ACC	adrenocortical carcinoma
BRCA	breast cancer supppressor gene
CDK	cyclin-dependent kinase
CGH	comparative genomic hybridization
CIN	cervical intraepithelial neoplasia
CIS	carcinoma in situ
DAPI	4',6-diamidino-2-phenylindole
DMSO	dimethyl sulfoxide
FITC	fluorescein isothiocyanate
GF	griseofulvin
HDF	human diploid fibroblast
HE	hematoxylin-eosin
HPV	human papillomavirus
IDT	intra- and interclonal intermitotic time
LOH	loss of heterozygosity
MTOC	microtubule-organizing center
NGS	normal goat serum
NPM	nucleophosmin
PCM	pericentriolar matrix
PCR	polymerase chain reaction
RB	retinoblastoma tumor suppressor protein
RA	retinoic acid
13cRA	13-*cis*-retinoic Acid

1 INTRODUCTION

1.1 CENTROSOME HISTORY

Figure 1.
Theodor Heinrich Boveri
(1862 – 1915)
Courtesy American
Philosophical Society

In 1875, a special organelle was first observed by Walther Flemming (Flemming, 1875) and subsequently Theodor Boveri found it and named "centrosome", a pair of centrioles surrounded by special material, which connects the chromosomes during cell division. (Boveri, 1887). In 1914, he wrote, "malignant tumors might be the result of a certain abnormal condition of the chromosomes, which may arise from multipolar mitosis. Abnormal (multipolar) mitoses may bring about an immense number of different chromosome combinations, such combinations as would make a cell into a tumor cell *must* occasionally occur. The more the abnormal (multipolar) divisions that take place in a tissue, the greater of course is the probability that the necessary combination (of chromosomes to become malignant tumors) shall appear." proposing that abnormal centrosome contribute to malignant progression (Boveri, 1914) (Figure 1, 2).

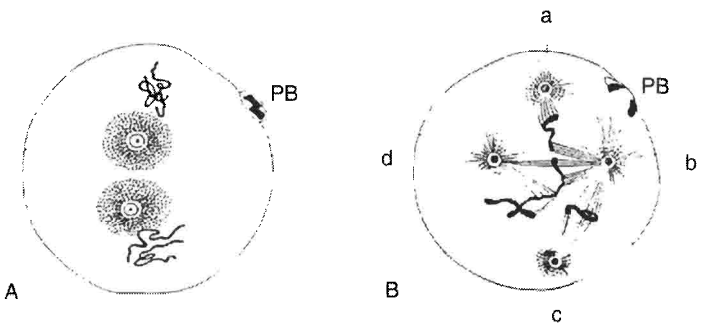


Figure 2. The centrosomes and chromosomes in an egg of *Ascaris megalocephala bivalens*.
PB: polar body. (Boveri, 1888).
A : The duplicated centrosomes and chromosomes.
B : Chromosomes arranged on tetrapolar spindles. a and c will receive only one chromosome, b three or four chromosomes and d two chromosomes.

However, his proposition about the role of centrosome has long been forgotten until the late of 1990s. Since centrosome detection became possible by improved immunohistochemical techniques, the centrosome has quickly become of central importance for cancer research and major breakthroughs have been made within the last decade (Doxsey *et al.*, 1994, Fukasawa *et al.*, 1996).

1.2 CENTROSOME STRUCTURE

The centrosome is a very tiny organelle in animal cells, about 1 μm in diameter, and consists of a pair of centrioles surrounded by a pericentriolar matrix (Figure 3). It is a major microtubule-organizing center, which nucleates and orients microtubules in interphase and thereby influences tissue architecture, and directs bipolar spindles during cell division. The equivalent in yeast is called "spindle body".

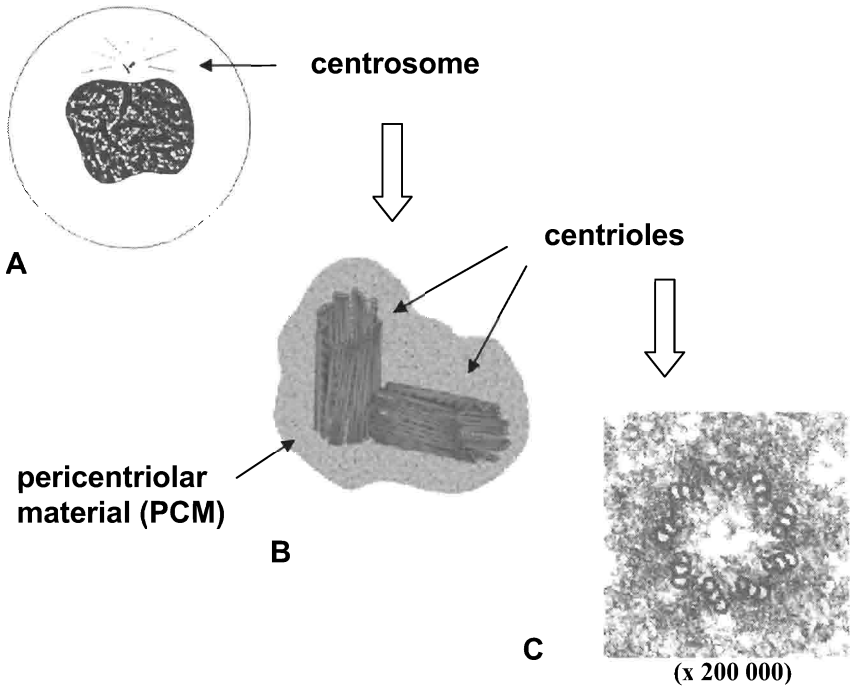


Figure 3. Schematic diagram of a centrosome in a normal interphase cell.
A centrosome consists of a pair of cylindrical centrioles surrounded by pericentriolar material (PCM) and is located mostly at the perinuclear region during interphase (A, B). Each centriole is made up of nine triplets microtubules (C). Figure B is adapted with permission from Fukasawa, 2002.

1.3 CENTROSOMAL PROTEINS

During the last several years, numerous proteins which are considered to be associated with centrosomes have been reported. These include both integral centrosomal/centriolar proteins and proteins associated with the centrosome, which are concentrated at the centrosome but disappear upon microtubule depolymerization (Andersen, 1999). Some of the integral centrosomal/centriolar proteins are γ-tubulin, pericentrin, centrin and antibodies against these proteins have been developed.

a) γ-tubulin is an ubiquitous and highly conserved protein (48-kDa) and forms a γ-tubulin ring complex together with other proteins (Zheng et al., 1995). It is a member of the tubulin family that also contains α- and β-tubulin. It nucleates

microtubules and links between microtubules and the centrosome (Oakley et al., 1989, 1992). Monoclonal and polyclonal antibodies against this protein are available and are widely chosen in centrosome-related studies.

b) Pericentrin is a 220 kDa protein and is localized to the pericentriolar material. Anti-pericentrin antibodies do not block gamma-tubulin assembly or microtubule nucleation from mature centrosomes (Doxsey et al., 1994). Recently, it has been suggested that pericentrin forms a complex with γ-tubulin independently of the γ-tubulin ring complex (Dictenberg et al., 1998).

c) Centrin is a 20 kDa protein and in interphase Hela cells, centrin is reported to be located at the pericentriolar material and redistributes to spindle poles during mitosis (Paoletti et al., 1996). It is phosphorylated from G2 phase to prophase and metaphase of the M phase, but not at later M phase and at interphase, suggesting that centrin phosphorylation triggers the separation of centrosomes at prophase (Lutz et al., 2001). However, in human cells, most of the centrin is not associated with the centrosome (Paoletti et al., 1996)

d) Nucleophosmin (NPM), which was originally found as a nucleolar phosphoprotein in the nucleolus (Feuerstein *et al.*, 1987, Schmidt-Zachmann *et al.*, 1987), is essentially associated with the centrosome and triggers initiation of centrosome duplication when phosphorylated by CyclinE/CDK2. (Okuda *et al.*, 2000).

1.4 CENTROSOME DUPLICATION

A normal animal cell in interphase has only one centrosome, which duplicates prior to the next cell division. To maintain constant chromosome numbers in proliferating cells, centrosome duplication and DNA replication must occur only once during each cell cycle in a coordinated way, resulting in equal chromosome segregation into two new daughter cells.

Around the G1-S transition, the centrosome duplication process initiates. First, the two centrioles within a centrosome separate, then a procentriole assembles next to the mature centriole. During S and G2 phase the daughter centriole elongates up to the length equal to the mother centriole. At the beginning of mitosis, duplicated centrosomes separate towards two opposite spindle poles of the cell and all microtubules nucleated from the centrosomes form a bipolar mitotic spindle (prophase). These microtubules bind to chromosomes at kinetochores that bind to specific DNA sequences at the chromosome centromeres and position them at the spindle center (prometa- and metaphase). As mitosis proceeds, chromosomes are pulled towards the spindle poles by kinetochore microtubules, allowing equal segregation of chromosomes into two daughter cells (ana-, telophase). The cytoplasm divides by cleavage, and ultimately centrosomes come to reside within the newly-formed cell and reform interphase arrays of microtubules (cytokinesis). Thus cellular bipolarity and the equal partition of chromosomes into two daughter cells are all dependent on normal centrosome function (Figure 4A).

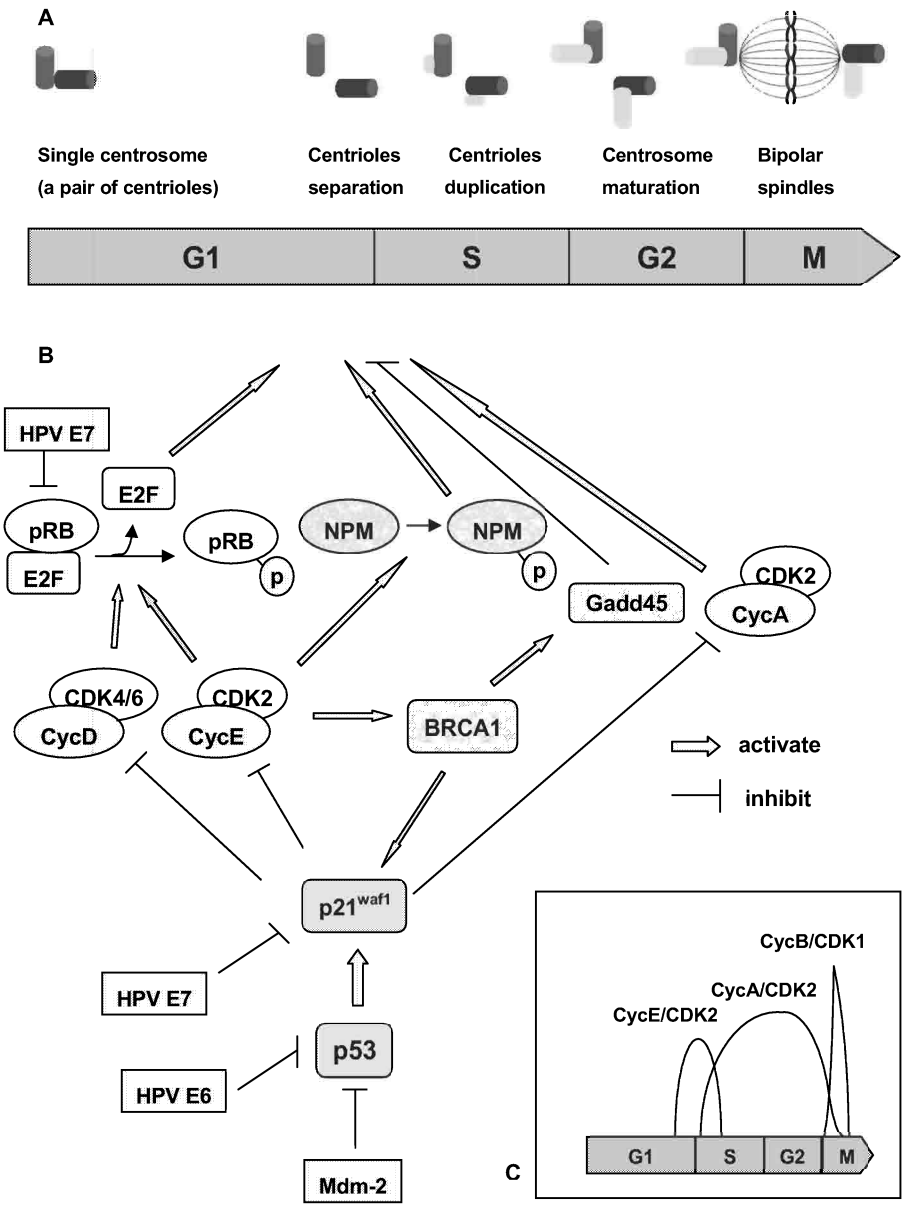


Figure 4. The centrosome cycle and its regulation by p53.
A: Centrosome duplication cycle. A pair of mother centrioles are shown in green and brown, procentrioles in light green and light brown, PCM in light gray.
B: Regulatory events on centrosome duplication cycle at G1/S transition.
C: Cyclin-CDK activity in the cell cycle.

1.5 CENTROSOME REGULATION AT G1/S TRANSITION

Many steps in cell cycle processes are controlled by the expression of kinases and phosphatases, and the binding to their substrates. The interaction and activity of specific cyclins to cyclin-dependent kinases (CDKs) play essential roles in progression through the cell cycle (Figure 4B, C). In general, hypophosphorylated pRB in early G1 phase represses transcription factor E2F and when phosphorylated, pRB releases E2F, which triggers the entry to S-phase. In case that $p21^{Waf1}$ is transactivated by induction of p53, it binds to and inhibits G1/S cyclins and also inhibits phosphorylation of pRB, and the cell is arrested at G1.

However, the regulation of the centrosome is poorly understood. Some studies have recently begun to reveal that the centrosome duplication is coordinated with cyclin/CDKs activity. Centrosome duplication in somatic cells is strictly dependent on the phosphorylation of the retinoblastoma protein (pRB) and requires both E2F transcription factors and cyclinA/CDK2 activity (Meraldi *et al.*, 1999) and loss of E2F directly cause centrosome amplification (Saavedra *et al.*, 2003). CyclinE/CDK2 activity promotes the G1/S transition and initiates centrosome duplication (Hinchcliffe *et al.*, 1999, Lacey *et al.*, 1999, Matsumoto *et al.*, 1999). Moreover, nucleophosmin (NPM)/B23 has been identified as a substrate of CyclinE/CDK2 in centrosome duplication (Okuda *et al.*, 2000). NPM/B23, which is associated with the centrosome, is released from the centrosome at late G1 phase upon phosphorylation by CyclinE/CDK2, leading to the separation of a pair of centrioles. Many in vitro studies have demonstrated that initiation of DNA replication is dependent on both the CyclinE/CDK2 complex and CyclinA/CDK2 activity. Cyclin E expression has a narrow maximal peak at G1/S transition (Jackson *et al.*, 1995, Krude *et al.*, 1997, Koff *et al.*, 1992). It is reasonable that the activation of CyclinE/CDK2 leads to both DNA replication and centrosome duplication.

Consequently, p53 as a gene transactivator of the CDK inhibitor, Waf1, has also been shown to be involved in the regulation of the centrosome duplication cycle. Loss or mutational inactivation of p53 disturbs the centrosome cycle, resulting in hyperamplification of centrosomes (Fukasawa *et al.*, 1996). Another study has revealed that in the absence of p53, centrosome duplication is initiated in early G1 instead of the late G1/early S phase as observed in p53 intact cells (Tarapore *et al.*, 2001).

Breast cancer suppressor gene (BRCA)1 is involved in various biological processes during tumorigenesis, including the inactivation of p53 and activation of ErbB2, c-Myc, p27. It has been demonstrated that BRCA1 is located in the nuclei of mitotic cells and binds to γ-tubulin (Hsu *et al.*, 1998). In centrosome duplication, it interacts with p53, Rb, cyclinA/CDK2, cyclinE/CDK2 (Deng *et al.*, 2000). Cdk2 phosphorylates p53 and inhibits centrosome regulatory function of p53. BRCA1 also transactivates p21 and Gadd45 (Mantel *et al.*, 1999, Harkin *et al.*, 1999).

It has been demonstarated that Human papillomaviruses (HPV) E6 and E7 oncoproteins disturb the centrosome duplication cycle. The HPV E7 oncoprotein rapidly drives abnormal centrosome duplication in phenotypically normal cells by inhibiting phosphorylation of pRB and inactivating $p21^{Waf1}$, whereas the E6

oncoprotein accumulates supernumerary centrosomes in multinucleated cells by degrading p53.

The *mdm-2* is an oncogene which can inhibit p53-mediated transactivation (Finlay, 1993) and promotes the rapid degradation of p53 through the ubiquitin-dependent proteosome pathway (Kubbutat *et al.*, 1997, Haupt *et al.*, 1997). Recently, mdm-2 overexpression was reported to induce centrosome amplification and chromosome instability has been reported in cultured cells (Carroll *et al.*, 1999). Thus, Mdm-2 can inactivate p53 and induce centrosome amplification.

1.6 FROM CENTOSOME DEFECTS TO ANEUPLOIDY

Centrosome defects have been reported in a wide range of human malignancies (Pihan *et al.*, 1998, Lingle *et al.*, 1998, Weber *et al.*, 1998, Sato, *et al.*, 1999, Kuo, *et al.*, 2000, Pihan *et al.*, 2001, Neben *et al.*, 2003, Kawamura *et al.*, 2003, Mayer *et al.*, 2003) and abnormalities of the centrosomes are classified into follwing groups (Krämer *et al.*, 2002),

(1) increase in centrosome number and volume
(2) accumulation of excess pericentriolar material
(3) supernumerary centrioles
(4) inappropriate phosphorylation of centrosome proteins
(5) functional abnormalities

Although disturbance in a lot of checkpoint functions including Mdm-2, p53 and cyclins are related to centrosome defects, the mechanisms, by which they arise in malignant tumors, are not clearly understood. Do the centrosome defects initiate malignant progression, or result from malignant development? Though this is still controversial, it seems reasonable to hypothesize that centrosome defects lead to aneuploidy, which is also a common feature in malignant tumors. Aneuploidy represents cells with increased number of chromosomes as compared to diploid cells. If cells get abnormal number of centrosomes, it could lead to the assembly of aberrant spindles and the improper segregation of chromosomes.

For example, increased numbers of centrosomes could result in multipolar spindles, leading to missegregation of the replicated chromosomes into more than two daughter cells. Moreover, multidirectional forces exerted on a single chromosome in a multipolar spindle could create chromosome breaks. If centrosomes fail to be duplicated or separated, the cell cannot generate bipolar mitotis spindles and the results will be monopolar spindles that would be unable to segregate chromosomes. Thus centrosome defects would lead to abnormal spindle formation and missegregation of chromosomes into daughter cells. Though most cells with aberrant chromosomes would be eliminated by apoptosis, cells which received enough chromosomes to survive could overcome such a problematic situation and become aneuploid (Figure 5). In tumor progression, endomitosis is a frequent event leading to tetraploidization. It is the result of the DNA division cycle without an intermediate cell division. Consequently, it leads to two

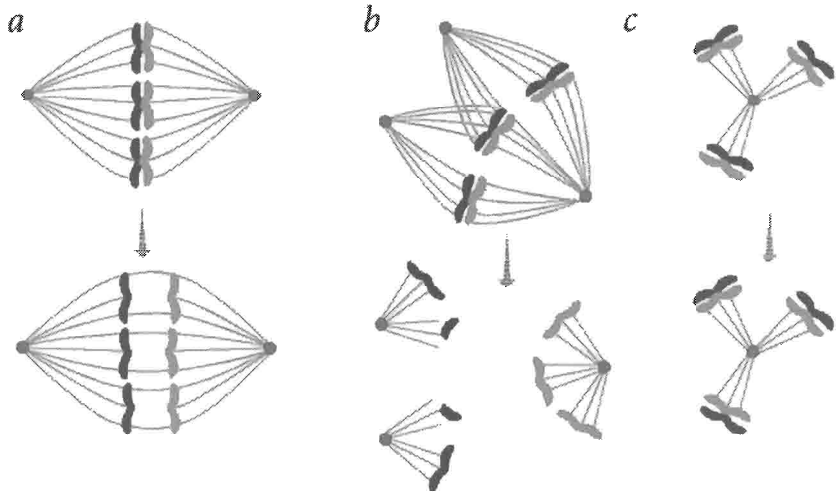


Figure 5. Mitotic spindles in normal and centrosome-defective cells. Adapted with permission from Doxsey, 1998.
Centrosomes (red) are located at the poles of the metaphase spindle. The replicated chromosomes (blue) are aligned at the spindle centre. Microtubule fibres (green) arise from centrosomes and contact chromosomes at kinetochores.
(*a*) Upon exit from mitosis, the replicated chromosomes are partitioned equally between the two resulting daughter cells and ultimately come to reside within the newly-formed nuclei of the nascent cells.
(*b*) Centrosome anomalies can lead to spindle disorganization and aneuploidy. Excessive duplication could lead to multipolar spindles and segregation of two sets of chromosomes into more than two daughter cells. The forces generated from pulling a single chromosome toward more than one spindle pole could also create chromosome breaks.
(*c*) Failure to duplicate or separate centrosomes could lead to monopolar spindles that are unable to segregate chromosomes.

centrosome cycles ending up with four centrosomes. When the tetraploid cells divide, there is a risk of missegregation of chromosomes.

1.7 CERVICAL CARCINOMA

Cervical carcinoma is the second most common cancer among women in the world. About 80 % of primary cervical carcinomas are squamous cell carcinoma and 20% adenocarcinoma.
Many studies have indicated beyond any doubt a strong and specific association relating HPV infection to cervical cancer (Bosch *et al.*, 2002) and more than 90% of squamous cell carcinoma contain HPV DNA (Waggoner, 2003). HPV genital types are divided into two groups; a low-risk group (e.g. HPV-6) which causes benign tumors such as condyloma acuminate and a high-risk group (e.g. HPV-16, 18 and 31) which has a big potentiality to undergo malignant development (Duensing *et al.*, 2002a). The

age-adjusted HPV-16 seroprevalence among pregnant women was 16 % in Stockholm and had increased to 21% by 1989 (af Geijersstam *et al.*, 1998).

Cervical carcinomas are graded from Stage 0 to IV based on the FIGO staging system. Stage 0 is the precancerous stage and further divided into intraepithelia neoplasia (CIN) grade I – III according to the severity of dysplasia. CIN I, II and III are corresponding to mild, moderate, severe dysplasia respectively. Carcinoma in situ (CIS) is included in CIN III. Natural history and follow up studies have clearly shown that HPV infections precede CIN lesions and cervical cancer by a substantial number of years and confirmed that sexual transmission is the predominant mode of HPV acquisition (Bosch *et al.*, 2002). However, most infected women do not develop cervical carcinoma.

As in many other human cancers, chromosomal changes are observed in cervical cancer. They include numerical chromosome imbalances such as gains and losses of whole chromosomes as well as structural chromosomal changes e.g, gains of chromosome arm 3q (Heselmeyer *et al.*, 1996).

1.8 ADRENOCORTICAL CARCINOMA

Adrenocortical carcinoma (ACC) is a very rare type of human malignant tumor, but once diagnosed, more than two-thirds of the patients are already at an advanced stage with metastasis and the prognosis is very poor. On the other hand, benign adrenocortical tumors are more frequent with an incidence of 1% (Copeland, 1983). Since most of the lesions csuse no symptoms, they are often incidentally detected. To distinguish malignant tumors from benign tumors, the size of tumors and histology are currently considered. However, the difficulty to obtain tumor tissue has detained the cytological analysis of the tumors.

Recent studies have reported molecular cytogenetic aspects of ACC. By comparative genomic hybridization (CGH) and loss of heterozygosity (LOH), frequent gross chromosomal aberrations in the carcinomas have been detected. Moreover, an increased number of alterations related to the tumor size was observed in carcinomas, whereas benign tumors were found to be genetically more stable. The detected chromosomal gains and losses involved most of the chromosomes and did not show any significantly predominant localizations. (Kjellman *et al.*, 1996). To the contrary, other studies have revealed numerous high-level amplifications of specific chromosome regions (Figueiredo *et al.*, 1999, Dohna *et al.*, 2000).

1.9 GRISEOFULVIN

Griseofulvin (GF), isolated as antibiotic (Oxford *et al.*, 1939) has been used as an oral antifungal agent to treat dermatophytoses in human since 1958 (Grove. 1963).
However, it has been reported as a spindle poison, which has become a major interest among researchers (Paget *et al.*, 1958, Gull *et al.*, 1974, Önfelt *et al.*, 1986). GF binds to microtubule associated proteins and affects their incorporation into microtubules, thereby influencing the stability of microtubules. Destabilized microtubules result in

structural changes of tubulin, leading to chromosome missegregation (Roobol *et al.*, 1977).
GF is produced by different species of Penicillium and in nature it may be producted by food contaminating fungi (De Carli *et al.*, 1988). Chemically, it is (2S)-trans-7-Chloro-2′,4,6-trimethoxy-6′-methylspiro(benzofuran-2[3H],1′-[2]cyclohexene)-3,4′-dione (Figure 6) and registered in Chemical Abstracts under serial (CAS) Number 126-07-8.

Though multipolar spindle formation in vitro after treatment with GF has been reported (Kochendorfer *et al.*, 1996), how GF effects on centrosome amplification is poorly understood.

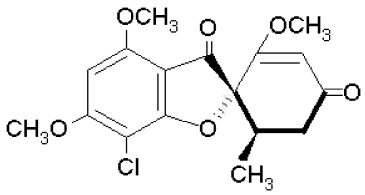


Figure 6. Structure of Griseofulvin
$C_{17}H_{17}ClO_6$ MW 352.77

1.10 RETINOIC ACID

Retinoic acid (RA), formed by oxidation from retinal, is one of the physiologically active forms of retinoid. It has been shown that cells treated with RA in vitro induce reorganization of the nucleus and cytoplasm, development of chemoattractant-directed migration and finally apoptosis by which RA inhibits cancer cell growth (Hsu *et al.*, 1999). RA is therefore considered as preventive and therapeutic agent and currently used to treat epithelial cancer and promyelocytic leukemia.

13-*cis*-retinoic acid (13cRA) is one of retinoic acid isomers and registered in CAS-number 4759-48-2 (Figure 7). According to a recent study, 13cRA is one of the most effective retinoids for the prevention of non-melanoma skin cancers in high-risk patients in clinical trials (Niles. 2002).

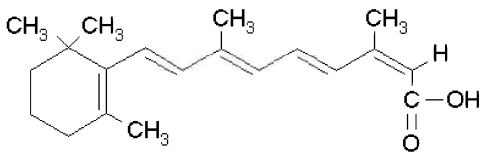


Figure 7. Structure of 13-*cis*-Retinoic acid
$C_{20}H_{28}O_2$ MW 300.4

2 AIM OF THE STUDY

The principle aim of this study is:

- to assess the relationship between centrosome status, malignant transformation grade of malignancy and genomic instability in human cancers
- to compare centrosome status, mitotic feature and DNA ploidy
- to test the effect of griseofulvin on centrosome status and genomic stability
- to investigate the stabilizing effect of retinoic acid on centrosome status
- to elucidate whether RA does interfere with cyclin up- and down- regulation in the MDA-231 cell line

3 MATERIALS AND METHODS

3.1 MATERIALS AND SAMPLE PREPARATIONS

3.1.1 Clinical materials

Cervical tumors and adrenocortical tumors were chosen in this study.
All clinical materials were obtained either after surgical resection or from archival frozen tissue collections at Karolinska Hospital in Stockholm and respective clinical data were also collected. The fresh tissue samples were stored at – 70°C until use. A representative section from each tissue sample was cut, fixed in 4% buffered formaldehyde, paraffin-embedded and stained to confirm the histopathological type and differentiation.
Informed consent was obtained from each patient and the studies were approved by the ethical committee of the Karolinska Hospital (KI forskningsetikkommitté Nord vid Karolinska sjukhuset). Ethical permission numbers (D-nr) for Paper I and II are 00-385 and 01-136 respectively.

Imprints

The imprints were prepared from either fresh tissues or archival frozen tissues. After surgical resection the fresh specimens were kept on dry ice until imprints were made.
Imprints for centrosome staining were immediately fixed in cold methanol (-20°C) and kept until further analysis. For DNA-ploidy measurement, imprints were air-dried.

Extraction of DNA for Polymerase Chain Reaction (PCR)

For extraction of DNA from the tissue samples, the samples were cut into small pieces, and then 500µL of lysisbuffer (10mM Tris HCL pH7.6, 1mM EDTA pH8.0, 1% sodium dodecyl sulfate, 50mM NaCl) and 400 to 600µg proteinase K/mL buffer were added to each sample in an Eppendorf tube and were incubated overnight at 50°C. After that 500µL phenol:chloroform:isoamylalcohol (25:24:1) were added and shaken carefully on a shaker for 10 min before centrifugation at 13,000 rpm for 15 min in an Eppendorf centrifuge. The water phase was taken and the former steps were repeated once. Then chloroform:isoamylalcohol (24:1) were added, shaken carefully for 5 min and centrifugated at 13,000 rpm for 5 min. This procedure was repeated once. The water phase was moved to a new tube; two volumes (about 1mL) ice-cold 95% ethanol and 3M NaAc pH5.2 to a final concentration of 0.3M were added and the tubes were stored overnight at -20°C to precipitate the DNA. Finally they were centrifuged at 13,000 rpm for 30 min before ice-cold 70% ethanol was added up to 1mL and centrifuged again for 15 min at the same rate to remove the phenol. After the DNA pellet was dried under vacuum, the DNA was dissolved in 20 to 30µL sterile distilled water and stored at -20°C until PCR analyses was performed.

3.1.2 Cell lines

Four different cell lines were chosen to carry out this study.

Three human breast cancer cell lines, MDA-MB-231 (MDA-231, ATCC-HTB-26), Hs578T (ATCC-HTB-126) and MCF7 (ATCC-HTB-22) obtained from American Type Culture Collection, USA and one human diploid fibroblast (HDF) cell line from Coriell Institute, USA were used.

The hypertriploid, MDA-231 was derived from a 51 years old woman. The modal chromosome number was 64. It is estrogen- and progesterone receptor negative.

The diploid-tetraploid Hs578T cell line was derived from a human ductal breast carcinoma of a 74 years old woman. It was initially hyperdiploid with a polyploid rate of 38%. The tumor cells were estrogen receptor negative.

Both the MDA-231 and Hs578T cell lines were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum, 2mM L-glutamine, 1.0mM sodium pyruvate, 50μg/ml bensylpenicillin and streptomycin.

The hypotetraploid estrogen receptor positive cell line MCF7 was originally derived from a 69 years old woman with breast adenocarcinoma and was grown in Eagle's Minimum Essential Medium (MEM) with 10% fetal calf serum, 2mM L-glutamine, 1.0mM sodium pyruvate, 50μg/ml bensylpenicillin and streptomycin and 0.1mM non-essential amino acids.

The diploid nonsenescent lung fibroblast cell line from human embryos (HDF) was grown in a 1:1 mixture of MEM and Ham's F-12 nutrient mixture, containing 10% fetal calf serum, 2mM L-glutamine, 50μg/ml bensylpenicillin and streptomycin.

All cell lines were cultivated in 80cm^2 culture flasks at 37°C in a humidified atmosphere of 5%CO_2 and grown in monolayers. They were subcultured when having reached confluence, using 0.05% trypsin-0.53mM EDTA solution. For further experiments, cells were seeded on glass slips in the culture dishes at densities with about 4×10^4 cells/ml and cultured in stated conditions.

3.1.3 Chemicals

a) Griseofulvin

In paper III, we used GF to test the effect upon polyploidization and dispersion of DNA distribution and centrosome amplification in relation to p53 mutation and analyzed normal and amplified numbers of centrosomes in interphase and metaphase of untreated and GF-treated cells. GF was purchased from Sigma, USA (G4753).

GF-Treatment of cultured cells

GF was reconstituted with dimethyl sulfoxide (DMSO) before use. The concentration of DMSO within the culture medium did not exceed 1%.

Cells cultivated on glass slips for 24 hours were moved into fresh growth medium containing GF and cultivated up to 72 hours. The final concentration of GF in the medium was 1- 40 μg/ml.

Based on these experiments, a further experiment was designed in order to assess which stage of mitotic progression GF inhibits and if its effect is reversible. MCF7 was treated with 10μg/ml GF for 72 hours, rinsed twice with GF-free medium and re-incubated in fresh culture medium for another 72 hours. For negative control, drug-free medium containing corresponding dose of DMSO was used instead of medium containing GF.

b) Retinoic acid

In Paper IV, we used 13cRA to investigate the in vitro effect of RA on centrosome amplification in relation to p53 status. 13cRA was purchased from Sigma, USA (R3255).

RA-Treatment of cultured cells

13cRA was dissolved in ethanol at the concentration of 1mM and stored at -20°C until use. The working concentration of 13cRA in cell culture medium was 1μM. As a negative control, drug-free medium containing a corresponding dose of ethanol was used.
MDA-231 cells were cultivated in medium containing 13cRA for up to 12 weeks.
At certain time points, cells were fixed for evaluation. For centrosome staining and Feulgen staining, cells were seeded onto glass slips in corresponding culture medium, incubated for another 24 hours and fixed in cold methanol at -20°C until use, or in 4% buffered formaldehyde at room temperature for 3 hours respectively. For flow cytometry, cells were collected by trypsinization, fixed in 95% ethanol and stored at 4°C until use.

3.2 METHODS

3.2.1 Hematoxylin-Eosin staining

To confirm the diagnosis of clinical material, sections of formalin-fixed, paraffin-embedded specimens were stained by Hematoxylin-Eosin (HE) staining and judged blindly by two independent pathologists.

3.2.2 Centrosome analysis

To examine centosome status by immunohistochemical detection, we have chosen monoclonal anti-γ-tubulin antibody (Sigma, T6557). The representativity of the staining using anti-γ-tubulin antibody has been confirmed by staining with anti-centrin antibody in some samples.

Samples fixed in cold methanol were post fixed in cold acetone at -20°C for 6 min and then incubated with blocking solution (1% normal goat serum, 0.1% Tween20 in PBS)

for 30 min at 37°C. Subsequently, monoclonal anti-γ-tubulin antibody diluted to 1:500 in PBS containing 2% normal goat serum, was applied to the imprint samples for 2 hours (1:1000, and for 1 hour for cultured cells). Antibody-antigen complexes were detected by FITC-conjugated anti-mouse IgG (Sigma, F5262), which was applied in 1:128 in PBS containing 2% normal goat serum for 45 min in the dark. The nuclei were counterstained with 5μM 4', 6-diamino-2-phenylindole (DAPI) and finally the samples were mounted with Vectashield mounting medium (Vector Laboratories, H-1000). All procedures were done in a moist chamber at room temperature unless otherwise stated and samples were extensively rinsed with PBS after each incubation.

Labelled cells were evaluated using a Zeiss Axioskop fluorescence microscope equipped with FITC and DAPI selective filter sets and a 50W mercury arc lamp using an oil immersion Zeiss objective lens (100×). For each experiment, all of the samples including positive and negative controls were coded, mixed, and scored blindly. For cultured cells, three areas with approximately 500 cells/area and 100 cells on each imprint sample were examined, and the numbers of centrosomes per cell were counted.

3.2.3 p53 and Waf1 analysis

To confirm the p53 protein status in the cell lines used, we performed immunohistochemical staining with the monoclonal antibody, DO-

7 to detect mutant p53 protein and another monoclonal antibody, AB-1 against *WAF1* gene product reflecting the wild type p53 transactivated gene product initiating growth arrest and involved in centrosome regulation.

Formalin-fixed, paraffin-embedded tissue sections were cut at a thickness of 4 μm. The sections were deparaffinized, rehydrated and antigen was retrieved by heating in 0.01M sodium citrate buffer for 5 minutes, 2 times, at 750W in a microwave oven. Prior to the primary antibody incubation, endogenous peroxidase activity was blocked by incubating in 0.5% hydrogen peroxide solution for 20 minutes, and non-specific binding was also blocked by incubating 1% bovine serum albumin in Tris-buffered saline (TBS) for 45 minutes. To detect p53 expression, monoclonal antibody, DO-7 (Novocastra Laboratories Ltd, NCL-p53-DO7) diluted to 1:100 in 1% BSA was applied to the slides at 4°C overnight and to detect Waf1, monoclonal antibody, Ab-1 (Oncogene Research Products, OP64) diluted 1:50 in 1% BSA was applied. The sections were washed thoroughly in TBS and avidin-biotin signal amplification technique was performed using biotinylated anti-mouse IgG (Vector BA-2000) diluted 1:200 in TBS for 30 minutes followed by avidin-biotin complex (Vector PK-6100). Diaminobenzidine was used as a chromogen. Finally, slides were counterstained with hematoxylin, dehydrated and mounted. To distinguish positively stained cells, which were immunoreactive, we exclusively counted cells with clearly brown-stained nuclei and the percentage of such positive cells in each sample was used for the further evaluation.

3.2.4 DNA content measurement and mitotic figure classification

DNA ploidy measurements by image cytometry were performed on Feulgen-stained samples.

Air-dried imprint samples and cells cultured on glass slips were fixed in 4% buffered formaldehyde overnight before staining, exposed in 5M HCl at 22°C for 1 hour and stained with Schiff's reagent for 2 hours in the dark at room temperature. Then the samples were washed in sulfide rinsing solution for 10 min, three times. Samples were rinsed in distilled H_2O between each step. Finally the samples were rinsed in running tap water for 5 min dehydrated in ascending ethanol, transferred into xylene and mounted using Entellan (Merck).

Stained samples were analyzed using a TV-based image analysis system. In each sample DNA contents on at least 200 cell nuclei were measured. As an internal standard at least 20 granulocyte or lymphocyte nuclei were assessed and their median value was considered as the diploid DNA value.

On Feulgen stained samples, mitotic figures were morphologically classified into three groups: prophase, metaphase and telophase.

3.2.5 Polymerase chain reaction (PCR) for HPV detection

For universal detection of HPV two sets of universal or consensus primer pairs from the highly conserved L1 region were used, one degenerated pair, My 11 and My 09, of Manos and coworkers (Manos *et al.*, 1989). Polymerase chain reaction (PCR) was performed on a total volume of 50µl containing 50mM KCl, 10mM Tris-HCl pH 8.3, 0.2mM of each dNTP, $MgCl_2$ optimized to 3.6mM, 1U of Taq polymerase (AmpliTaq;Perkin Elmer Cetus) and 20 pmoles of each primer including ~0.5µg of each sample. Forty cycles amplification were performed, consisting of one initial cycle of denaturation at 95°C for 5 minutes, primer annealing at 45°C for 30 seconds and primer extension at 72°C for 60 seconds; this was followed by 38 similar cycles, except that denaturation was for 30 seconds only. The last (40^{th}) cycle differed only with regard to the time for primer extension, which was 5 minutes.
Using the concensus primers, GP5+/GP6+, the amplification solution was changed to 25 pmoles of each primer and the concentration of magnesium chloride optimized to 3.5mM. Forty cycles of amplification were carried out, each cycle included a denaturation step at 94°C for 1 minute, an annealing step to 38°C for 1 minute and an extension step to 71°C for 2 minutes. The first cycle was preceded by a denaturation step at 94°C for 4 minutes and the last cycle was extended for 4 minutes at 71°C.
PCR, with type-specific primers, was performed for 35 – 40 cycles. The primer annealing was set at 55°C for these primers, otherwise the temperatue and the time were the same as for the consensus primers My11 and My09. The magnesium chloride concentration was optimized to 1.5mM compared to 3.6mM for the My primers. Type-specific HPV primers for HPV 6, 16, 18, 31, and 33 from E5-E6-E7 gene regions were used according a procedure described earlier (Skyldberg *et al.*, 1991). Primers for HPV6 and 16 were usually run together using only one reaction tube ("multiplex PCR"). The other HPV types were assayed one by one. As a control of DNA extension and amplification i.e. to exclude false negative reactions, a set of HLA DQ primers

(GH26/27) were used (Hagmar et al., 1995, de Roda Husman et al., 1995, Skyldberg et al., 1999, Ehrlich *et al.*, 1989).
For analysis of the PCR product, 15 µL of the amplified product was run on a 3% agarose gel and stained with ethidium bromide (0.5µg/mL).

3.2.6 Expression level of Cyclins A, D and E

To analyze cyclin A, D, and E expression pattern in G1 phase by flow cytometry, cells were stained with cyclin A mouse IgG_1 monoclonal antibody (Nova castra Laboratories, NCL-CYCLIN A), FITC-conjugated cyclin D3 mouse IgG_1 monoclonal antibody (Santa Cruz Biotechnology, sc-6283 FITC) and cyclin E rabbit IgG polyclonal antibody (Santa Cruz Biotechnology, sc-481). Cyclin A and Cyclin E detection were performed using FITC conjugated anti-mouse IgG (Sigma, F0257) and FITC conjugated anti-rabbit IgG (Sigma, F1262), respectively.

Cells, collected by trypsinization followed by centrifugation, were fixed in 95% ethanol and stored at 4°C until use. The following steps were all executed at room temperature and samples were extensively washed using 1 ml washing buffer (PBS containing 1% bovine serum albumin) by centrifugion at 2000 rpm, for 5 minutes between each step unless otherwise stated. Prior to staining cells were washed twice and incubated in washing buffer added 0.25% TritonX 100 on ice for 5minutes and washed. Thereafter they were incubated with primary antibodies at 4°C overnight. After washing twice, secondary antibodies were applied respectively for 1 hour in the dark. Samples were washed and incubated with 10µg/ml propidium iodide in PBS containing 0.1% RNase A for 20 minutes.

The stained samples were kept in the dark for 20 minutes before running them in the fluorescence-activitated cell sorter (FACS). The FACS was equipped with an argon laser; the FITC fluorescence was analyzed at 515nm and the PI at 488nm. 10 000 events were stored for each sample. The Cell Quest (Becton Dickinson) program was used for the two parameter dot plot histogram analysis.

3.2.7 TV-video time lapse filming

The cells were seeded at a relatively low density onto a 5 cm plastic Petri dish and placed in the incubator. The next day the dish was rapidly sealed with a rubber ring and then removed from the incubator. The sealed dish was placed in an inverted microscope (DIA-PHOT-TDM, Nikon) with an incubator constructed from a plastic case and temperature adjustment by an inhalation and exhalation tube. A video camera (Panasonic CCTV) was adapted to the microscope and linked to a time-lapse recorder (Panasonic NV-8050). The cells were observed on a monitor (Panasonic WV-5410) equipped with an internal timer. Cells growing in 13cRA supplemented medium were prepared for time-lapse filming after 10-14 days of treatment.
Individual cells were followed during subsequent cell divisions as long as possible and intermitoic times recorded. Problems to trace cells are related to moving of the cells out of the visual field or cell clustering.

4 RESULTS AND DISCUSSION

4.1 CENTROSOMES IN HUMAN MALIGNANCIES

The majority of cells both in clinical material and cultured cells showed only one or two centrosomes. On the other hand, amplified centrosomes showed often enlarged and irregular shape. In negative controls, in which the primary antibody was omitted, no specific signal was detected (data not shown). In contrast, in positive controls (fibroblasts), 99.7% of the cells showed one or two signals. These findings confirmed the specificity and the sensitivity of the methodology applied.

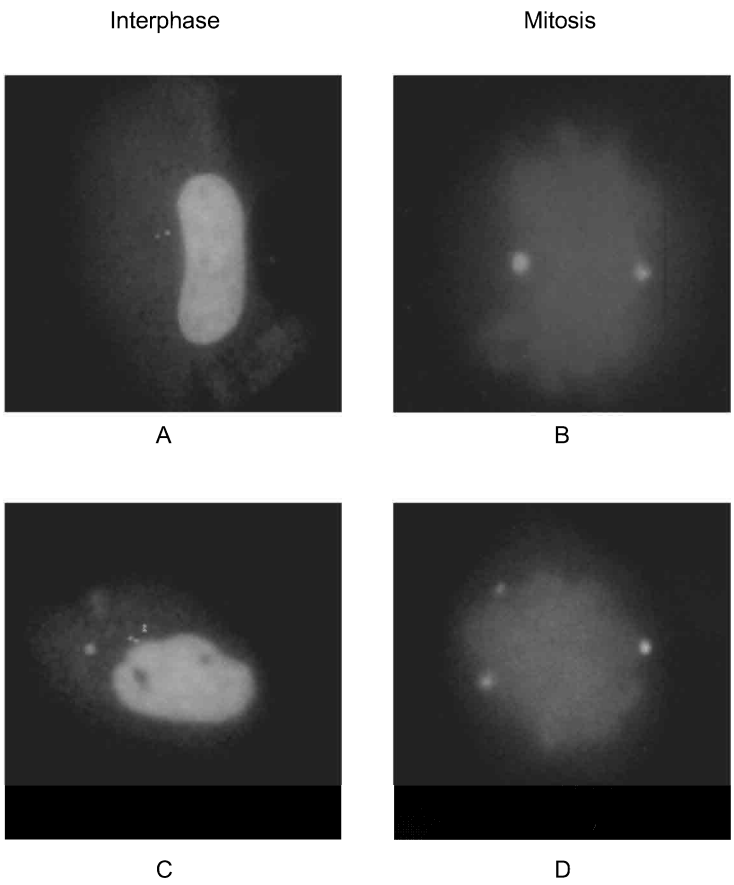


Figure 8. Representative immunofluorescence staining of centrosomes in MDA-231 cells in interphase and mitosis. Labelling with mouse anti-γ-tubulin monoclonal antibody and antibody-antigen complexes were detected by FITC-conjugated anti-mouse IgG. An expected number of centrosomes are shown in interphase (A) and mitosis (B) and numerical centrosome aberrations are seen in interphase (C) and mitosis (D).

4.1.1 Cervical carcinoma (paper I)

In cervical cancers, centrosome status, DNA ploidy pattern and HPV infection were studied.

Increasing number of aberrant centrosomes is significantly correlated with both grades of atypia and DNA ploidy aberrations. None of the condylomas or normal cases had centrosome aberrations. Amplified centrosomes were first observed in CIN I lesions (0.25%). It increased with grade of atypia and were highest in carcinoma. DNA histograms also showed an increasing number of aneuploid cells as tumors become more atypical (CIN II, III and carcinoma). In contrast, only diploid cells were observed in normal epithelium and condylomas. Interestingly, tetraploid cells were detected in CIN lesions, which indicated tetraploidy to be an intermediate stage during tumor development. These results are consistent with another study (Levine, *et al.*, 1991), which indicated that formation of a tetraploid intermediate in the diploid – tetraploid – aneuploid sequence of pancreatic tumor formation is accompanied by cells with 5 or more centrioles. All CIN lesions and carcinomas were infected with high-risk types HPV (16, 18 and 31). Interestingly, HPV infection was also found in non-malignant lesions including four of five condylomas and one of five normal cases, in which neither centrosome aberrations nor disturbed DNA distribution was observed. Three of five condylomas were HPV-16 positive, one was infected with low-risk HPV-6 and one normal epithelium was HPV-16 positive.

These observations are in agreement with the epidemiological evidence of a causal relation between HPV infection and cervical carcinomas and that the infection precedes the malignant development by years. Once carcinogenesis is initiated by HPV, the viruses interfere with essential regulatory mechanisms of cellular growth and cause after some time aberrant centrosome and disturbed DNA distribution. In our study, those malformations were first observed in CIN I lesions. It is likely that numerical chromosome aberrations result from centrosome defects, which is in line with the stepwise formation of DNA ploidy patterns (diploidy – tetraploidy – aneuploidy) during tumor development. The centrosome duplication cycle perturbed by HPV infection might undergo the next centrosome cycle without a mitotic event and produce double duplicated centrosomes. Those cells having two centrosomes and duplicated chromosomes could be detected as tetraploid. If such cells enter another endomitotic event, they could become octaploid. Another possibility is that the cells fail to divide in a proper way and they receive aberrant chromosome numbers. Most of those cells would be eliminated by apoptosis. However, cells that received enough chromosomes to survive exhibit qualitative and quantitative aberrant centrosomes.

The mechanism from HPV infection to aneuploidy remains to be clarified. Though the p53 tumor suppressor gene is the most frequently mutated gene in human malignancies, p53 mutation is very rare in cervical cancer (Olivier *et al.*, 2002). Previous work has shown that a limited number of cell lines derived from HPV-positive cervical cancers exhibits wild-type p53. However, recent studies have revealed the functional inactivation of p53 associated with HPV during carcinogenesis (Duensing *et al.*, 2002b). The two high-risk HPV E6 and E7 oncoproteins target tumor suppressor genes in different ways. HPV E6 abrogates p53-mediated checkpoint response by

inducing degradation of p53 (Scheffner *et al.*, 1990, Werness *et al.*, 1990). On the other hand, HPV E7 binds and degrades pRB (Boyer *et al.*, 1996, Dyson *et al.*, 1989) and inactivates the CDK inhibitor $p21^{Waf1}$ (Funk *et al.*, 1997, Jones *et al.*, 1997), thereby E2F transcription factors, which are abnormally disassociated from the E2F-pRB complex, triggers G1/S transition leading to aberrant centrosome duplication. Thus, even though the p53 gene is wild-type and functionally intact, it can be indirectly inactivated by other means leading to the establishment of malignant tumors.

We conclude that centrosome defects are significantly related to malignant development in cervical cancer. By interference with the p53 pathway, HPV can cause abnormal centrosome duplication, followed by stepwise tetraploidization and aneuploidy.

4.1.2 Adenocortical carcinoma (paper II)

Centrosome status in ACC was examined in relation to tumor size and chromosome aberrations.

Both adenomas and carcinomas exhibited a significantly increased frequency of cells with amplified centrosomes as compared to the normal controls ($p<0.0001$) and in ACC, the aberrant cells carried three or four positive signals in nine of the ten tumors and six signals in one tumor, while in the adenomas more than three signals were only recorded in one of the cases. The assessment of centrosome aberration in relation to tumor size showed a higher degree of centrosome amplification in the larger tumors. Tumor size is generally used as a main indicator of malignant potential of adrenocortical tumors. Preoperative diagnosis by tumor size of all tumors examined were confirmed by postoperative histopathological diagnosis. The borderline between adenoma and carcinoma in the tumors included in this study was 5.0-7.5 cm.

Furthermore, we have previously reported, using CGH and LOH techniques, a strong relationship between chromosome imbalances and both tumor size and malignancy. No alterations were observed in the smaller adenomas (<5 cm), wheares larger adenomas (≥5 cm) and carcinomas (7 – 20 cm) showed increased numbers of genetic alterations (Kjellman *et al.*, 1996). Taken together, centrosome status in adrenocortical tumors is highly related to tumor size, malignancy and aneuploidy.

At present, there is no clear evidence for a malignant progression from adenoma to carcinoma. The regulatory function of p53 on centrosome cycle has been demonstrated. However, this model can not be readily applied to adrenocortical tumors, since p53 mutation is not detected in benign tumors, while various percentages (5-70%) of p53 mutations in ACC has been reported (Stojadinovic *et al.*, 2002, Barzon *et al.*, 2001).
The expression of cell cycle regulators including p53, $p21^{Waf1}$ and Mdm-2 have been simultaneously assessed (Stojadinovic *et al.*, 2002). P53 mutations were observed in only 5.4% of ACC. $p21^{Waf1}$, which is a CDK inhibitor that can be induced by p53, was overexpressed in 70% of ACC, but only in 5.4% of the normal adrenal tissues. Mdm-2, which inhibits the transactivation function of p53, was positive in only 20% of ACC and in 92% of normal tissues. These results might indicate that down-regulated Mdm-2

could achieve hyperactivation of $p21^{Waf1}$ in the p53 pathway, which might give a possible explaination for the mechanism of malignant progression of ACC showing slightly positive p53, centrosome aberration and chromosome instability. However, p53 expression is not observed in benign adrenocortical tumors, which makes that this model can not be readily applied to tumor progression from benign to maliganant tumor. The role of Mdm-2 in malignant development of adrenocortical tumors remains to be elucidated.

4.2 CENTROSOME ABERRATION IN VITRO

4.2.1 P53 and Waf1 (paper III and IV)

Immunohistochemicl technique using the monoclonal antibody DO-7 for p53 detection, and another monoclonal antibody, Waf1, for p21 detection confirmed the p53 status in the four cell lines included in this study.
HDF and MCF7 presented with wild-type p53, whereas Hs578T and MDA-231showed mutated p53.

4.2.2 Centrosome status in cell lines (paper III)

The proportion of interphase cells containing amplified centrosomes, i.e. more than the expected number of one or two centrosomes was investigated in four cell lines (Figure 1 in paper III).
In each of the four cell lines, more than 500 cells were investigated. The p53 mutated and Waf1 negative lines had the highest number of > 2 centrosomes/cell (Hs578T had 6.1% and MDA-231 8.9%). In the fibroblast cell line HDF, we found 0.3% of the cells with centrosomes of >2/cell and in MCF7 1.3%. Comparing the two p53 mutated lines with the p53 wild type ones, the difference was statistically significant ($p<0.0001$). However, put it in the opposite way, all four lines had more than 90% of interphase cells with the expected normal number of one or two centrosomes.
The three human breast cancer cell lines showed significantly increasing proportions of cells containing multiple centrosomes as cells became more aneuploid. These observations support the results from our earlier studies (paper I) showing that a strong correlation exists between DNA ploidy and centrosome amplification.

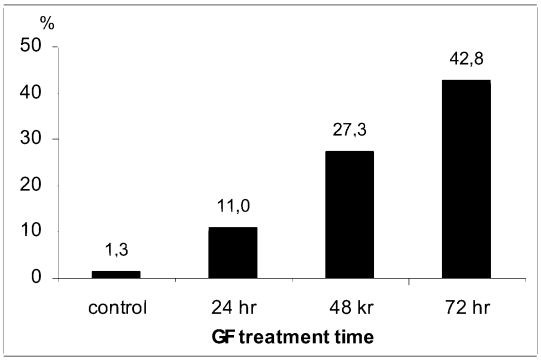
4.2.3 Cytogenetic effects of griseofulvin (paper III)

We perturbed the four cell lines by adding GF, which inhibits the polymerization of tubulin (Olmsted *et al.*, 1973) stopping cytokinesis and assembling the cells in prophase. GF can also split the polarity of the spindle particularly in giant cells generating more than two daughter cells (Gualandi *et al.*, 1984) To proceed to prometaphase and metaphase, the mitotic spindle built up of tubulin has to catch the condensed chromosomes at the kinetochore and by balanced forces form the metaphase plate having the chromosomes on a line in the middle of the spindle before segregation to the anaphase position. In progression to telophase, the two new nuclei surrounded by a new nuclear envelope are generated. Mitosis is normally ended after telophase by cell cleavage that is a division of the cytoplasm initiated by the cleavage furrow formed by the actin ring, built up of actin filaments and myosin. The nuclear division and cytoplasmic cleavage are normally very tightly synchronized but can occur uncoupled.

To define the experimental variables associated with GF-induced aneuploidy in culture cells, we have first examined both the exposure time- dependent effects and the dose-dependent effects of GF on these four cell lines.

4.2.3.1 Time-dependent effect

Abnormal centrosome numbers were increased in an exposure-time dependent way. After 72 hours' treatment with GF of MCF7 cells, more than 40 % of the cells contained multiple centrosomes and the DNA histpgram showed a very scattered pattern. (Figure 9) Furthermore after 72 hours' exposure to GF, cells were replaced in drug-free medium for another 72 hours. However, the number of aberrant centrosomes did not decrease significantly (will be discussed later).



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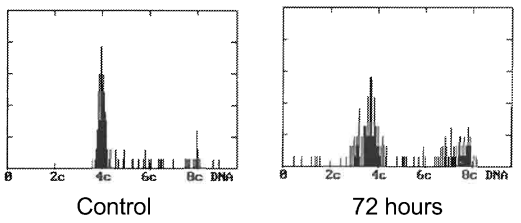

b

Figure 9. Time-dependent GF effect on MCF7 cells.
GF : 40μg/ml
(a) centrosome amplification
(b) DNA histogram

4.2.3.2 Dose-dependent effect

Increasing doses of GF resulted in statistically significant increases in the frequency of abnormal centrosomes (Figure 10).

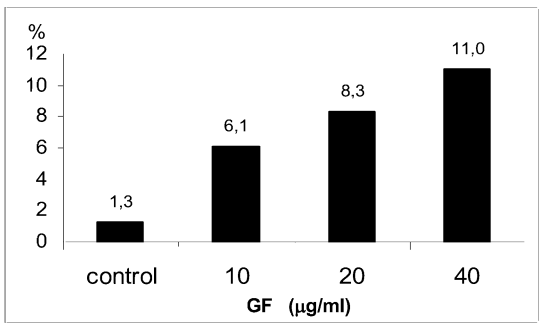

Figure 10. Dose-dependent GF effect on MCF7 cells.
GF-Treatment : 24 hrs

4.2.3.3 GF-induced centrosome amplification in different cell lines

Though GF had aneugenic effects on the cell lines included in this study, the disturbing effect of GF on each cell line was very variable. Aneuploid cell lines were more vulnerable by the drug compared to diploid fibloblasts, demonstrating that aneuploid cells are more genetically unstable as compared to diploid cells (Figure 11).

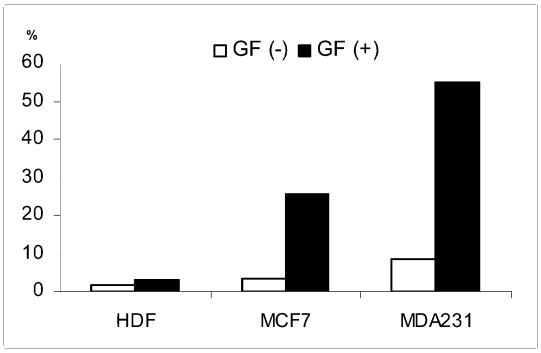

Figure 11. GF dose-dependent effect on HDF, MCF7 and MDA-231 cells. GF-treatment : 24 hours

4.2.3.4 GF-effect on interphase cells (paper III)

After exposure of interphase cells to GF, there was a significant increase, in all tumor cell lines, of cells containing >2 centrosomes. This was most evident in the p53 mutated and Waf1 negative lines, Hs578T and MDA-231. In the DNA histogram, an increase of cells in higher ploidy regions appeared. However, the disturbing effects on different cell lines were very variable.

The relative increase of cells with 4 centrosomes as a result of GF treatment in the four lines were assessed. The two p53 wild type lines showed the most pronounced increase in relative units (MCF7, 56-fold and HDF, 37-fold), which is a multiple of the number of two in normal late G2 and mitotic cells, indicating an endomitotic progress due to the mitotic block of GF. On the other hand, the relative increase in the number of cells with > 4 centrosomes was highest in Hs578T and MDA-231, 10-fold respectively 20-fold. These findings are supported by the DNA histograms revealing an increase of G2 peaks, a GF effect reported in the literature (Larizza *et al.*, 1974, Kinsella *et al.*, 1982). In MDA-231 and Hs578T, the relative increase of interphase cells with more than four centrosomes was much more evident than in HDF and MCF7, which was related to increased DNA histogram scattering. It has been shown that there is a p53-dependent arrest of non-transformed mammalian cells that have reached a tetraploid G2 state when mitosis is blocked by dihydrocytochalasin B or nocodazole (Andreassen *et al.*, 2001). This is an arrest called the tetraploid G1 checkpoint. Our data show that in all four lines cells pass their G2 phase during GF block of mitosis and enter another S-phase to reach a doubling of the genome to a higher ploidy level including the non-transformed HDF line. After the GF block, the chromosomes must become decondensed again, as in the G1 phase, and be triggered for another DNA cell cycle.

Similar results have been found after overexpression of Aurora-A kinas, where an amplification of centrosomes in cultured cells was found through defects in cell division and leading to tetraploidization (Meraldi *et al.*, 2002). P53 mutation

exacerbates this phenomenon. However, the causal relation between p53 mutation and centrosome amplification is not fully understood (Lingle, *et al.*, 2002). Both a spindle assembly defects and tetraploidization checkpoint failure are discussed in the literature and the tetraploidization checkpoint is suggested to be controlled by cdk2 kinase, hypophosphorylated retinoblastoma protein, and elevated levels of Waf1 and cyclin E, both of which require p53 (Andreassen *et al.*, 2001). It might be that the very common intermediate state of tetraploidization in tumor progression per se enhancing the number of centrosomes is exacerbated in p53 mutated cells.

In MDA-231, an increased number of cells were found in the G2-region after 48 hours of GF treatment, but 24 hours later, the DNA distribution was much more dispersed, without an evident assemble of cells at a G2 position. This might be due to the fact that when the cells having reached the hexaploid position and start to divide, they do it with an increased frequency of failure.

4.2.3.5 GF-effect on mitosis progression (paper III)

To assess mitotic cells for centrosome staining is hazardous, because cells in the mitotic figure are easily detached from the slide during the fixation and staining procedure. To avoid loss of cells, we reduced the time of GF treatment to 24 hours and increased the concentration of GF to 40 μg/ml and compared MCF7 and Hs578T, two lines with relative stable mitotic figures.
In the Hs578T line, a p53 mutated type, treatment with GF caused a 23-fold increase of mitotic cells with > 2 centrosomes, from 2.5% to 59.0%, and a 3-fold increase from 9.8% to 31.3% in the MCF7, p53 wild type. This might be due to the p53 mutation in Hs578T and can also reflect the larger relative increase in size of the tetraploid population in Hs578T.

To investigate in detail the blockage of GF on the mitotic progress, we followed the MCF7 cells through prophase, metaphase to telophase after GF-treatment. After GF-treatment, the percentage of telophase cells was reduced from 24% to 2% and the prophase cells increased from 35% to 60%. After release of GF, the normal frequency of telophase cells was restored 72 hours thereafter. The frequency of metaphase cells was not changed significantly. The DNA histograms showed a clear-cut change due to GF treatment towards more scattered values compared to untreated cells and the effect remained 72 hours after stop of treatment.
This indicates that the separation and withdrawal of the chromatids from the metaphase plate towards the anaphase position was the crucial site of GF effect (Mullins, *et al.*, 2001).

We conclude that the GF effect in all four lines cause a stop in mitosis and force some cells to proceed to higher ploidy levels (endomitosis). In the p53 mutated lines an increased number of more than four centrosomes per cell was found (Fig. 5), increasing the DNA content heterogeneity. Thus endomitosis is a risky event in p53 mutated cells that might generate more aneuploidy after division of the polyploid cells.

4.2.4 Effect of retinoic acid on centrosome status (paper IV)

In MDA-231, our previous study has shown an effect of a more even distribution of mass and DNA to daughter cells during mitosis after adding 13cRA to the culture medium (Sennerstam *et al.*, 1995). In this investigation, the effects of 13cRA on centrosome duplication and cyclin activity was studied.

4.2.4.1 Centrosome

In untreated MDA-231, there was a slightly but significantly less number of mitotic figures with amplified centrosomes as compared to interphase cells. The 13cRA effect on centrosome number in MDA-231 metaphase and interphase cells appeared after a time elapse of several weeks. After two weeks of 13cRA treatment, there were significantly reduced numbers of mitotic figures with amplified centrosomes comparing control cells and 13cRA treated cells. However it took up to 12 weeks before a statistically significant reduction was found among interphase cells. The reason for such a long time-lag might be due to that it takes time to rinse the cell population from cells with increased numbers of centrosomes, in particular when p53 is still mutated. The centrosomes go along with the cell through subsequent cell divisions. It is even not immediately deleterious to carry extra centrosomes. In spite of that mitosis works in many cases. Furthermore, 13cRA exerts an effect upon intermediate filament and actin cytoskeleton and increases content and organization of F-actin fibers (Olins, *et al.*, 2000, Bruel *et al.*, 2001, Zitterbart *et al.*, 2001, Ng *et al.*, 1985). Changes in actin cytoskeleton also take place during 13cRA induced apotosis (Bruel *et al.*, 2001). The results presented in this study might reflect an apoptotic activity, not triggered via p53/Waf1/cdk2/cyclinE. It is reported that 13cRA induces apoptosis in MDA-231 in a clearly time-dependent way. After one week of 13cRA treatment a moderate increase of apoptosis was seen (Toma *et al.*, 1997).

4.2.4.2 Cyclins

Cyclin A, D and E were evaluated by indirect immunofluorescence in flow cytometry. We looked into three size gates of G1 cells from small, medium to large cells and a quite similar increase of expression in all three cyclins occurred during G1 cell growth in size. No significant differences between control cells and 13cRA treated ones were found. Comparing small and large G1 cells for cyclin E counts no significant difference appeared neither with increasing size nor between treated and untreatred cells. Based on these observations, cells from the gate with the largest size group, i.e. cells late in G1 phase, from 5 days, 1 week and 2 weeks of 13cRA treatment were further assessed. The total amount of the three cyclins A, D and E between control and 13cRA treated cells did not differ statistically over this time period. Cyclin D was unaffected by 13cRA treatment at a relative high level of counts.

These findings indicate that the checkpoint functions reflected by the cyclins investigated were not restored by 13cRA and with an unchanged p53 mutation status that was less expected. A similar level of cyclin E in early and late G1 indicates a mutated state, since cyclin E has a narrow peak of expression in late G1 in untransformed cells. An endogenous p53 independent up-regulation of Waf1 due to 13cRA treatment in human hepatoma, Hep3B cells has been reported (Hsu *et al.*,

1999). In our experiments, we found no increase in Waf1 gene expression in MDA-231 cells after RA treatment.

4.2.4.3 Mitotic time duration, intra- and interclonal heterogeneity

Our previous study has reported mitotic time duration in MDA-231 to be extended significantly as compared to other cell lines (Sennerstam *et al.*, 1995) In this report, we compared a mean time duration of mitotis in MCF7, MDA-231 control cells and 13cRA treated cells and they were exposed for 32, 69, 70 minutes respectively. Thereby no effect upon duration of mitosis was seen due to 13cRA treatment.

Both intra- and interclonal intermitotic time (IDT) duration varied extensively in MDA-231 control cells. After 13cRA treatment a marked reduction was seen in both aspects. We compared to a diploid-tetraploid p53 mutated human breast cancer cell line Hs578T, reflecting an intermediate in intra- and interclonal IDT variation. The marked reduction in interclonal IDT heterogeneity might point to a survival of a homogenous group of clones or that 13cRA exerts an effect on the cytoskeleton of the cell (Olins, *et al.*, 2000).

5 CONCLUSIONS

The normal cell cycle can be subdivided into three important events: the DNA-division cycle (DDC), the cell growth cycle (CGC) and the centrosome cycle (CSC). These three subcycles are coupled to each other by a network of links including checkpoints, inhibitors and triggers, gene product reducers and enhancers creating important timers for the three subcycles to start and end. The CSC has to be coordinated with both DDC and CGC. Otherwise, the doubling of DNA and mass will not be transmitted to the daughter cells in a proper way. To give the next generation of cells a fair chance to survive and proliferate, the CSC – investigated in the present work – must do its job in the very tightly regulated way planned for in the cell cycle genes.

In the present thesis, we found a strong correlation between centrosome aberrations and the transformation of the cell towards neoplasia.

In paper I, we found that centrosome aberrations occurred already in cervical epithelial dysplasia in increasing frequencies following higher grade of dysplasia to carcinoma in situ (CIS) and invasive cancer. The intermediate appearance of tetraploid cells found in dysplasia and CIS indicates a diploid – tetraploid – aneuploid developmental pathway of cervical carcinoma. Through an endomitotic progression from a diploid G2 cell to the tetraploid stage, the centrosomes amplifies twice and end up in a mitotic situation with four centrosomes, causing a substantial risk for mitotic failure. Most of the cervical cancers reported in the literature reveal a low frequency of p53 mutation. However, the virus involved in cervical carcinogenesis (high-risk HPV) reveals p53 function disturbance. Therefore the carcinogenesis for cervical cancer might be written as:
HPV-infection – centrosome amplification – tetraploidy – aneuploidy.

In paper II, we could show that an enhanced number of centrosomes above the normal number of one or two appeared first in the very common adrenocortical benign adenomas in a mean frequency of 4% and in the very rare invasive adrenocortical carcinoma in a mean frequency of 10%. In fibroblasts the normal number of one or two centrosomes was registered in 99.7%. The adrenocortical carcinomas in our study were all aneuploid and in the literature few p53 mutated carcinomas have been found.
However, a disturbed function of Mdm-2 that inhibits the p53 gene transactivation ability has been reported and can give the same net effect as in a situation with mutated p53. Overexpression of Mdm-2 has been found to cause both amplification of centrosomes and extensive chromosome alterations. The carcinogenesis for adrenocortical cancers might be expressed as:
adenoma – *mdm2* mutation – inhibition of p53 – centrosome amplification – chromosome aberration – aneuploidy.

In the in vitro studies from the human breast cancer cell lines a p53 mutation and Waf1 negativity revealed the highest number of cells with centrosomes above the normal numbers of two. After perturbing mitosis by griseofulvin various frequencies of cells in all lines including the fibroblast line were lifted up in ploidy level. As a consequence of that the centrosome number was amplified. The euploid (diploid and tetraploid) lines were shifted to another even multiple of the normal G2 centrosome number to four signals in γ-tubulin staining but the p53 mutated aneuploid lines to centrosome numbers above four. This reflects a vulnerability of the CSC in p53 mutated aneuploid lines.

Retinoic acid (13cRA) was found to reduce the total number of centrosomes in the aneuploid line after some time of treatment. The effect was first seen in mitosis and later in interphase cells after weeks of treatment. In a system where p53 was unaffected by 13cRA the apoptotic function was out of order and can explain the long time duration it took to rinse the cell from aberrant centrosomes.
No effect was seen on cyclinA, E and D levels due to treatment indicating that checkpoint functions driven by these cyclins were unchanged by the treatment. The in vitro studies reveal that the centrosome amplification above even multiples of the normal was correlated to p53 mutation and abolished Waf1 expression.

How the centrosomes are regulated is not fully revealed by these studies. Some correlations have been confirmed, but the causal relation has to be further analyzed.

6 FUTURE PROSPECTS

The present thesis has opened the field for further studies. The network of contributing factors working in either an enhancing or a degrading way for the establishment of a proper centrosome cycle will give several ideas to go further.

The MDA-231 breast cancer cell line mutated in many genes involved in checkpoint function can be used in a gene transfecting prospect. Genes to be transfected to the cell might be wild type *p53*, *WAF1*, *mdm-2* and the three genes expressing the cyclins A, D and E.

Another experiment to design is to further understand how 13cRA exerts its effect upon various cell components to stabilize structures as it actually does. We will identify cytoskeleton and mitotic spindle components in treated and untreated cells. Furthermore, we plan to investigate which apoptotic pathway 13cRA use to eliminate defective cells.

The MCF7 breast cancer cell line that has retained a tetraploid DNA-index (DI) position over decades without high frequency of aberrant centrosomes occurs to be extremely useful for further studies in this field. During many years of in vitro growth, this cell line has not changed its modal DNA value, e.g. to a hypertriploid position as found in many tumors. Why not?

Finally we should continue to further study the relationship between centrosome status and clinical stage of malignant tumors in order to make centrosome staining meaningful in clinical practice.

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