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Met amplification and tumor progression in *Cdkn2a*-deficient melanocytes

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Summary

While many genetic alterations have been identified in melanoma, the relevant molecular events that contribute to disease progression are poorly understood. Most primary human melanomas exhibit loss of expression of the *CDKN2A* locus in addition to activation of canonical MAPK signaling including RAS, RAF, MEK, and ERK. In this study, we used a *Cdkn2a*-deficient mouse melanocyte cell line to screen for secondary genetic events in melanoma tumor progression. Upon investigation, intrachromosomal gene amplification of *Met*, a receptor tyrosine kinase implicated in melanoma progression, was identified in *Cdkn2a*-deficient tumors. RNA interference (RNAi) targeting *Met* in these tumor cells resulted in a significant delay in tumor growth *in vivo* compared with the control cells. *MET* expression is rarely detected in primary human melanoma but is frequently observed in metastatic disease. This study validates a role for *Met* activation in melanoma tumor progression in the context of *Cdkn2a*-deficiency.

Keywords

Met; Melanocytes; *Cdkn2a*; Transformation; Progression

INTRODUCTION

The incidence of melanoma has increased significantly over the last forty years (Jemal et al., 2008). If detected early, the disease is treatable; however, following metastasis it is largely resistant to most conventional therapies and is associated with a high mortality rate (Ahmed, 1997). Several loci mutated in familial or sporadic melanoma have been identified, providing targets for possible therapeutic intervention [reviewed in (Chin et al., 2006)]. The *CDKN2A* locus has been linked to numerous cancers, including melanoma and encodes two distinct tumor suppressor proteins: p16^{INK4A}, which inhibits CDK4/6 and cell cycle progression, and p14^{ARF} a key element involved in p53 regulation (Chin et al., 1998). With less frequency, mutations in tumor suppressors such as *p53*, *PTEN*, and *Rb* have also been reported (Chin et al., 2006). With mutually exclusive mutations in *NRAS* and *BRAF*, the mitogen-activated protein kinase (MAPK) signaling pathway is constitutively activated in over 80% of sporadic malignant melanomas (Davies et al., 2002).

NRAS and *BRAF* mutations are frequently found in primary melanomas, suggestive of an important initiating role in melanoma genesis, whereas hyperactive receptor tyrosine kinase (RTK) signaling by the epidermal growth factor receptor (EGFR) or MET RTK has been associated with melanoma progression and metastasis (Koprowski et al., 1985; Natali et al., 1993; Wiltshire et al., 1995; Bastian et al., 1998). Abnormal MET signaling has been implicated in the progression and maintenance of many different cancer types, including melanoma (Natali et al., 1993; Puri et al., 2007). Tissue-specific overexpression of Met or its ligand hepatocyte growth factor (HGF) in mice leads to malignant transformation of a variety of cell types including melanocytes (Otsuka et al., 1998; Vande Woude, 2008). HGF expression also enhances melanoma formation in mice devoid of *Ink4a/Arf* (Recio et al., 2002; Ha et al., 2007). Gain-of-function germline mutations in the *MET* tyrosine kinase domain are implicated as the cause of hereditary papillary renal carcinoma in humans (Schmidt et al., 1997). Furthermore, genomic amplification of *MET* has been found to occur in up to 10% of gastric cancers (Smolen et al., 2006), 4% of lung cancers (Zhao et al., 2005), 4% of esophageal adenocarcinomas (Miller et al., 2006), and 47% of metastatic melanomas (Moore et al., 2008). Both lung and gastric cancer cell lines with *MET* amplification have been shown to be dependent on the amplified MET kinase for growth, indicating a critical role for MET in cell growth and survival (Natali et al., 1993). Furthermore, *MET* gene amplification and overexpression has been associated with poor clinical outcome in a number of human cancers (Birchmeier et al., 2003).

In this study, we used a *Cdkn2a*-deficient mouse melanocyte cell line to screen for spontaneous secondary genetic events in melanoma tumor progression *in vivo*. Cytogenetic analysis of cell lines established from these tumors revealed high-level intrachromosomal amplification of the RTK *Met*. Subcutaneous injection of these cells into nude mice resulted in rapid tumor formation with no delay. *In vivo* growth of these tumor cells is dependent on *Met* expression as RNA interference (RNAi) targeting *Met* resulted in a significant delay in tumor growth *in vivo* compared with the control cells. *MET* expression is rarely detected in primary human melanoma, but is frequently observed in metastatic disease. This study demonstrates that *Met* amplification induces tumor progression in *Cdkn2a*-deficient melanocytes.

RESULTS AND DISCUSSION

We have previously reported the generation and characterization of a pure mouse *Cdkn2a*-deficient melanocyte cell line, D6-MEL (Whitwam et al., 2007). These cultures were found to be immortal but non-transformed. Since these cells are *Cdkn2a*-deficient, transformation requires only a single genetic hit. As such, expression of constitutively active NRAS in these cells resulted in numerous colonies in soft agar and rapid tumor formation when injected into nude mice (Whitwam et al., 2007). As early as three weeks post-injection, tumors were visible in 16/16 mice injected with melanocytes expressing constitutively active NRAS (Figure 1). In contrast, tumors were not visible in mice injected with the parental *Cdkn2a*-deficient mouse melanocyte cell line, D6-MEL, during this time frame. Therefore, we used this cell line to screen for spontaneous secondary genetic events involved in tumor progression *in vivo*. After a long latency, tumors became visible in 6/16 mice (Figure 1). The slow kinetics of tumor formation in mice injected with *Cdkn2a*-deficient melanocytes suggested that additional genetic alterations were required for growth of these cells *in vivo*.

To define the putative genetic alteration(s) that mediated the *in vivo* growth of *Cdkn2a*-deficient melanocytes, the subcutaneous tumors were isolated and a portion of the tumor was formalin fixed and paraffin embedded for histological analysis. Cultured cell lines were also successfully established for two of the six tumors. All of the tumors, including the NRAS-driven tumors, displayed high-grade histology and were comprised of spindle cells that exhibited moderate to

severe nuclear pleomorphism, abundant mitotic figures, and variable extents of tumor necrosis (Figure 2).

Tumor cells have increased *Met* expression

The *c-MET* proto-oncogene encodes the receptor for hepatocyte growth factor/scatter factor (HGF/SF), which is known to mediate mitogenic, motogenic, and invasive responses. While infrequent in nevi and primary melanomas, 38%–47% of metastatic lesions have significant *MET* expression suggesting a role in tumor progression (Natali et al., 1993; Moore et al., 2008). Since amplification and/or overexpression of the receptor tyrosine kinase *MET* has been implicated in melanoma progression (Koprowski et al., 1985; Natali et al., 1993; Wiltshire et al., 1995; Bastian et al., 1998), we evaluated the expression of *Met* in both tumor sections and the established tumor cell lines, subcutaneous isolates 303 and 305. The expression in the tumor sections was evaluated by immunohistochemistry for *Met* protein and was compared to the expression in tumors expressing *NRAS* (Figure 2). High *Met* expression was detected in all of the tumors that formed after a long latency. *Met* expression was also detected in the *NRAS* expressing tumors but at a significantly lower level. The expression in the tumor cell lines was visualized by Western blot analysis. A significant increase in *Met* protein levels was detected in the tumor cells as compared with the pre-injected cells and *NRAS* expressing tumor cells (Figure 3). These results were in agreement with the immunohistochemistry data (Figure 2). This demonstrates that changes in *Met* did not occur as a result of growth in culture. The increase in *Met* protein levels also correlated with significantly enhanced *Met* activity as detected by Western blot analysis of Y1234/1235 phosphorylated *Met* (Figure 3). Active *Met* was not detected in the D6-MEL cells or in the *NRAS* expressing tumor cells (Figure 3).

Increased *Met* protein levels result from gene amplification

Gene amplification of the RTK *MET* can be found in 10–20% of primary human gastric cancers (Smolen et al., 2006) and 47% of metastatic melanomas (Moore et al., 2008). In a *Brcal/Trp53* mouse model, high level *Met* gene amplification was observed in 73% of the mammary tumors (Smolen et al., 2006) providing further support for the relevance of secondary genetic events in tumorigenesis. The *Met* expressing tumor cell lines and the parental D6-MEL melanocytes were stained with propidium iodide and analyzed by flow cytometry as an initial quantification of nuclear DNA content. The DNA content of the parental D6-MEL cells was normal. In contrast, the DNA content of cell lines established from the tumors that developed indicated that between 55% and 65% of the cells were aneuploid (Table 1). The genomic integrity of the D6-MEL melanocyte cell line was also evaluated using spectral karyotyping (SKY). Eighteen metaphases were analyzed and 18/18 metaphases had an additional chromosome 6 and 15, 18/18 metaphases had an extra derivative chromosome 4 translocated with chromosome X, but only 1/20 was near tetraploid (Figure 4A).

To determine if the mechanism of increased *Met* protein levels in the tumors was the result of gene amplification, we analyzed both interphase and metaphase cells by fluorescent in-situ hybridization (FISH) using a probe specific for the *Met* locus. FISH analysis showed 3 copies of both the *Met* specific probe and the control probe in the pre-injected D6-MEL parental melanocytes (Figure 4B–C). The 3 copies of chromosome 6 correspond with the additional chromosome 6 detected by SKY in the D6-MEL cells (Figure 4A). In contrast, a high level of intrachromosomal *Met* gene amplification was detected by FISH in the tumor cells (Figure 4D–E); ~46 and ~28 copies of *Met* were detected in the 2n tumor cells and ~92 and ~44 copies of *Met* were detected in the 4n tumor cells from 303 and 305, respectively. The *Met* cDNA was cloned from both of these cell lines and sequenced. No mutations were identified indicating that the amplified *Met* was wild-type.

Inhibition of Met protein expression slows the growth of the tumor cells

To confirm that Met was responsible for mediating tumor progression in the D6-MEL melanocytes, lentiviral vectors were used to deliver M2 short hairpin RNA (shRNA) targeting *Met* to the 303 and 305 tumor cells (Lutterbach et al., 2007). Efficient reduction in Met protein expression was detected in both tumor cell lines compared with the parental and luciferase (Luc) targeted control cells (Figure 3). A significant reduction in the level of active phosphorylated Met was also observed in the cells expressing the M2 shRNA (Figure 3). Following confirmation of Met knockdown, these cells were injected subcutaneously into nude mice and the growth rate of the tumors was compared with the control Luc cells (Figure 5). Importantly, the control cells formed tumors rapidly. The 303 cells grew at a faster rate than the 305 cells and this may be due to the greater number of copies of Met in 303 compared with 305. The M2 Met shRNA significantly reduced the growth of both the 303 and 305 cells. The M2 Met shRNA reduced tumor growth by greater than 50% by the end of the experiment. The difference was greater than 75% at earlier time points, suggesting that cells that had maintained Met expression may have been selected for *in vivo*. The differences between the Luc and M2 cells became significant ($P < 0.05$) beginning at day 14. The level of Met activity was also evaluated in growing tumors with or without Met knockdown by immunohistochemistry for phosphorylated Met. High levels of membranous phosphorylated Met were detected in the parental uninfected and Luc tumors compared with the M2 tumors (Figure 6). These data strongly suggest that the *Met* amplification observed in the explanted tumor cells contributed to tumor progression *in vivo* and was required for tumor maintenance.

This is the first report to demonstrate that spontaneous *Met* amplification induces tumor progression in melanoma. Through its ability to promote tumor progression, MET is being developed as a therapeutic target for drug intervention in many metastatic tumors including melanoma. Recently, a MET-specific RTK inhibitor displayed promising anti-tumor activity *in vitro* against melanoma cell lines expressing *MET* (Puri et al., 2007). We further demonstrate that targeting *Met* by RNAi slows the growth of tumor cells with amplified *Met* *in vivo*.

METHODS

Cell culture

The D6-MEL *CDKN2A*-null mouse melanocyte cell line has been described (Whitwam et al., 2007). Mouse melanocytes and B16 melanoma cells were cultured in 254 media containing HMGS (Cascade Biologics, Portland OR), 5% FBS, and 50 µg/ml gentamicin at 37 °C with 5% CO₂. Single cells were obtained from subcutaneous tumors by mincing the tissue with a razor blade followed by incubation in 0.05% trypsin for 45 min at 37 °C. Cells were established in culture and maintained in 254 media supplemented with 10% fetal bovine serum. DF-1 cells and 293FT cells were grown in DMEM-high glucose (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 100 units of penicillin per ml, and 100 µg of streptomycin per ml, and maintained at 39 °C with 5% CO₂ (Schaefer-Klein et al., 1998).

Short hairpin RNAi lentiviral constructs

The shRNA sequence targeting Met (M2) was GATCTGGGCAGTGAATTAGTTCAAGAGACTAATTCAGTCCCCAGATC, and targeting Luc was CACCGGTGTTGTAACAATATCGACGAATCGATATTGTTACAACACCAAAA as described (Lutterbach et al., 2007). The oligonucleotides were annealed, cloned into pENTR™/U6 and recombined into pLenti6/Block-iT™-Dest (Invitrogen, Carlsbad, CA) per the manufacturer's specifications. Infectious virus was produced in 293FT cells following the manufacturer's instructions for the BLOCK-iT™ Lentiviral RNAi Expression System (Invitrogen, Carlsbad, CA) with the following exceptions. Viral supernatant was collected at

48 and 72 hours, subjected to 0.45 μ m filtration, and used to infect 60% confluent target cells in 6-well plates. Following multiple rounds of infection the cells were washed in PBS and fresh growth medium was added containing 10% FBS. After passing the cells once (4 days post first infection) a set of each infected group was harvested for Western blot analysis while the other set was expanded for further experimentation.

Vector constructs

The RCASBP(M)-NRAS^{Q61R} retroviral vector has been described (Whitwam et al., 2007).

Western blotting

The protein lysates were separated on Tris-glycine polyacrylamide gels, transferred to nitrocellulose, and incubated for 1 h at room temperature in blocking solution (0.05% Tween-20 in TBS with 5% non-fat dry milk). All antibodies were diluted in blocking solution. Blots were immunostained for Met using a mouse monoclonal anti-Met antibody (3127; Cell Signaling, Beverly, MA) at a 1:1000 dilution; for phosphorylated Met using rabbit monoclonal anti-Phospho-Met Tyr1234/1235 (D26; Cell Signaling, Beverly, MA) at a 1:1000 dilution; and for tubulin using a mouse monoclonal anti- α -tubulin antibody (AB-1) at a 1:1000 dilution (Oncogene, San Diego, CA). Western blots were incubated in the primary antibody overnight at 4 °C with constant shaking and then washed three times in TBS-T wash buffer (0.05% Tween-20 in Tris-buffered saline). The blots were then incubated with anti-mouse or anti-rabbit IgG-HRP secondary antibody diluted 1:3000 (Sigma, St. Louis, MO) for 1 h at room temperature. The blots were washed three times in TBS-T wash buffer, incubated with ECL solutions per the manufacturer's specifications (Amersham, Piscataway, NJ), and exposed to film.

In vivo studies

Four-week old female athymic nude (NCr *nu/nu*) mice were injected subcutaneously (s.c.) in each flank with 1×10^7 cells/mouse in 100 μ l Hanks' balanced salt solution (HBSS). Tumor size was evaluated by caliper measurements, and tumor volume was calculated by length $\times$ width $\times$ depth. All experiments were performed in compliance with the guiding principles of the "Care and Use of Animals" (available at <http://www.nap.edu/books/0309053773/html/>) and were approved by the IACUC prior to experimentation.

Histology

Tumor tissue from all injected mice was fixed in 10% neutral buffered formalin overnight and subsequently processed on a Microm STP 420D tissue processor (Thermo Fisher, Kalamazoo, MI). Tissues were then embedded in paraffin, and 5- μ m sections were adhered to glass slides and stained with hematoxylin and eosin (H&E) or left unstained for immunohistochemistry. Histologic evaluation was performed independently by a board certified anatomic pathologist. Images were captured using a BX51 Olympus microscope equipped with an Infinity camera.

Immunohistochemistry

Sections were de-paraffinized and treated with 3% hydrogen peroxide for 30 min to quench endogenous peroxidase activity. Antigen retrieval was performed in 'Diva Decloaking' buffer by boiling for 10 min in a Diva Decloaking chamber (Biocare Medical, Concord, CA). Sections were blocked in Background Sniper (Biocare Medical, Concord, CA) for 5 min at room temperature. Met expression was detected using a rabbit polyclonal primary antibody to a peptide mapping within a C-terminal cytoplasmic domain of Met (Sc-162; Santa Cruz Biotechnology, CA) diluted 1:200 in Renaissance diluent for 1 hour at room temperature. Detection of Met phosphorylation was performed overnight at 4 °C using a 1:100 dilution of a rabbit polyclonal antibody to phosphorylated Met (ab61024; Abcam, Cambridge, MA) in

Renaissance diluent. The sections were washed with TBS-T and then probed with Mach 4 polymer reagent (Biocare Medical, Concord, CA) for 30 min at room temperature. The signals were visualized with 3,3'-diaminobenzidine tetrahydrochloride (DAB; Biocare medical, Concord, CA).

Cell cycle analysis

Cell cycle analyses were performed using propidium iodide (PI) (Sigma) in a modified Vindelov's preparation (containing 50 µg/ml PI solution, 10 mM NaCl, 10 mM Tris-base, 0.1 % IGEPAL, and 0.7 U/ml RNase) using a 4-color FACSCalibur flow cytometer (Vindelov et al., 1983). Data analysis was conducted using ModFIT LT software v3.0 (Verity Software House, Topsham, ME).

Metaphase spreads

Cells were incubated overnight with colcemid (12 ng/ml; Invitrogen) to arrest cells in metaphase. Cells were detached by trypsin, washed twice in PBS and then gently resuspended in 5 ml 37 °C, 75 mM KCl for 10 min. to permeabilize the plasma membrane, and fixed in 10 ml ice cold fixative (75% methanol, 25% glacial acetic acid).

Reverse 4'-6-diamidino-2-phenylindole (DAPI)

Metaphase spread slides were prepared from cell cultures that were harvested and fixed with methanol:acetic acid (3:1), as described above. Slides were aged overnight at 55 °C. Once slides were cooled to room temperature, DAPI was applied and covered with a glass cover slip. Image acquisition was performed with a COOL-1300 SpectraCube® camera (Applied Spectral Imaging-ASI, Vista, CA) mounted on an Olympus BX51 epifluorescence microscope and analyzed using FISHView® software (EXPO v4.0, ASI). DAPI images were inverted to a black and white reverse DAPI image that allows for visualization of chromosome centromeres and bands similar to G-banding. Chromosomes were counted and obvious aberrations were noted for at least 20 metaphases per cell line.

Spectral karyotyping (SKY)

Metaphase spreads were prepared from cell cultures as described above and SKY was performed according to the standard supplied protocol (ASI) on freshly prepared metaphase slides to investigate cytogenetic abnormalities. Image acquisition was performed as described above and analyzed using SKYView® software (EXPO v2.1.1, ASI). Eighteen metaphases were analyzed.

Fluorescent *in situ* hybridization (FISH)

FISH probes were prepared from purified BAC clones RP23-73G15 and RP24-462C10 (Children's Hospital Oakland Research Institute; <http://bacpac.chori.org>) and labeled with SpectrumOrange™ and SpectrumGreen™ (Abbott Molecular Inc, Des Plaines, IL), respectively, by nick translation. These probes were used to detect *Met* amplification within region 6A2 and region 6D3 as a control, respectively. Metaphase spread slides were prepared from cell cultures that were harvested and fixed with methanol:acetic acid (3:1). The slides were pretreated with 2× saline sodium citrate (SSC) at 37 °C for 10 min, 0.005% pepsin at 37 °C for 4 min, and 1× PBS for 5min. The slides were then placed in 1% formaldehyde for 10 min at room temperature, washed with 1× PBS for 5 min, and dehydrated in an ethanol series (70%, 85%, and 95%) for 2 min each. Samples were denatured in 70% formamide in 2× SSC at 73 °C for 5 min, washed in a cold ethanol series (70%, 85%, 95%) for 2 min each, and air dried. FISH probes were denatured at 75 °C for 5 min before applying to each dried slide and mounted with a glass cover slip. The slides were hybridized overnight at 37 °C, washed with 2× SSC at 73 °C for 2 min, and rinsed briefly in H₂O. Slides were then air-dried, counterstained

with anti-fade DAPI, and covered with cover slips. Image acquisition was performed as described above and analyzed using FISHView® software (EXPO v4.0, ASI). Hybridization signals were analyzed for at least 10 metaphases per cell line.

Significance

The development of cancer from a pre-malignant primary tumor to a metastatic cancer that develops at secondary sites is a multi-step process, thought to require many genetic and epigenetic events that provide a growth advantage to cells. It is still unclear, however, which of the many genetic changes are required late in tumor progression and in the metastatic pathway. Therefore, it is critical to validate candidate genes thought to be involved in this process. In an *in vivo* screen for spontaneous secondary genetic events initiated by loss of the *Cdkn2a* locus in melanoma, we observed high level amplification of *Met*, which encodes a growth factor receptor tyrosine kinase. MET expression has been implicated in tumor progression in human melanoma and this study validates *Met* amplification as a tumor promoting factor in melanoma.

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Figure 1. *In vivo* tumor formation

Volumes are shown for tumors induced by subcutaneous inoculation of 1×10^7 cells/mouse for the cell lines indicated. Tumor size was evaluated by caliper measurements, and tumor volume was calculated by length $\times$ width $\times$ depth (data are represented as mean $\pm$ SEM; $n = 16$).

Figure 2. Histological examination of tumor tissue and immunohistochemical staining for Met expression

Hematoxylin (blue nuclei) and eosin (pink cytoplasm) stained tumor tissue from all mice was analyzed histologically by a board certified pathologist. (Left Panel) Representative images from tumors that developed after long latency in mice injected with D6-MEL cells (A–F) and mice injected with D6-MEL cells expressing activated NRAS (G–H). (Right Panel) Serial sections from the same tumors shown in the left panel immunohistochemically stained with an antibody directed against Met. The bars represent 100 μ m.

Figure 3. Analysis of Met expression and activity

303 and 305 represent lysates from individual tumor cell lines established in culture from long latency D6-MEL tumors. Luc represents cells infected with the lentiviral vector containing an shRNA sequence targeting luciferase (control) and M2 represents cells infected with the lentiviral vector containing an shRNA sequence targeting *Met*. 100 and 101 represent lysates from individual tumor cell lines established in culture from D6-MEL/NRAS tumors. B16 melanoma tumor cell lysate was included as a control. The blots were incubated with antibodies to specific proteins as indicated to the right of each panel. MW, molecular weight.

Figure 4. Spectral karyotyping and *Met* FISH of melanocytes and tumor cells

A. Reverse DAPI (upper left panel), classified image (upper right panel), and karyogram showing both spectral and classified colors of each chromosome (lower panel) of D6-MEL melanocytes. B. Interphase D6-MEL cell pre-implantation. C. Metaphase D6-MEL cell pre-implantation. D. Interphase 303 tumor cell. E. Metaphase 305 tumor cell. Ten metaphases were analyzed per cell line. A representative image is shown of each. Orange, probe detecting the *Met* locus on chromosome 6; green, chromosome 6 control probe.

Figure 5. Reduced *Met* expression in 303 and 305 tumor cells delays tumor growth *in vivo*

Volumes are shown for tumors induced by subcutaneous inoculation of 1×10^7 cells for the cell lines 303/M2, 305/M2 (cell lines with M2 shRNA targeting *Met*), 303/Luc, 305/Luc (cell lines with Luc shRNA targeting luciferase). Tumor size was evaluated by caliper measurements, and tumor volume was calculated by length $\times$ width $\times$ depth (data are represented as mean $\pm$ SEM; $n = 10$ for each group). The differences between the M2 and Luc tumors became significant ($P < 0.05$) beginning at day 14.

Figure 6. Immunohistochemical staining for phosphorylated Met expression

(A–C) Tumor tissue from mice injected with the 305 uninfected parental (A), Luc infected (B), and M2 infected (C) cells were analyzed histologically by hematoxylin (blue nuclei) and eosin (pink cytoplasm) staining. Representative images from tumors that developed are shown. (D–F) Serial sections from the same tumors shown in the left panel immunohistochemically stained with an antibody directed against phosphorylated Met. The bars represent 50 μ m.

Table 1

DNA content of melanocytes and tumor cells.

Cell line	% of cells*	
	2n	4n
D6-MEL	100%	0%
303	34%	66%
305	44%	56%

* Quantification of DNA content by propidium iodide staining of nuclear DNA and analysis by flow cytometry.