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Inhibition of Aurora-A kinase induces cell cycle arrest in epithelial ovarian cancer stem cells by affecting NFκB pathway

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Abbreviations: EOC stem cells, epithelial ovarian cancer cells; mOCCs, mature ovarian cancer cells; OSEs, ovarian surface epithelial cells; Aurora-A, aurora-A kinase

Recurrent ovarian cancer is resistant to conventional chemotherapy. A sub-population of ovarian cancer cells, the epithelial ovarian cancer stem cells (EOC stem cells) have stemness properties, constitutive NFκB activity, and represent the chemoresistant population. Currently, there is no effective treatment that targets these cells. Aurora-A kinase (Aurora-A) is associated with tumor initiation and progression and is overexpressed in numerous malignancies. The aim of this study is to determine the effect of Aurora-A inhibition in EOC stem cells. EOC stem cells were treated with the Aurora-A inhibitor, MK-5108. Cell growth was monitored by Incucyte real-time imaging system, cell viability was measured using the Celltiter 96 assay and cytokine levels were quantified using xMAP technology. The intracellular changes associated with MK-5108 treatment are: (1) polyploidy and cell cycle arrest; (2) inhibition of NFκB activity; (3) decreased cytokine production; and (4) nuclear accumulation of IκBα. Thus, inhibition of Aurora-A decreases cell proliferation in the EOC stem cells by inducing cell cycle arrest and affecting the NFκB pathway. As EOC stem cells represent a source of recurrence and chemoresistance, these results suggest that Aurora-A inhibition may effectively target the cancer stem cell population in ovarian cancer.

Introduction

Epithelial ovarian cancer (EOC) is a highly lethal disease usually diagnosed in a very late stage. In 2010, in the United States, an estimated 21,880 new patients were diagnosed with ovarian cancer and 13,850 died from the disease. First-line standard treatment for ovarian cancer has not changed since 1996 [1] and includes intravenous administration of a platinum agent (carboplatin or cisplatin) and paclitaxel (Taxol). Initially, most patients respond, but the disease usually recurs within five years. Thus, fewer than one in ten patients survive beyond five years following standard salvage chemotherapy treatment.[2] Therefore there is a need to find new therapeutic modalities that can help improve patient survival.

Recent evidence suggests the existence of heterogeneous cancer cell populations in the tumor mass. A minor subpopulation of cancer cells, the cancer stem cells (CSC), has been implicated as the putative mediators of tumor initiation and chemoresistance.[3,4] We demonstrated in ovarian cancer that the $CD44^+$ epithelial ovarian cancer stem cells (EOC stem cells) have tumor-initiating and chemoresistant properties.[5,6] Additionally, these cells have the capacity to acquire different phenotypes, for example, to acquire the classical endothelial markers, CD34 and VE-cadherin.[6]

Nuclear Factor KappaB (NFκB) has been shown to be important in cancer biology, and especially in the EOC stem cells.[5,7,8] The p65/p50 NFκB complex is localized in the cytoplasm when bound to inhibitor of kappaBα (IκBα). Upon phosphorylation, IκBα undergoes degradation releasing the p65/p50 complex, which then translocates to the nucleus and activate target genes.[9] NFκB target genes include inflammatory cytokines such as IL-6, TNFα, MCP-1 and others; as well as genes associated with the regulation of cell survival and apoptosis. EOC stem cells are characterized by constitutive NFκB activity as well as constitutive cytokine secretion.[5,7,8] Interestingly, NFκB inhibition is a potent inducer of cell death in the chemoresistant EOC stem cells.[10]

Aurora-A kinase (Aurora-A) (also known as STK15, STK6, STK7 or BTAK) is involved in centrosome separation, duplication and maturation, as well as in bipolar spindle assembly and stability.[11] Additionally, Aurora-A contributes to the completion of cytokinesis—the process by which the cytoplasm of the parent cell is split into two daughter cells. The Aurora-A gene is located in chromosome 20q13, an area that is commonly amplified

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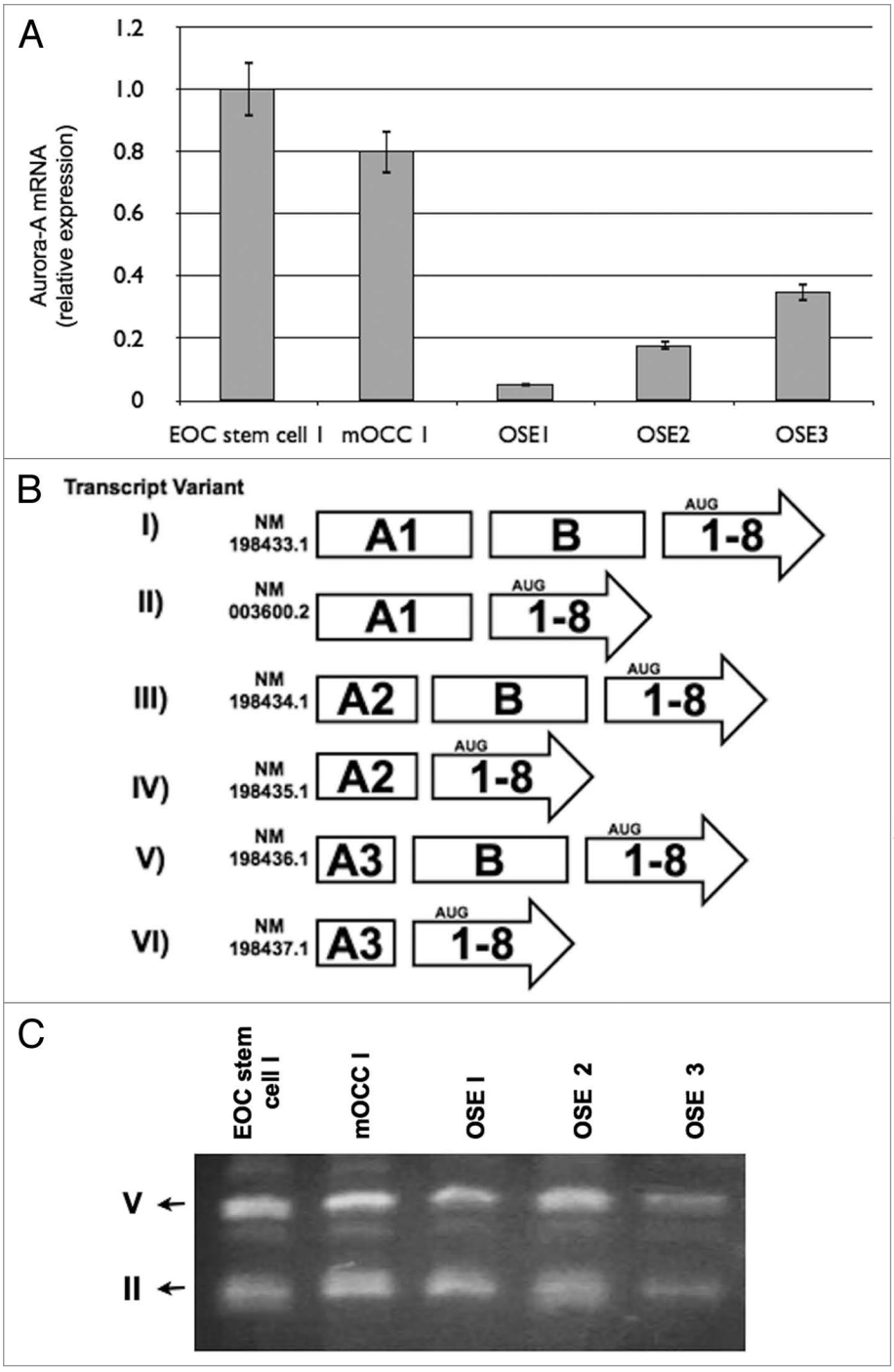

Figure 1. Aurora-A kinase expression in ovarian cancer cells and normal ovarian surface epithelial cells. (A) qRT-PCR measurement of Aurora-A. Data represent the average from three independent experiments, measured in triplicate and normalized to GAPDH mRNA. Error bars indicate standard errors of the means. Data shown is for EOC stem cell 1 and mOCC 1. Similar results were obtained with other cell cultures; (B) Alignment of exons in six Aurora-A transcript variants is shown with 5′ end on the left and 3′ end on the right. The translation initiation site (ATG) is marked; (C) Aurora-A isoform expression was analyzed by RT-PCR. EOC stem cell, epithelial ovarian cancer stem cell; mOCC, mature ovarian cancer cells; OSE, normal ovarian surface epithelial cells.

in various human cancers.[12] Overexpression of Aurora-A was implicated in promoting cell proliferation and inhibiting apoptosis in esophageal squamous cell carcinoma cell line[13] and glioblastoma.[14] Moreover, it was shown that elevated Aurora-A expression, at levels that reflect cancer-associated gene amplification, overrides the checkpoint mechanism that monitors mitotic spindle assembly, inducing resistance to the chemotherapeutic agent paclitaxel.[15]

In this study, we investigated the effect of the Aurora-A inhibitor, MK-5108 on EOC stem cells. We document that MK-5108 treatment can induce cell cycle arrest in the EOC stem cells. Furthermore, we demonstrate that MK-5108 inhibits the constitutive NFκB activity in these cells. Our study identify a regulatory circuit where Aurora-A inhibition can inhibit NFκB activity by promoting the accumulation of the IκBα in the nucleus. These findings indicate the possible value of Aurora-A inhibitors in ovarian cancer patients.

Results

Aurora-A kinase is overexpressed in ovarian cancer cells. We first determined Aurora-A kinase expression in a panel of EOC stem cells, CD44-negative mature ovarian cancer cells (mOCCs) and normal ovarian surface epithelial cells (OSEs) using quantitative real-time PCR (qRT-PCR). As shown in **Figure 1A**, Aurora-A mRNA expression levels were significantly elevated in the ovarian cancer cells tested compared to the OSEs. Six transcript variants of Aurora-A have been identified, which differ in the structure of first and second exons from non-coding region (**Fig. 1B**). To determine which of these transcript variants are expressed in the cells we tested, we designed specific pairs of primers for each isoform. All cells tested express isoforms II and V (**Fig. 1C**). Thus, OSEs and ovarian cancer cells do not differ in the isoforms of Aurora-A expressed, the difference is in the level of expression.

Inhibition of Aurora-A kinase induce cell death in EOC stem cells. Since the EOC stem cells represent the chemoresistant cell population, we focused on the possible effect of Aurora-A inhibition in these cells. Thus, a panel of EOC stem cells was treated with a novel Aurora-A kinase inhibitor, MK-5108, which exhibits potent activity against Aurora-A in an ATP-competitive manner.[16] The effect of MK-5108 on

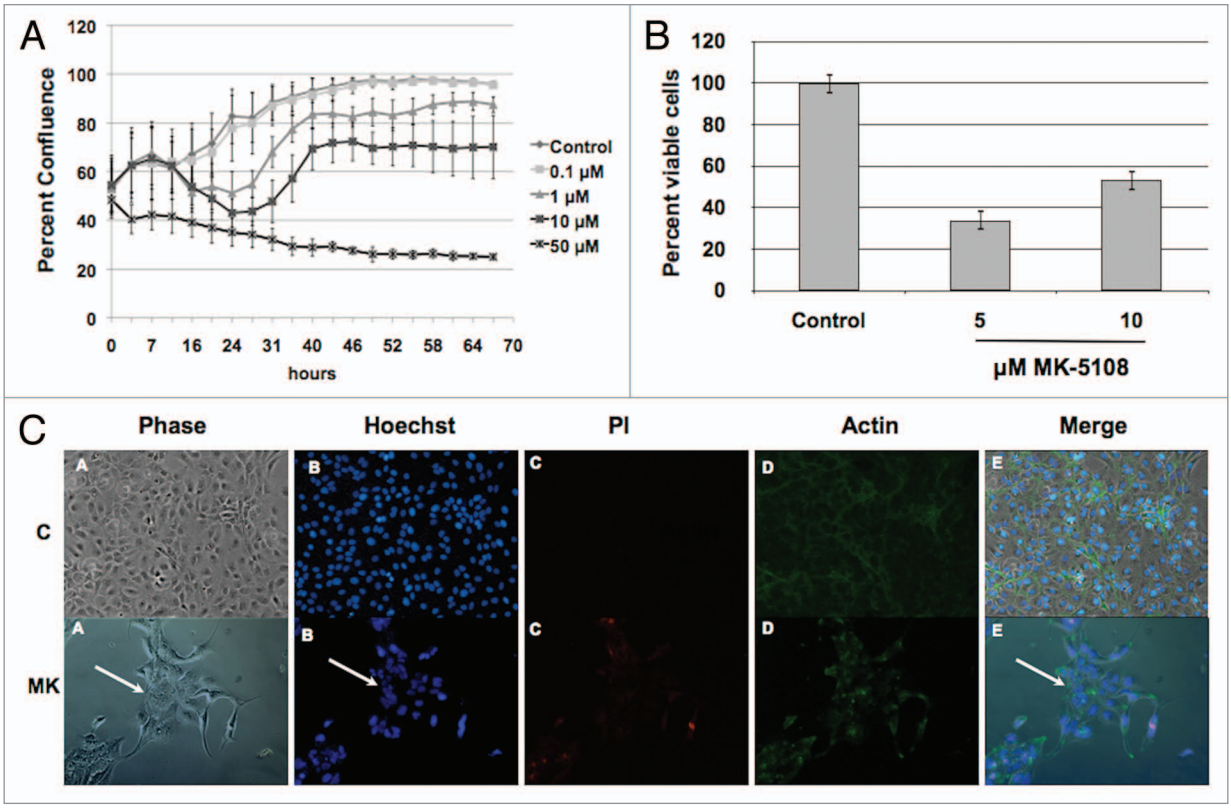

Figure 2. MK-5108 induce cell death in EOC stem cells. EOC stem cells were treated with DMSO vehicle or increasing doses of MK-5108 for 72 hr. (A) Growth rate was determined using the Incucyte real-time video imaging system; (B) Cell viability was determined using CellTiter 96 assay; (C) Representative immunofluorescence images of control and MK-5108-treated EOC stem cells. Cells were stained with propidium iodide (red) and F-actin (green). Nuclei were visualized by 4′,6-diamidino-2-phenylindole (DAPI) staining (blue). Representative data shown are for EOC stem cell 1.

the growth rate of the EOC stem cells was determined by the Incucyte real-time video imaging system. Inhibition of Aurora-A by MK-5108 had a significant inhibitory effect on the growth of EOC stem cells in a dose and time dependent manner (**Fig. 2A**), compared to the vehicle-treated cells. Moreover, quantification of viable cells after 72 h of treatment showed that MK-5108 significantly decreased the percentage of viable EOC stem cells (**Fig. 2B**). When EOC stem cells were stained with Hoechst 3332 for nuclear evaluation, propidium iodide to determine plasma cell integrity and actin-phalloidin for evaluation of cytoskeleton structure, we saw multiple, aggregated, Hoechst 3332-positive cells in cultures treated with MK-5108 but not in the vehicle control (**Fig. 2C**). Similarly, a high number of propidium iodide-positive cells with rearranged cytoskeletal structure were found in the treated cultures (**Fig. 2C**). Taken together, these results provide evidence that EOC stem cells undergo cell death as a result of Aurora-A inhibition.

MK-5108 induces polyploidy in EOC stem cells. Our next objective was to further characterize the cell death process in the EOC stem cells upon Aurora-A inhibition. Apoptosis or programmed cell death leads to the formation of apoptotic cells with contracted cytoplasm, condensed chromatin and culminates in the formation of apoptotic bodies. However, treatment with MK-5108 did not result in significant increase in caspase-3 activity (**Fig. 3A**). In addition, microscopic analysis of the EOC stem cells after treatment with MK-5108 did not reveal morphological changes associated with apoptosis, instead we observed the presence of multi-nucleated cells or polyploidy morphology (**Fig. 3B**).

MK-5108 induced cell death is associated with cell cycle arrest. The presence of multi-nucleated cells suggests cell cycle arrest. Indeed, Aurora-A has been shown to be involved in cell cycle regulation, particularly in cytokinesis.[11] Cell cycle analysis after MK-5108 treatment revealed a significant decrease in the number of cells at G_1 with high percentage of cells that were arrested in G_2/M phase (**Fig. 4**). This correlates with the observed appearance of multi-nucleated cells after treatment with MK-5108.

MK-5108 inhibits constitutive NFκB activity in EOC stem cells. EOC stem cells are characterized by constitutive NFκB activity and constitutive secretion of pro-inflammatory cytokines.[5,7,8] In addition, it has been shown previously that Aurora-A can affect NFκB activity by direct phosphorylation of IκBα.[17] To determine the effect of Aurora-A inhibition on the NFκB pathway in EOC stem cells, we used a luciferase reporter system with two NFκB promoter elements.[7] MK-5108 treatment resulted in a significant reduction in the baseline constitutive NFκB activity (**Fig. 5A**). To further confirm the inhibitory effect on the NFκB pathway, we measured the levels of cytokines and chemokines that has been shown previously to be constitutively secreted by the EOC stem cells.[5,7] Treatment of EOC stem cells with

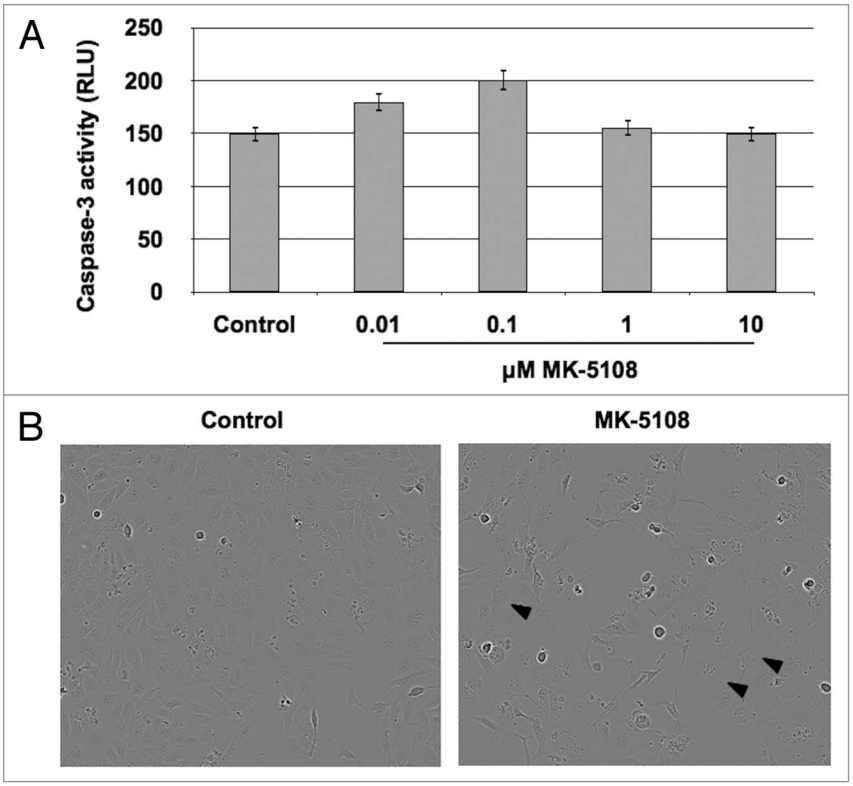

Figure 3. MK-5108's effect on the caspase cascade in EOC stem cells. (A) EOC stem cells were treated with DMSO vehicle or increasing doses of MK-5108 and activity of caspase 3/7 measured using CaspaseGlo. (B) Microscopic analysis showed multi-nucleated cells (black arrow). Representative data shown are for EOC stem cell 1.

MK-5108 resulted in a significant reduction in all cytokines/chemokines tested, compared to control (**Fig. 5B**).

To further understand the molecular mechanism by which Aurora-A may affect the NFκB pathway in the EOC stem cells, we evaluated the expression of IκBα in the cytoplasmic and nuclear fractions of EOC stem cells after treatment with MK-5108. Western blot analysis showed a time-dependent increase in nuclear IκBα upon inhibition of Aurora-A (**Fig. 6**). Interestingly, there was no significant change in the levels of cytoplasmic IκBα upon inhibition of Aurora-A (**Fig. 6**).

MK-5108 inhibits TNFα-induced NFκB activity in EOC stem cells. Since we showed that MK-5108 can inhibit baseline NFκB activity in EOC stem cells, we also determined if it can inhibit TNFα-induced NFκB activation. Thus, EOC stem cells were treated with TNFα in the presence or absence of MK-5108. TNFα was able to significantly increase luciferase activity compared to control cells, however, the addition of MK-5108 significantly inhibited TNFα-induced NFκB activation (**Fig. 5A**). These results were confirmed by immunofluorescence, which showed that compared to control, EOC stem cells treated with TNFα have higher nuclear p65, which was significantly

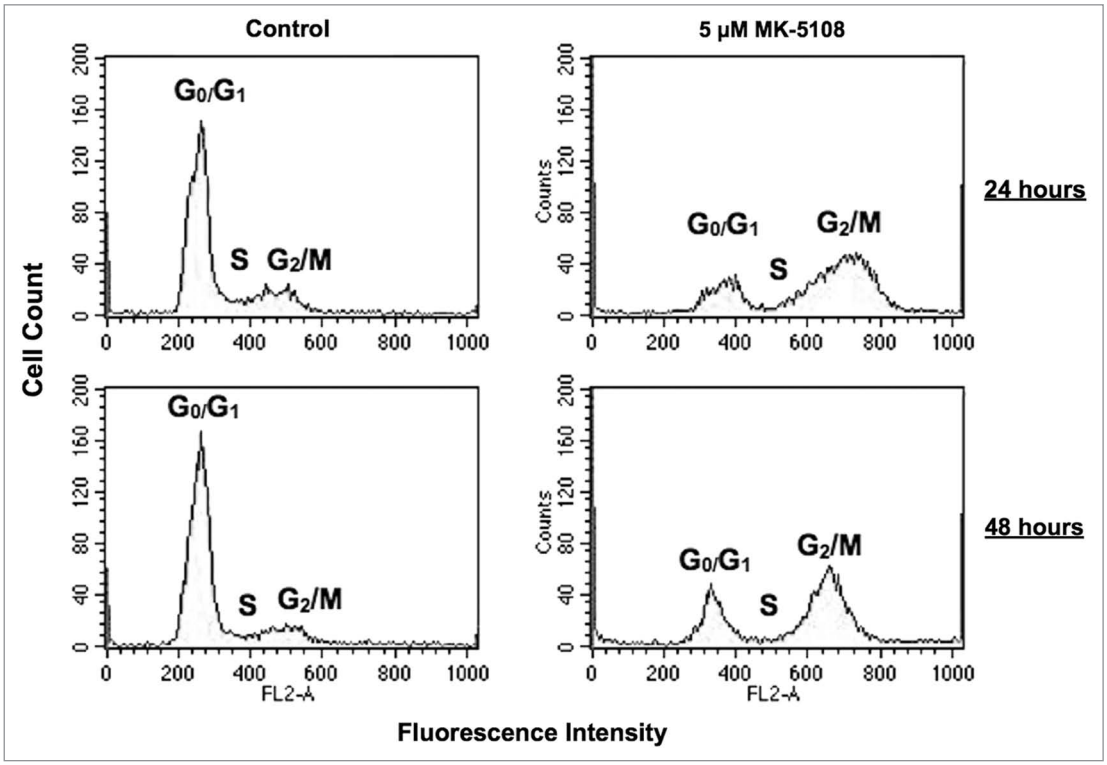

Figure 4. MK-5108 arrests EOC stem cells in G_2/M phase. EOC stem cells were treated with DMSO vehicle or 5 μM MK-5108 for 24 h or 48 h and cell cycle analysis performed with propidium iodide staining and flow cytometry. Representative data shown are for EOC stem cell 1.

downregulated in the presence of MK-5108 (**Fig.** 7). Taken together, these results show that the inhibition of Aurora-A can inhibit both basal and induced NFκB activity in the EOC stem cells.

Discussion

In this study we describe the effect of Aurora-A inhibition in EOC stem cells using a specific inhibitor, MK-5108. We showed, that MK-5108 can decrease the growth of EOC stem cells, induce the formation of multi-nucleated cells and arrest the cells in G_2/M phase. Moreover, we demonstrate that MK-5108 can abrogate NFκB activity, as well as cytokine and chemokine secretion in the EOC stem cells.

The CD44$^+$ EOC stem cells represent the population with tumor-initiating as well as chemoresistant properties.[5] Further characterization of these cells showed high levels of genes associated with pleuripotency such as β-catenin, Sox-2 and SSEA-4. The plasticity of these cells was shown with the demonstration that they can serve as tumor vascular progenitors.[6] EOC stem cells can form vessel-like structures in vitro when cultured in high density matrigel, and this process is associated with the acquisition of endothelial cell markers, CD-34 and VE-cadherin. This role is further shown in vivo with the demonstration that xenografts obtained from human EOC stem cells contained CD-34-positive cells of human origin.[6] Another important characteristic of the EOC stem cells is the constitutive secretion of pro-inflammatory cytokines such IL-6, IL-8, MCP-1 and GRO-α, which is brought about by its constitutively active NFκB pathway.[7,8] The pro-inflammatory microenvironment created as a result impacts not only cell growth and response to chemotherapy but also interaction with the immune system. Taken together these suggest that targeting the EOC stem cells is essential for disease-free prognosis.

Aurora-A is a key regulator of mitosis and plays an important role in centrosome function, spindle assembly and mitotic entry.[12] Its overexpression was reported in various malignancies, including breast, colorectal, pancreatic and gastric cancer.[18-20] In this study, we showed that Aurora-A is overexpressed in ovarian cancer cells compared to OSEs. In addition, we showed that variants II and V are predominant. This is important in view of several studies showing that the composition and length of the splice variants influence efficiency of transcription processing and half-life of the protein.[21]

MK-5108 is a novel ATP-competitive inhibitor of Aurora-A. This compound showed robust selectivity against other members of the family (Aurora-B: 220 fold and Aurora C:190 fold).[16] Screening with MK-5108 showed inhibition of growth in 14 cell lines from breast, cervix, colon and pancreas cancer with IC_{50} values between 0.16 and 6.4 μM.[16] Intriguingly, MK-5108 was less effective on three different types of breast cell lines, without showing a potential correlation between Aurora-A expression and the response to MK-5108 treatment. This differential response

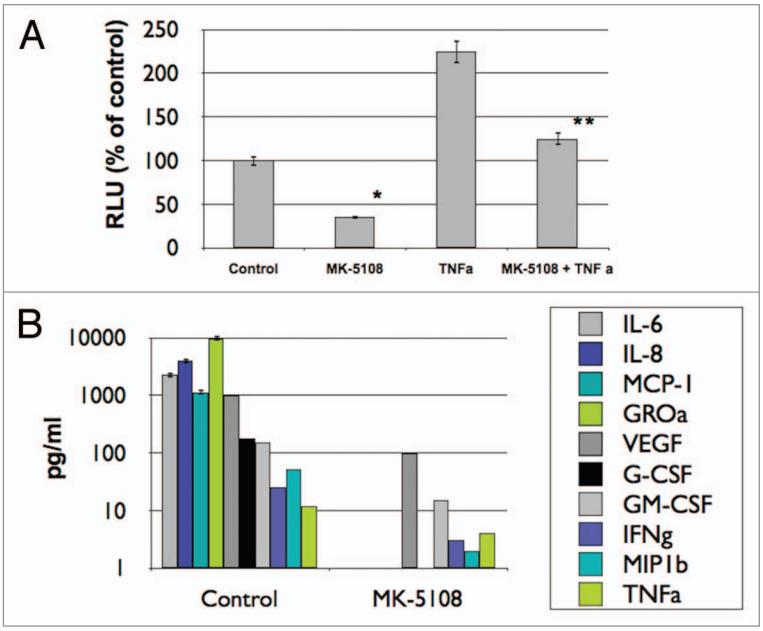

Figure 5. MK-5108 inhibits constitutive NFκB activity and constitutive cytokine secretion in the EOC stem cells. (A) EOC stem cells were treated with 5 μM of MK-5108 prior to treatment with 10 ng/ml TNFα. NFκB activity was measured using a luciferase reporter system. Bars show mean ± SEM. *p < 0.01 compared to control, **p < 0.01 compared to TNFα alone; (C) levels of secreted cytokines and chemokines were quantified in cell supernatants using xMAP technology. Representative data shown are for EOC stem cell 1.

may be associated with the differentiation status of the cells rather than the tumor type. If that is the case, the presence of a higher percentage of cancer stem cells in the total cell population could affect the overall response to the treatment.

In this study we show that MK-5108 can inhibit growth and induce G_2/M arrest in a panel of EOC stem cells at micromolar levels. These findings are in agreement with previous studies describing the effect of Aurora-A inhibitors in multiple myeloma, medulloblastoma and gastroenteropancreatic neuroendocrine tumor cell lines.[22-24]

An interesting morphological change observed in the EOC stem cells treated with MK-5108 was the presence of multi-nucleated cells. Multi-nucleation or polyploidy results from the inhibition of cytokinesis in the presence of normal DNA replication.[25] This morphological finding correlates with data showing an arrest in the G_2/M phase after MK-5108 treatment. It is known that cell cycle arrest and polyploidy can induce apoptosis or senescence in cancer cells and that normal cells are relatively more resistant to induced polyploidy.[25,26] Thus, it is plausible that the mechanism by which Aurora-A inhibition leads to cell death is related to its ability to induce polyploidy. This property may be important in the potential use of MK-5108 for the treatment of ovarian cancer where it can target cancer cells without affecting normal proliferating cells.

Another interesting observation is the deranged cytoskeleton structure after MK-5108 treatment. Considering the critical role of cytoskeleton structure in cell migration and hence invasion and metastasis, Aurora-A inhibition may result in diminished

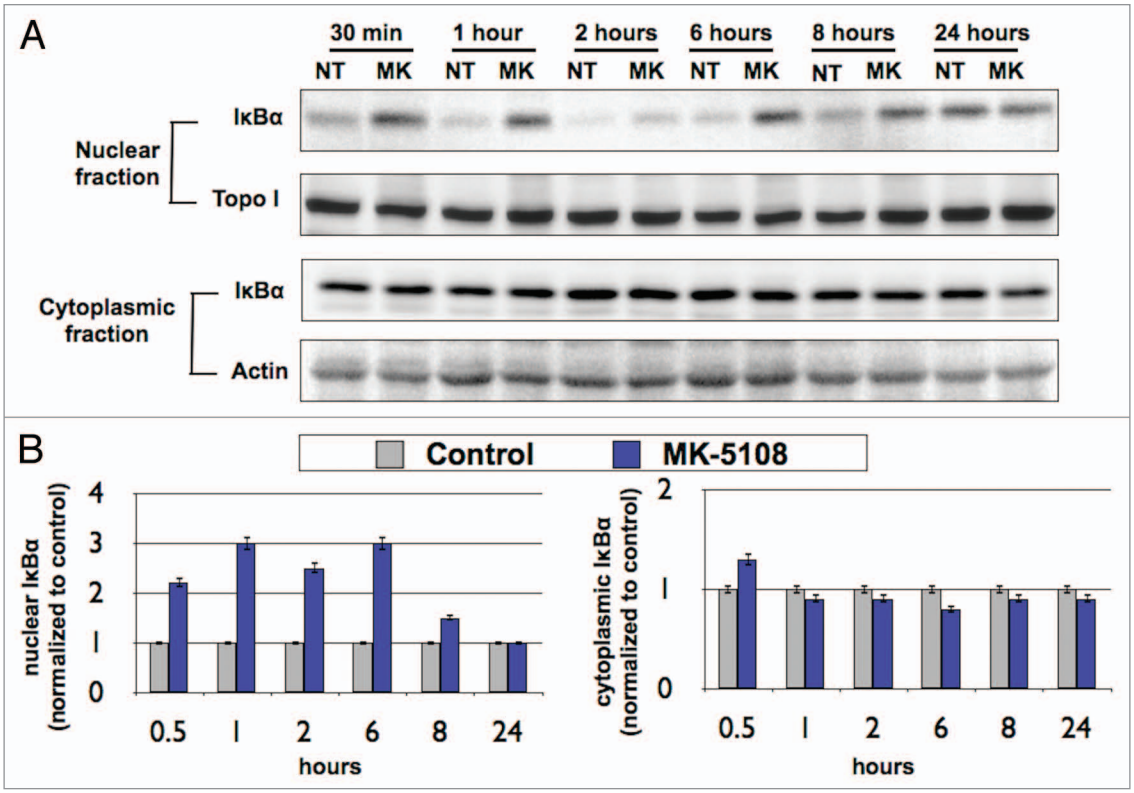

Figure 6. MK-5108 promotes accumulation of nuclear IκBα. (A) EOC stem cells were treated with 5 μM MK-5108 at time points shown and levels of IκBα determined in cytoplasmic and nuclear fractions by western blot analysis. (B) Densitometer graphs depicting IκBα levels normalized to topoisomerase (for nuclear fraction) and to actin (for cytoplasmic fraction). Representative data shown are for EOC stem cell 1.

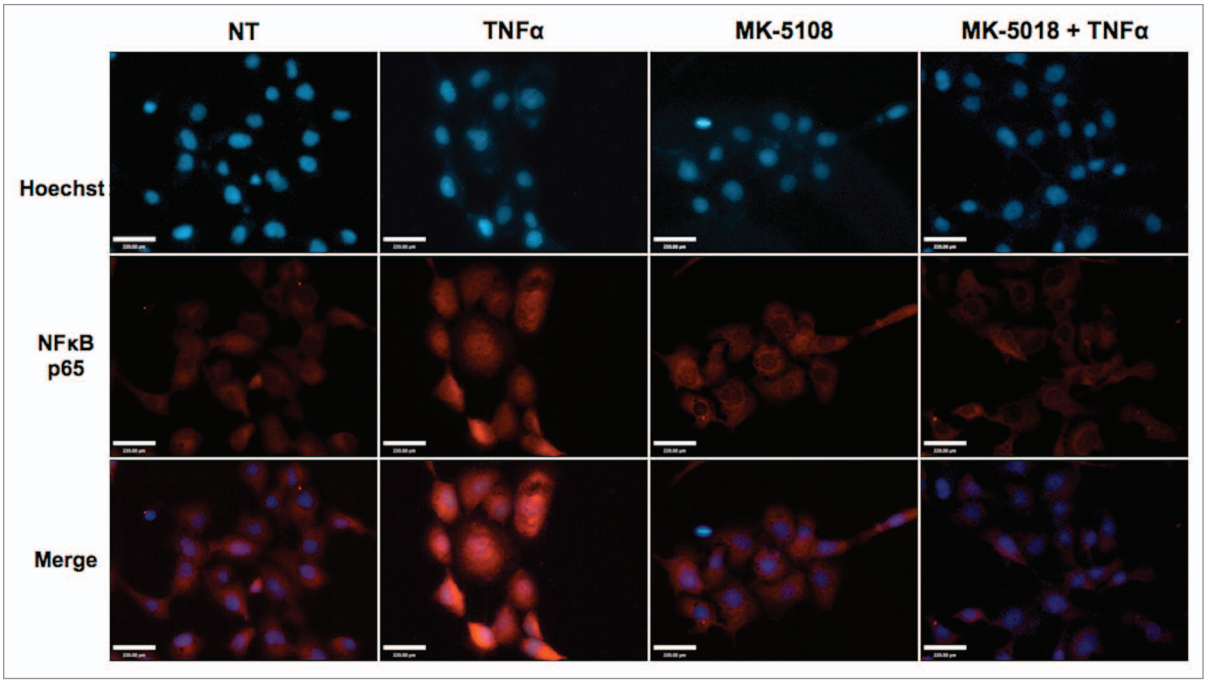

Figure 7. MK-5108 inhibits TNFα-induced NFκB activity in the EOC stem cells. (A) EOC stem cells were treated with 5 μM of MK-5108 prior to treatment with 10 ng/ml TNFα. Levels of nuclear p65 was determined by immunofluorescence with Hoechst dye.

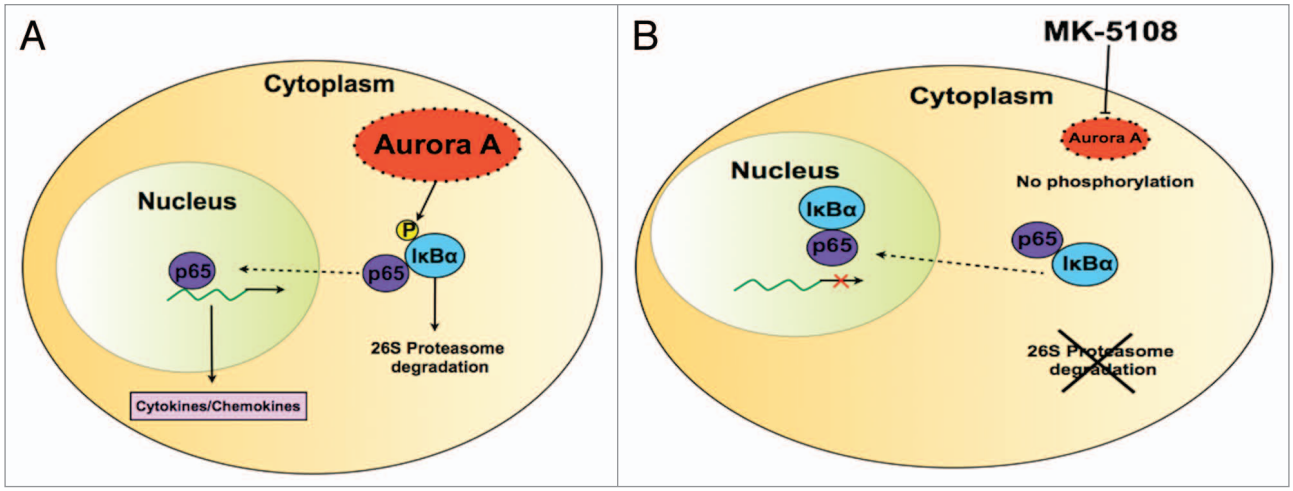

Figure 8. Proposed model for the role of MK-5108 in EOC stem cells on NFκB activity, see text for description.

metastatic capacity. Consistent with this suggestion is the demonstration that Aurora-A inhibition can suppress tumorigenicity in a pancreatic cancer model.[27]

Another major impact of MK-5108 in the EOC stem cells is the inhibition of the constitutively active NFκB pathway. Inhibition of this pathway has implications not only in growth, response to therapy, but also as mentioned above, can affect the tumor-immune cell interaction in the tumor microenvironment.[7,8,10] A new finding in this study is the accumulation of nuclear IκBα during MK-5108 treatment. It has been shown that IκBα is a target of Aurora-A and this interaction determines the stability of IκBα.[12] Upon phosphorylation of the residues Ser32 and Ser36, IκBα gets degraded leading to the activation of NFκB.[28] Thus, the importance of Aurora-A in EOC stem cells might be associated with the regulation of NFκB activity. By inhibiting Aurora-A we can increase the levels of IκBα and consequently inhibit NFκB (**Fig. 8**).

In light of the present results, we propose the following model for the role of Aurora-A in the EOC stem cells. Aurora-A induces phosphorylation of IκBα and its subsequent cytoplasmic degradation, as a result, EOC stem cells have constitutive NFκB activity, which creates a pro-inflammatory and anti-apoptotic environment. Upon Aurora-A inhibition, IκBα accumulates in the nucleus where it can bind to NFκB and inhibit its activity.

In summary, we report the characterization of Aurora-A inhibition in EOC stem cells as a potential target responsible for the inhibition of cell growth and proliferation. Our data suggest that Aurora-A may be an important link to the NFκB pathway, which is an important factor in tumor initiation and progression. The regulation of Aurora-A expression may be used as a potential therapeutic approach in EOC patients.

Materials and Methods

Cell cultures and culture conditions. Cells used in these studies were isolated from either ascites or cancer tissue from ovarian cancer patients and grown as previously described in reference 29. All patients signed consent forms and the use of patient samples was approved under the Yale University's Human Investigations Committee (HIC # 10425).

Reagents. Carboplatin and Paclitaxel were purchased from Sigma (St. Louis, MO). Aurora-A Kinase inhibitor MK-5108 was provided by Merck (Whitehouse Station, NJ). Rabbit anti-human β-actin antibody was purchased from Sigma (St. Louis, MO). Mouse anti-human NFκB-p65 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-human IκBα antibody was purchased from Cell Signaling Technology (Danvers, MA). Mouse anti-human caspase-2 and mouse anti-human Topoisomerase I antibodies were purchased from BD Pharmigen (Franklin Lakes, NJ).

Growth curves and cell viability assay. Cells (5,000 cells/well for EOC stem cells) were plated in a 96-well plate. After 24 hr, the medium was replaced with OptiMEM (Gibco, Invitrogen, Carlsbad, CA) for 4 hr followed by treatments in OptiMEM. Growth curves were constructed by imaging plates using the Incucyte system (Essen Instruments, Ann Arbor, MI), where the growth curves were built from confluence measurements acquired during round-the-clock kinetic imaging. Cell viability was determined using CellTiter 96 Assay (Promega, Madison, WI).

Caspase-3 activity assay. The activity of caspase-3 was measured using Caspase-Glo™ 3 (Promega, Madison, WI,), as previously described in reference 8.

Immunofluorescence. Cells were cultured in four well chamber slides and fixed with 4% paraformaldehyde for 20 min at room temperature, washed with PBS and then permeabilized in cold 100% methanol at -20°C for 10 minutes. To study cell membrane integrity, cells were incubated with Propidium iodide for 5 minutes and washed thoroughly prior to permeabilization. To visualize the actin cytoskeleton, cells were incubated with fluorescein-phalloidin dye (Biotium, Hayward, CA). For NFκB localization, cells were stained with mouse anti-p65 antibody as previously described in reference 10. Next, slides were incubated with Alexa Fluor546 anti-mouse IgG and

counterstained with Hoechst 33342 dye (Molecular Probes, Eugene, OR).

Examination of cell cycle. Cells (1–2 x 10^6) were harvested, washed twice using PBS and resuspended in 1 ml of PBS. The cells were fixed by adding drop-wise cold 100% ethanol at 4°C for 15 min. Next, the cells were stained with Propidium iodide (50 μg/ml) and RNase (100 μg/ml) at RT in the dark for 20 minutes. Cell cycle was determined by BD FACSCalibur (BD Biosciences, San Jose, CA) and analyzed by CellQuest software. The assay was repeated three times in duplicates.

Protein preparation and cellular fractionation. Protein extraction was carried out as previously described in reference 29. For total protein extraction, cell pellets were lysed on ice in 1x phosphate-buffered saline with 1% NP40, 0.1% SDS and freshly added 20 ml/ml protease inhibitor cocktail (Sigma Chemical) and 2 mM phenylmethylsulfonyl fluoride (Sigma Chemical). Cytoplasmic and nuclear fractions were separated using the NE-PER Nuclear and Cytoplasmic Extraction kit (Pierce Biotechnology, Inc., Rockford, IL) according to manufacturer's instructions. Protein concentration was determined by BCA Protein Assay (Pierce Biotechnology, Rockford, IL) and proteins were stored at -80°C until further use.

SDS-polyacrylamide gel electrophoresis and western blots. A quantity of 20 μg of each protein sample was denatured in sample buffer and subjected to 12% SDS-polyacrylamide gel electrophoresis as previously described in reference 29. The following antibody dilutions were used: mouse anti-human NFκB (1:1,000), mouse anti-human IκBα (1:1,000), mouse anti-human Topoisomerase (1:1,000), rabbit anti-human Caspase-2 (1:1,000) and rabbit anti-human β-actin (1:10,000). Specific protein bands were visualized using enhanced chemiluminescence (Pierce Biotechnology).

Quantification of NFκB activity. NFκB activity was measured using a luciferase reporter construct, pBII-LUC containing two κB sites before a FOS essential promoter (a gift from Dr. S. Ghosh, Yale University). Cells were stably transfected and luciferase activity measured as previously described in reference 10.

Cytokine profiling. Levels of cytokines and chemokines were measured from cell-free supernatants using the Bioplex Pro Cytokine Assay (Biorad, Hercules, CA). Data were acquired using the Bioplex system (Biorad) and analysis was carried out using the Bioplex software as previously described in reference 7.

Isoform specific RT-PCR. Total RNA was isolated using the high pure RNA isolation kit (Roche, Indianapolis, IN). One μg of total RNA isolated from each samples was then used as a template for cDNA synthesis, prepared with a first-strand cDNA synthesis kit (Thermo Scientific, Epsom, Surrey, UK). The expression of various isoforms was assessed by RT-PCR amplification 95°C for 10 min; (95°C for 15 s, 55.4°C for 45 s; 40 cycles) using Taq polymerase (Qiagen, Valencia, CA), using the gradient PCR system (Bio-Rad, Hercules, CA). To identify various isoforms, the PCR products were resolved by 4% agarose gel electrophoresis.

Quantitative RT-PCR. Total RNA was isolated using the high pure RNA isolation kit (Roche, Indianapolis, IN). One μg of total RNA isolated from each samples was then used as a template for cDNA synthesis, prepared with a first-strand cDNA synthesis kit (Thermo Scientific, Epsom, Surrey, UK). The expression of various transcripts was assessed by real-time PCR amplification 50°C for 2 min; 95°C for 10 min; (95°C for 15 s, 62°C for 45 s; 40 cycles) with Kapa-Sybr Fast qPCR mix (Kapa Biosystems, Boston, MA), using the CFX96-Real-Time System (Bio-Rad, Hercules, CA). All primers are designed as intron-exon spanning primers; primers sequences are available upon request. All PCR reactions were carried out in triplicate and validated by the presence of a single peak in the melt curve analysis. Changes in gene expression were calculated relative to GAPDH using the $2^{-\Delta Ct}$ method. After the quantification procedure, the products were resolved by 2.5% agarose gel electrophoresis to confirm that the reaction had amplified DNA fragments of expected size.

Statistical analysis. Data is presented as mean ± SD. Statistical significance ($p < 0.05$) was determined using one-way analysis of variance with the Bonferonni correction.

Acknowledgments

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