Aurora-A Is Essential for the Tumorigenic Capacity and Chemoresistance of Colorectal Cancer Stem Cells

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Abstract

Colorectal cancer stem cells (CR-CSC) are responsible for the generation and maintenance of intestinal tumors and are highly resistant to conventional chemotherapeutic agents. Aurora-A, a serine-threonine kinase involved in mitosis regulation, plays multiple key functions in tumor initiation and progression. We found that Aurora-A is overexpressed in primary colorectal tumor cells, in the CR-CSC fraction, and in stem cell–derived differentiated cells, compared with normal colon tissue. Aurora-A expression was functionally linked to centrosome amplification in CR-CSC, as indicated by the decrease in cells with multiple centrosomes that followed Aurora-A silencing. Knockdown of Aurora-A resulted in growth inhibition of CR-CSC, alteration of cell cycle kinetics, and downregulation of the expression levels of antiapoptotic Bcl-2 family members, strongly sensitizing to chemotherapy-induced cell death. Moreover, Aurora-A silencing compromised the ability to form tumor xenografts in immunocompromised mice and reduced the migratory capacity of CR-CSC. Altogether, these results indicate that Aurora-A is essential for CR-CSC regeneration and resistance to cytotoxic stimuli and suggest that therapies directed against Aurora-A may effectively target the stem cell population in colorectal cancer. Cancer Res; 70(11); OF1–11. ©2010 AACR.

Introduction

Emerging evidence suggests the existence of a functional heterogeneity in different cell populations shaping the tumor mass. A small subpopulation of cells within the tumor mass called “cancer stem cells” (CSC) or “cancer-initiating cells” was shown to sustain tumor growth upon injection into immunocompromised mice, in contrast to more differentiated cells that are nontumorigenic (1). CSCs were first identified in human hematologic malignancies (2) and later in several solid tumors (3-7). Colorectal CSC (CR-CSC) isolated from human tumors can be enriched in vitro through culture in nonadherent conditions in the presence of basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF), and subsequently propagated as “colospheres.” When injected into immunocompromised mice, sphere-derived CSCs retain the ability to generate tumor xenografts with morphologic structure and antigenic profile that reproduce those of the parental human tumors (1, 7).

The question remains open of whether CSCs originate from normal tissue stem cells or from partially differentiated progenitors, acquiring unlimited self-renewal potential as a consequence of genetic and/or epigenetic alterations. Because the tumorigenic process requires the accumulation of multiple mutational events, stem cells seem to be the ideal candidates to support this model due to their long life span (8, 9). Genomic instability (GIN) is a key molecular step in neoplastic transformation. The mechanisms underlying GIN have been investigated in embryonic stem cells, in which it has been shown that the mitotic-spindle checkpoint, although functional, does not initiate apoptosis, thus contributing to karyotypic instability (10). However, mechanisms of GIN in CSC represent a largely unexplored field. It was recently shown that embryonic germ cells transplanted in the testis of severely immunodeficient mice could generate a highly metastatic CSC population characterized by new nonclonal genomic rearrangements (11). GIN can be broadly classified into microsatellite instability and chromosomal instability. Chromosomal instability is observed in ~85% of colon cancers occurring as the consequence of mutation or amplification of several genes such as hBUB1, BRCA1, BRCA2, and Aurora-A (12).

Aurora-A (also known as STK15/BTAK) is a member of a serine/threonine kinase family involved in mitosis entry, formation of spindle bipolarity, control of centrosome maturation, and segregation during mitosis (13). Repression of
Aurora-A by RNA interference delays mitotic entrance in several cancer cells (14–16), whereas its overexpression leads to centrosome amplification, cytokinesis inhibition, and aneuploidy (17, 18).

The Aurora-A gene maps to chromosome 20q13.2, a region frequently amplified in several human epithelial tumors (19, 20). Overexpression of Aurora-A is sufficient to transform rodent fibroblast cell lines giving rise to aneuploid cells (18). Moreover, it was recently shown that Aurora-A overexpression in mouse mammary epithelium induced malignant transformation preceded by centrosome amplification, chromosome tetraploidization, and premature sister chromatid segregation (21). This evidence establishes Aurora-A as an oncogene that causes neoplastic transformation through the induction of GIN.

Aurora-A is also a key regulatory component of the p53 pathway as it phosphorylates p53 at Ser215 and Ser315, abrogating p53 DNA binding/transactivation activity and leading to p53 proteolytic degradation after MDM2-mediated ubiquitination (22, 23). Consequently, Aurora-A overexpression abrogates wild-type p53 functions, such as growth arrest and/or apoptosis, entailing a checkpoint-response deregulation and thus contributing to GIN and malignant transformation.

Due to its role as a spindle checkpoint regulator, Aurora-A has been implicated in conferring resistance to chemotherapy in cancer cells. Increased expression of Aurora-A confers resistance to apoptosis induced by chemotherapeutic agents (24, 25), whereas interference with Aurora-A expression sensitizes tumor cells to antineoplastic treatments (26, 27).

In this study, we show that Aurora-A is essential to maintain CR-CSC tumorigenicity, chemoresistance, and migratory ability, indicating it as an attractive target for the development of new therapeutic approaches for colorectal cancer treatment.

Materials and Methods

Tumor specimens and cells

Colon cancer specimens were obtained from patients undergoing surgical resection, in accordance with ethical standards of the Institutional Committee of University of Palermo on human experimentation. Normal colon specimens were obtained from peritumoral tissue. Enzymatic digestion was performed using collagenase (1.5 mg/mL; Invitrogen) and hyaluronidase (20 μg/mL; Sigma-Aldrich) for 6 hours, dissociated by trypsinization, swollen in 75 mmol/L KCl, fixed with 3:1 methanol/acetic acid, and dropped on slides from a microtome using cryosections of human normal and cancer tissues, on primary cancer cells and SDAC (grown on polylysine-coated glass coverslips), and on CR-CSC (cytospun on microscope slides). Tissue sections were fixed in ice-cold acetone, whereas cells were fixed in 2% parafomaldehyde, permeabilized with 0.2% Triton-X, and blocked with 0.5% bovine serum albumin. Samples were exposed to antibodies against γ-Tubulin (Sigma-Aldrich), Aurora-A (CST), CD133 (Miltenyi Biotec), CD29 (Calbiochem), cytokeratin 20 (DAKO), epithelial antigen (Ber-EP4, DAKO), and β-Catenin (Santa Cruz), or isotype-matched controls. Then, cells were treated with fluorochrome-conjugated anti-mouse or anti-rabbit antibodies (Invitrogen) plus RNase A (200 ng/mL, Sigma-Aldrich) and counterstained using Toto-3 iodide (Invitrogen). For centrifuge counting, at least five randomly selected regions for slides were analyzed and a minimum of 500 nuclei was counted for each sample. Samples were analyzed on a Nikon confocal microscope equipped with ×40 oil objectives and EZ-C1 software.

Ploidy determination

CR-CSCs were treated with 0.2 μg/mL colcemid (Sigma-Aldrich) for 6 hours, dissociated by trypsinization, swollen in 75 mmol/L KCl, fixed with 3:1 methanol/acetic acid, and dropped onto glass microscope slides. Slides were dried and stained with 3% Giemsa. Chromosome numbers were evaluated using a BX60 Olympus microscope under a ×100 oil objective.

Cell cycle analysis

Dissociated CR-CSCs were fixed overnight in ice-cold 70% ethanol. Cells were resuspended in PBS containing 50 μg/mL.
propidium iodide (Sigma-Aldrich), 3.8 mmol/L sodium citrate, and 10 μg/mL RNase. Samples were analyzed by a FACSCalibur cytometer and CellQuest Software (Becton Dickinson).

**Real-time PCR**

Total RNA was obtained using the Rneasy Mini kit (Qiagen GmbH) and retrotranscribed using the High-Capacity cDNA Archive kit (Applied Biosystems) according to the manufacturer’s instructions. Quantitative PCR analysis was performed with the ABI PRISM 7900HT (Applied Biosystem). The probes and primer sets (all from Applied Biosystems) used were as follows: Hs01582072_m1 (Aurora-A), Hs00195591_m1 (Snail), Hs00195591_s1 (Twist), and Hu glyceraldehyde-3-phosphate dehydrogenase. Relative quantitation of gene expression was calculated on triplicate reactions using the ΔΔCt method. Data processing was performed using the ABI PRISM SDS software v2.1 (Applied Biosystems).

**Cell viability**

Cell viability was evaluated on primary cancer cells, CR-CSC and SDAC-transduced PLK0.1-shAurora-A, or control shRNA and treated with 50 μg/mL 5-fluorouracil (5-FU) and/or 100 μmol/L oxalaplatin (both from Sigma-Aldrich) for 24 hours. Viability was assessed using the CellTiter Aqueous Assay kit (Promega Corp.) according to the manufacturer’s instructions. Cell death was analyzed with orange acridine (50 μg/mL) and ethidium bromide (1 μg/mL) staining.

**Lentiviral transduction**

Gene transfer of primary tumor cells, CR-CSC, and SDAC was performed with the PLK0.1 lentiviral vector (Sigma-Aldrich) and sequenced-verified short hairpin RNA (shRNA) for Aurora-A or an equivalent scrambled sequence. Lentiviral supernatants were collected 48 hours after calcium phosphate transfection of HEK-293T packaging cell line and centrifuged at 141,000 g at 4°C for 90 minutes to obtain a virus-containing pellet that was resuspended in DMEM or stem cell medium. Conditions. Cell death was analyzed with orange acridine (50 μg/mL) and ethidium bromide (1 μg/mL) staining.

**Invasion assay**

Dissociated CR-CSCs (1.5 × 10⁶) were plated on solidified growth factor–depleted Matrigel (BD) diluted 1:3 in serum-free medium in 8-μm pore size Transwell (Corning). 3T3 stem–conditioned medium was used as chemoattractant. Chambers were incubated at 37°C for 48 hours.

**Generation of tumor xenografts**

Five- to 6-week-old female athymic (nu+/nu+) mice were obtained from Charles River Laboratories and maintained according to the institutional guidelines for animal experimentation. CR-CSCs (5 × 10⁵) transduced with PLK0.1-shAurora-A or control shRNA were suspended in 100 μL of PBS/Matrigel and injected s.c. Tumor mass size was measured weekly for up to 8 weeks according to the formula: (π/6) * larger diameter * (smaller diameter)².

**Statistical analysis**

Statistical significance was determined by ANOVA (one way or two way) with the Bonferroni posttest using Graph-Pad Prism (GraphPad Software, Inc). Results were considered statistically significant when P values were <0.05. Asterisks indicate P < 0.05 (*) and P < 0.01 (**).

**Results**

**Aurora-A is expressed in human colon CSC**

CR-CSCs were previously characterized by our and other groups through surface expression of CD133 and absence of differentiation markers such as CK20 (1, 7, 30). Colon tumor specimens (Table 1) were phenotypically characterized by H&E and Alcian blue staining, and analyzed by immunohistochemistry for the expression of CK7, CK20, and CD133 (Fig. 1A). CR-CSCs were characterized through the CD133⁻/CD29⁻/CK2₀⁻ phenotype (Fig. 1A) and further validated through the capacity to form colon tumor xenografts in immunodeficient mice (data not shown; ref. 1).

RNA analysis performed on five different samples showed that Aurora-A was barely detectable in normal colon tissue, whereas it was clearly expressed in primary tumor cells, CR-CSC, SDAC, CD133⁻, and CD133⁺ colon tumor populations (Fig. 1B). Immunoblot analysis revealed a high expression of Aurora-A in tumor specimens compared with normal tissue (Fig. 1C, top), as subsequently confirmed in matched samples derived from five different patients (Fig. 1C, bottom). Among samples derived from the same patient, the highest Aurora-A expression was displayed by SDAC, possibly reflecting their high proliferative rate. Further immunofluorescence analysis showed that Aurora-A expression was barely detectable in normal specimens, whereas a strong signal was detected in cancer tissue sections and in all tumor cell populations (Table 1; Fig. 1D). A diffuse cytoplasmic immunoreactivity was observed in primary tumor cells, whereas in CR-CSC and SDAC, the expression was also nuclear and more intense in dividing cells. Centrosome localization of Aurora-A in G2/M cells also revealed mitotic spindle alterations (Fig. 1D). Altogether, these observations point to a deregulation of Aurora-A expression in colon cancer. In addition, the presence of Aurora-A in CR-CSC and in CD133⁺ cells suggests that it plays an unpredicted role in the CR-CSC compartment. Because it was hypothesized that CSC could derive from normal stem cells, we investigated the expression of Aurora-A and CD133 both on normal and tumoral tissues. Accordingly (31), in normal colon, Aurora-A was expressed only in a small population (3.6 ± 0.6%) located in proximity to colon cells with the high proliferative index (Supplementary Fig. S1A, top). The rare CD133³ population present in the normal colon was mostly negative for Aurora-A (Supplementary Fig. S1A, top), whereas Aurora-A was detected in CD133⁻ and CD133⁺ epithelial cell populations.
in colon cancer (Supplementary Fig. S1A, bottom). To verify if Aurora-A was able to transform normal colon epithelial cells, we transduced the NCM460 normal colon cell line with Aurora-A and assessed their ability to form colonies in semi-solid culture and xenografts in immunocompromised mice (Supplementary Fig. S1B–E). In contrast to what we observed in other cellular systems (17, 18, 21), Aurora-A expression was not capable per se to induce cell transformation, suggesting a tissue-specific influence of this gene on growth regulation.

Because loss-of-function mutations of p53 may induce a phenotype analogue to Aurora-A overexpression such as centrosome amplification, GIN, and oncogenic transformation (32, 33), we analyzed p53 status in CRC samples to select specimens in which the effects of Aurora-A overexpression were not masked by those of p53 mutations. We found wild-type p53 in 8 of the 15 Aurora-A overexpressing CR-CSC lines analyzed (53%; Supplementary Fig. S2A; Table 1) and we selected such samples for further analyses.

### Aurora-A overexpression is responsible for centrosome amplification in CR-CSC

Aurora-A has been reported to regulate centrosome number and genomic stability (15, 18). To investigate whether altered centrosome numbers were found in CR-CSC overexpressing Aurora-A, we evaluated the number of centrosomes in primary tumor cells, CR-CSC, and SDAC (Fig. 2A, left). We observed supernumerary centrosomes in 20 ± 5% of primary colon cancer cells, in 15 ± 6% of CR-CSC, and in 19 ± 2% of SDAC (Fig. 2A, right), whereas a regular number of centrosomes was observed in normal colon sections (data not shown).

Transduction of the Aurora-A silencing construct, but not of a control scrambled shRNA, resulted in the efficient knockdown of Aurora-A expression at the RNA and protein levels in all the treated tumor cell populations (Fig. 2B). γ-Tubulin immunostaining of cells expressing Aurora-A shRNA revealed a significant reduction in the number of cells with more than two centrosomes and a simultaneous increase of cells with one centrosome (Fig. 2C), indicating that centrosome amplification is a reversible process linked to Aurora-A expression levels.

Cytogenetic analysis revealed a high percentage of aneuploidy (≥90%) in all cell populations analyzed (primary cells, CR-CSC, and SDAC derived from the same patient; Fig. 2D, left). Freshly isolated CD133+ cells showed a percentage of aneuploidy comparable with that observed in CR-CSC, thus

<table>
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<tr>
<th>Patient</th>
<th>Age/sex</th>
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<th>Aurora-A protein expression*</th>
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*+ indicates immunohistochemical expression of Aurora-A in at least 20% of tumor cells.
†Mut, Mutated p53. Wt, wild-type p53.
excluding any effect of in vitro long-term culture. (Fig. 2D, left). Then, we investigated whether Aurora-A silencing was able to affect the number of chromosomes present in hyper-diploid colon tumor cells. However, we did not find a significant decrease in the number of cells with chromosomal alterations following Aurora-A knockdown. (Fig. 2D, right). These observations suggest that although Aurora-A over-expression may be involved in the initial development of 

Figure 1. Aurora-A is expressed in human CRC specimens and tumor cell subpopulations. A, immunohistochemical staining with H&E, CK7, and CK20, and Alcian Blue and immunofluorescence analysis of CD133 on colorectal cancer tissue sections (top) and CD133, CD29, and CK20 on CR-CSC (bottom). Nuclei of immunohistochemical sections were revealed by hematoxylin, whereas nuclei of immunofluorescence analyses were counterstained with Toto-3 (pseudocolored blue). B, mRNA expression of Aurora-A on peritumoral tissue (normal), cultured primary colon tumor cells (primary), CR-CSC, SDAC, CD133+, and CD133− cells freshly purified from cancer tissue. HeLa cells were used as positive control. Columns, mean of five independent experiments performed with five matched sets of samples, each derived from a different patient; bars, SD. C, immunoblot analysis of Aurora-A on samples as above. Columns, mean of five independent experiments, expressed as Aurora-A/β-Actin absorbance ratio; bars, SD. D, confocal microscopy analysis of Aurora-A on cryostat sections of normal (normal) and tumor (parental) colon tissue, cultured primary colon tumor cells (primary), CR-CSC, and SDAC. Arrowheads, supernumerary centrosomes. One representative experiment of 20 performed with cells derived from different patients is shown.
Figure 2. Aurora-A controls centrosome numbers in CR-CSC. A, immunofluorescence analysis of cultured primary colon tumor cells (primary), CR-CSC, and SDAC stained with anti-γ-Tubulin antibody. Arrowheads, supernumerary centrosomes. One representative experiment of five performed is shown (left). Percentage of cells with one (1 centr), two (2 centr), or more than two centrosomes (>2 centr) assessed in five different cultures of primary colon tumor cells (primary), CR-CSC, and SDAC (right). B, mRNA (left) and protein (right) levels of Aurora-A on primary colon tumor cells (primary), CR-CSC and SDAC transduced with control shRNA (SCR), or Aurora-A shRNA (sh-A). Columns, mean of the results obtained from five independent experiments performed with cells from different patients; bars, SD. C, percentage of cells with one (1 centr), two (2 centr), or more than two centrosomes (>2 centr) in primary colon tumor cells (primary), CR-CSC, and SDAC transduced as in B. *, P < 0.05. D, representative Giemsa-stained metaphases obtained from cultured primary colon tumor cells (primary), CR-CSC, CD133+ freshly purified cells (CD133*), and SDAC (left). Quantification of chromosome numbers, expressed as hyperdiploid (hyper), diploid (diploid), and hypodiploid (hypod) present in five samples of primary colon tumor cells (primary), CR-CSC, and SDAC transduced as in B (right).
aneuploidy, once chromosome alterations have been established, it becomes dispensable for their maintenance.

Knockdown of Aurora-A alters the cell cycle kinetics of CR-CSC and inhibits their growth in vitro

Targeting Aurora-A by means of chemical inhibitors or RNA interference has been shown to reduce tumor cell proliferation in several cancer types (14, 34). We found that Aurora-A silencing effectively inhibited the growth of all the tumor cell populations examined, indicating that Aurora-A is essential for the proliferation of colon cancer cells including the CR-CSC fraction (Fig. 3A). Furthermore, we observed a modest increase Aurora-A–silenced CR-CSC in G2/M (from 12–19%), a simultaneous decrease of cells in G0/G1 (from 69–53%), and an increase of the sub-G1 population (from 2–12%; Fig. 3B and C). These results suggest that Aurora-A silencing can induce G2 arrest in part of CR-CSC and apoptosis in another part of the population, possibly reflecting a heterogeneity in p53 levels within the same CR-CSC population.

In support of this hypothesis, we found that the expression levels of p53 were increased in CR-CSC transduced with Aurora-A shRNA compared with control shRNA (Supplementary Fig. S2B). Additional flow cytometry analysis revealed that CR-CSC (which are 100% positive for the epithelial antigen Ber-EP4) show a heterogeneous expression of p53 and such expression was increased by 55 ± 13.5% following Aurora-A knockdown (Supplementary Fig. S2C).

Aurora-A silencing decreases antiapoptotic protein expression and sensitizes CR-CSCs to chemotherapy-induced death

We have previously reported that CD133+ CR-CSCs are widely resistant to chemotherapeutic drugs (1). Because Aurora-A overexpression correlates to chemotherapy resistance in cancer cell lines (22, 35), we investigated whether Aurora-A silencing was able to overcome CR-CSC resistance to chemotherapy. Aurora-A silencing resulted in a slight decrease of basal cell viability in the three populations examined. As expected, control CR-CSCs were highly resistant to chemotherapeutic drugs, whereas the viability of primary cells and SDAC was moderately affected. Importantly, chemotherapy treatment of Aurora-A–silenced CR-CSC resulted in a significant increase in cell death compared with control CR-CSC, with a 75% reduction in cell viability obtained with the combination of both drugs (Fig. 4A and B). A similar sensitization to drug-induced death by Aurora-A knockdown was observed in primary tumor cells and SDAC (Fig. 4A). To investigate whether resistant cells would emerge from chemotherapy treatment despite Aurora-A knockdown as a possible consequence of increased p53 expression, we exposed CR-CSC that survived chemotherapy to a second round of drug treatment. Although Aurora-A–silenced CR-CSC died after 48 hours of drug restimulation, CR-CSC transduced with control shRNA showed only a modest effect on cell viability (Supplementary Fig. S2D), indicating that Aurora-A–mediated p53 inhibition is essential for CR-CSC chemoresistance. Altogether, these results show that Aurora-A silencing enhances the combined cytotoxic effect of 5-FU and oxaliplatin, thus representing a valid approach to sensitize CR-CSC to conventional treatment. We found a strong decrease in MDM2, Mcl-1, and Bcl-2 expression upon Aurora-A silencing, whereas Bcl-xL levels were moderately decreased (Fig. 4C). These findings suggest that Aurora-A overexpression may confer resistance to apoptosis both by promoting MDM2-dependent p53 degradation and by upregulating survival factors involved in the mitochondrial apoptotic pathway.

Aurora-A regulates the migratory ability and the tumorigenic capacity of CR-CSC

It has been hypothesized that CSCs are the only cells able to migrate from primary tumors to generate distal lesions (36). Because Aurora-A overexpression was shown to enhance the
migratory capacity of cancer cell lines (37), we investigated its involvement in cell motility of CR-CSC. Aurora-A silencing reduced the migratory capacity of CR-CSC after only 24 hours (Fig. 5A). Quantitative real-time PCR analysis showed that Aurora-A knockdown reduced the expression of Twist and Snail (Fig. 5B), two genes involved in epithelial-mesenchimal transition, suggesting that Aurora-A may modulate CR-CSC invasiveness through the regulation of key factors responsible for epithelial-mesenchimal transition. Finally, we investigated the role of Aurora-A in the production of tumor xenografts by CR-CSC. Nude mice were s.c. inoculated with CR-CSC transduced with control shRNA (SCR) or Aurora-A shRNA (sh-A) and treated for 24 h with 50 μg/ml 5-FU, 100 μmol/L oxaliplatin, or a combination of both drugs. Columns, mean of five independent experiments; bars, SD. **, P < 0.01 between control and Aurora-A–silenced CR-CSC. B, immunofluorescence staining with orange acridine/ethidium bromide of CR-CSC treated as in A. One representative experiment of five performed with cells from different patients is shown. C, immunoblot analysis (left) and relative band quantification (right) of MDM2, Bcl-xl, Mcl-1, and Bcl-2 on CR-CSC treated as in A. Columns, mean of five independent experiments performed with cells from different patients; bars, SD.

Discussion

Aurora kinases are critical for cell division and have been closely linked to tumorigenesis and cancer susceptibility. Aurora-A is overexpressed in colorectal tumors (17, 38–40) and is strongly related to chromosomal instability. We have found Aurora-A overexpression in 60% of colon tumor specimens examined, a percentage compatible with that observed previously (17, 39). Then, we have analyzed Aurora-A expression in different cell populations within colon cancer and found that Aurora-A was overexpressed in primary tumor cells, in CSC, in CD133+/- fractions, and in SDAC compared with nontumor tissue.

Although Aurora-A did not show a transforming ability per se in normal colon cells, the presence of an aberrant Aurora-A expression in the CR-CSC fraction suggests its contribution to neoplastic transformation by facilitating centrosome amplification and G1N with a subsequent occurrence of additional oncogenic mutations. In line with this hypothesis, several studies have shown a correlation between Aurora-A overexpression and aneuploidy (15, 18). In particular, a recent analysis of Aurora-A expression in chromosomally-unstable versus microsatellite-unstable sporadic colorectal cancers showed that higher Aurora-A gene copy numbers were found only in chromosomally-unstable colorectal carcinomas (41). Accordingly, we found that 90% of Aurora-A–overexpressing colon tumor specimens displayed a marked aneuploidy that could not be reverted by Aurora-A silencing.
However, we have observed a dynamic balance between Aurora-A expression and centrosome amplification in colon cancer cell populations, as Aurora-A silencing was able to restore normal centrosome numbers. According to studies by Katayama and colleagues (22), we found that Aurora-A regulates the expression levels of MDM2 in CR-CSC. Increased Aurora-A levels in CR-CSC could therefore contrast with the G1 checkpoint arrest by inactivating p53, allowing aneuploid cells to proceed during the S-phase of the cell cycle.

Our observation that Aurora-A controls MDM2 expression also supports a general model in which Aurora-A levels contribute to determine p53 status in cells with wild-type p53. An initial overexpression of Aurora-A would determine p53 activation as a consequence of GIN, although later, its constitutive overexpression would promote p53 degradation, thus creating a favorable environment for the establishment of additional genetic alterations. In accordance, Aurora-A silencing provoked an increased p53 stabilization in CR-CSC.

Our finding that Aurora-A is expressed in the stem cell fraction of colon tumors has important therapeutic implications. The CSC population is particularly resistant to conventional therapies due to high expression of ATP-binding cassette proteins, to hyperactive DNA repair pathways, or to decreased production of reactive oxygen species (42–44). Additionally, CR-CSCs have been shown to possess an autocrine and/or paracrine mechanism of interleukin-4 production that confers cell death resistance through the upregulation of antiapoptotic molecules (1). Our results show that Aurora-A silencing decreases the expression of antiapoptotic Bcl-2 family members (Bcl-2, Mcl-1, and, to a minor extent, Bcl-xL) in CR-CSC. Accordingly, we found that Aurora-A silencing sensitized CR-CSC to chemotherapy-induced cytotoxicity, at least in part by stabilizing p53 levels. Although the molecular mechanisms that link Aurora-A to antiapoptotic protein expression and chemoresistance remain to be fully clarified, Aurora-A therapeutic inhibition may increase the efficacy of chemotherapeutic drugs toward the stem cell population, possibly preventing tumor relapse and metastatic dissemination.

Overexpression of Aurora-A has been correlated with the occurrence of metastasis, whereas Aurora-A knockdown inhibited the proliferation and invasion of human esophageal cancer cells (16, 45). Our finding that Aurora-A knockdown compromised the migratory capacity of CR-CSC in vitro may have important implications given that CSC may play a central role in metastasis formation (36). Besides suggesting a nonmitotic role of Aurora-A in CSC, these observations suggest that pharmacologic Aurora-A inhibition may reduce the incidence of metastasis in cancer patients.

Finally, we found that Aurora-A silencing completely abrogates the ability of CR-CSC to form tumor xenografts.

**Figure 5.** Aurora-A silencing inhibits the in vitro migratory capacity and in vivo tumorigenicity of CR-CSC. A, migration assay of CR-CSC transduced with control shRNA (SCR) or Aurora-A shRNA (sh-A). Columns, mean of five independent experiments performed with cells from different patients; bars, SD. B, mRNA expression of Twist and Snail of CR-CSC treated as in A. Columns, mean of five independent experiments performed with cells from different patients; bars, SD. C, in vivo growth of CR-CSC transduced as in A expressed as the size of subcutaneous tumor xenografts derived from the injection of 5 × 10⁶ colosphere-derived cells. Points, mean obtained with five mice for each group (SCR and sh-A); bars, SD. D, representative analysis of tumor xenografts obtained as in C, showing a xenograft-bearing nude mouse 8 wk after CR-CSC injection (top) and H&E and CK20 staining of xenograft-derived paraffin-embedded sections.
highlighting its essential role in supporting CR-CSC tumorigenicity in vivo.

Although the overexpression of Aurora-A is not restricted to CR-CSC, our results support its involvement in the exclusive properties of CR-CSC such as resistance to conventional drugs and tumorigenic potential. Recently, Cicales and colleagues (46) showed that p53 stabilization in tumor mammospheres switches stem cell division from symmetrical to asymmetric, representing a potential mechanism of tumor suppression. By regulating p53 stability, Aurora-A could indirectly contribute to tumor growth in vivo through an increase of CSC self-renewal. Preclinical studies indicate that Aurora-A inhibitors lead to tumor stabilization and/or regression (47, 48), in accordance with a possible role of Aurora-A in driving colon cancer progression from the stem cell compartment. Future clinical studies will definitely clarify the therapeutic potential of Aurora-A inhibition in countering progression and metastasis of colon tumors.

Disclosure of Potential Conflicts of Interest

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