Aurora A Is Essential for Early Embryonic Development and Tumor Suppression

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Aurora A is a serine/threonine kinase that functions in various stages of mitosis. Accumulating evidence has demonstrated that gene amplification and overexpression of Aurora A are linked to tumorigenesis, suggesting that Aurora A is an oncogene. In addition, Aurora A overexpression has been used as a negative prognostic marker, because it is associated with resistance to anti-mitotic agents commonly used for cancer therapy. To understand the physiological functions of Aurora A, we generated Aurora A knock-out mice. Aurora A null mice die early during embryonic development before the 16-cell stage. These Aurora A null embryos have defects in mitosis, particularly in spindle assembly, supporting critical functions of Aurora A during mitotic transitions. Interestingly, Aurora A heterozygosity results in a significantly increased tumor incidence in mice, suggesting that Aurora A may also act as a haploinsufficient tumor suppressor. Consistently, Aurora A heterozygous mouse embryonic fibroblasts have higher rates of aneuploidy. We further discovered that VX-680, an Aurora kinase inhibitor currently in phase II clinical trials for cancer treatment, could induce aneuploidy in wild type mouse embryonic fibroblasts. We conclude that a balanced Aurora A level is critical for maintaining genomic stability and one needs to be fully aware of the potential side effects of anti-cancer therapy based on the use of Aurora A-specific inhibitors.

First identified in Drosophila, Aurora kinases are evolutionarily conserved serine/threonine kinases in all eukaryotes, which function mainly during mitosis. Although there is only one member of Aurora in yeast and two in Drosophila, three were identified in mammals (Aurora A/B/C) with highly conserved catalytic domains (1). Aurora A exerts its function at multiple steps during mitosis and is particularly important for centrosome maturation and spindle assembly (2). Aurora B localizes to centromeres during prometaphase and is mainly responsible for chromosome bi-orientation (3, 4). Aurora C is specifically expressed during meiosis and its function is less understood (5, 6).

The expression of Aurora A fluctuates during the cell cycle, being low in G1/S, and quickly rises in G2 and peaks at early mitosis (7–9). This expression pattern is consistent with its role as a key mitotic kinase. As a kinase, Aurora A has multiple substrates. The consensus phosphorylation site of Aurora A was first identified in yeast as [KR][XTS][ILV] (10), which was later confirmed in mammalian Aurora A substrates TACC, cdc25B, and NDE1 (11–13).

Aurora A is mainly localized at duplicated centrosomes and promotes centrosome maturation. Several cofactors for Aurora A including Bora (14), Ajuba (15), Tpx2 (16), and PAK1 (17) have been identified, which stimulate Aurora A kinase activity by promoting autophosphorylation of Thr288 at its T-loop (18). Aurora A also promotes mitotic entry, presumably through its centrosomal function. For examples, Aurora A targets the CDK1-Cyclin B1 complex to centrosome during prophase (15) and facilitates the activation of this complex by phosphorylating and degrading Cdc25B (13, 19), which is crucial for promoting mitotic entry and nuclear envelope breakdown. Recent evidence also suggests that Aurora A may directly participate in nuclear envelop breakdown (20). Besides its role in early mitotic phases, Aurora A also operate at other stages of mitosis including centrosome separation, spindle assembly, asymmetric cell division, and cell fate determination (2).

Accumulating evidence has demonstrated that Aurora A is an oncogene. Human Aurora A resides at chromosome 20q13, a region frequently amplified in breast cancers (21). Overexpression of Aurora A has also been identified in various tumors, and also leads to disruption of the spindle checkpoint and causes cells to become resistant to anti-mitotic agents (7, 8, 21). Cellular studies also demonstrate that overexpression of Aurora A leads to genomic instability and aneuploidy (8, 22), further lending credence to the claim that Aurora A promotes tumor formation. Many inhibitors of Aurora kinases have been designed and their potency in preventing tumor growth has...
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been tested widely both in vitro and in vivo (23, 24). VX-680, one of the first Aurora inhibitors, was shown several years ago to be an effective anti-tumor agent in xenograft models and eradicate leukemia cells (25). VX-680 binds to the ATP binding cleft of the kinase domain, which is shared in all 3 Aurora kinases, thus inhibiting kinase activity. Because Aurora A is highly expressed in tumors, it is believed that these small molecule inhibitors of Aurora kinases could be potent anti-cancer agents. Indeed, multiple clinical trials have been initiated studying the efficacy of these inhibitors in AML, colon cancer, and gleevec-resistant CML (26, 27).

Although Aurora A has been studied for many years, most of the knowledge was obtained in vitro based on cell culture studies. To understand the physiological function of Aurora A in vivo, we generated Aurora A knock-out mice (maintained by L. Y. and K. M.-D.). Aurora A deletion in mice results in embryonic lethality. Surprisingly and interestingly, heterozygous mice develop spontaneous tumors at a higher incidence than wild type mice, suggesting that Aurora A may also act as a haploinsufficient tumor suppressor. The implication of this study will be discussed.

EXPERIMENTAL PROCEDURES

Generation of Aurora A Heterozygous Mutant Mice—Gene trap ES cell line XQ0149 was obtained from Bay Genomics. The insertion site of the gene trap vector was mapped using genomic PCR and sequencing. Chimeras were generated through blastocyst injection of ES cells and germline chimeras were backcrossed to C57BL/6 to obtain mice heterozygous for Aurora A. We established a colony of Aurora A+/− and Aurora A +/− mice and maintained them on a normal diet. We euthanized mice ranging from 50 to 70 weeks by CO2 asphyxiation and subjected them to necropsy. Tissue samples were collected and fixed in formalin and embedded in paraffin blocks prior to cutting and hematoxylin and eosin (H&E) staining. Statistical analysis was performed using chi-square analysis.

In Vitro Culture and Genotyping of Preimplantation Embryos—Preimplantation embryos were obtained from intercross of mice heterozygous for Aurora A. E3.5 embryos were flushed out of the uterus using M2 medium (Sigma) and cultured in Dulbecco’s modified Eagle’s medium supplemented with 15% fetal bovine serum, 0.1 mM D-glucose, 4 mM glutathione, 4 mM glutamine, and 10 ng/ml recombinant LIF. Nested PCR was used to genotype the embryos using the methods described previously (28) with the following primers. First round sense primer for both WT and KO, 5′-GGCTCTGCATGATTTTGTTTAG-3′; first round antisense primer for WT, 5′-CTGTGGCCTTTAAGGACCAGGC-3′; first round antisense primer for KO, 5′-TAGAAGCGGAAGGGTGGGAAAGG-3′; second round sense primer for both WT and KO, 5′-CGCTGAGCTGCATGCTGGTCTG-3′; second round antisense primer for WT, 5′-AACGGCTTCTGAGAAGGGAGG-3′; second round antisense primer for KO, 5′-CGCTGACGCGGATGTGGGAGAAACC-3′. RNA was extracted from embryos using TRIzol reagents, and cDNA was generated using SuperScript III (Invitrogen). Nested PCR was used to amplify the cDNA of WT and KO alleles with the following primers: first round sense primer for both WT and KO, 5′-TACCTCTAGGCTGTATGGCTATTT-3′; second round sense primer for both WT and KO, 5′-CCGTACCCGAGTTCATCTGGCTTCA-3′; antisense primer for WT for both KO, 5′-CGTTGTGGGACGAGCTAAGGCTTC-3′; antisense primers for KO for both rounds, 5′-ATCGGCACCATATCTGTGGATTCA-3′.

Antibodies, Western Blotting, and Immunostaining—Mouse embryonic fibroblasts (MEF) were lysed by NETN (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40) to remove the plasma membrane and nuclei. The cleared lysates were then centrifuged at 14,000 × g for 10 min and cleared lysates were collected. Samples were boiled in 2× Laemmli buffer and run on SDS-PAGE. Membranes were blocked in 5% milk-TBST and then probed with antibodies as indicated. Aurora A antibody was raised in rabbit against mouse Aurora A (residues 1–11). Monoclonal β-actin was purchased from Sigma. Standard immunostaining procedures were used. Anti-phosphorylated histone H3 Ser10 antibody was obtained from Millipore and anti-α-tubulin antibody was from Sigma.

Flow Cytometry and Metaphase Spread—For analyzing cell cycle profiles, cells were harvested and fixed in 70% ethanol overnight. Cells were washed with phosphate-buffered saline and incubated in 40 μg/ml propidium iodide and 100 μg/ml RNase A for 30 min. DNA content was then analyzed using the BD LSR II system. Doublet discrimination was done before the analysis. For metaphase spread, cells were incubated with 50 ng/ml colcemid (Invitrogen) for 12 h. Cells were then washed with phosphate-buffered saline and treated with 75 mM KCl for 15 min. They were fixed in Carnoy’s solution (75% methanol and 25% acetic acid) and a 15-μl aliquot was dropped onto a pre-cleaned slide, which was stained with 5% Giemsa solution (Invitrogen). Chromosome numbers were determined under light microscope.

Drug Treatment—VX-680 was synthesized by Ryss Lab Inc., and dissolved in dimethyl sulfoxide at a concentration of 2 mM. For all studies, 100 nM VX-680 was used and incubated with cells for 72 h.

RESULTS

Generation of Aurora A Knock-out Mice—To study the physiological function of Aurora A in vivo, the gene trap ES cell clone XQ0149, in which a copy of Aurora A was disrupted by a gene trap vector inserted at intron 6 (Fig. 1a), was used to generate Aurora A knock-out mice. The exact position of gene trap vector integration was identified through genomic PCR and sequencing. Chimeras were generated through standard blastocyst injection and mice heterozygous for Aurora A were obtained by backcrossing the chimeras with C57BL/6 mice. We predicted a fusion product of the N terminus of Aurora A and β-gal from the gene trap integration site, however, we did not detect any fusion protein using β-galactosidase antibody or truncated protein using Aurora A N-terminal antibody in the heterozygous mice (data not shown), suggesting that the gene trap vector totally abolished the proper expression from the
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Aurora A Null Embryos Fail to Survive Due to a Defect in Mitosis—Intercross from heterozygous mice yielded no mice with Aurora A−/− genotype, suggesting that Aurora A null mice were embryonic lethal. The death occurred at early stages of embryonic development as no Aurora A null embryos could be obtained at 10.5 day post coitum (Fig. 1c). We then attempted to collect pre-implantation embryos at 3.5 dpc. We consistently found some abnormal embryos that contain much fewer cells than other normal morula or blastocyst stage embryos. Genotyping indicated that all of them, but none of the normal ones were null for Aurora A (Fig. 1B). Reverse transcriptase-PCR also confirmed that these abnormal embryos did not contain any wild-type Aurora A mRNA (Fig. 1D). The cell number varies from 8 to 16 in these abnormal embryos (normal embryos have more than 50 cells at this stage), which suggested that they were developmentally delayed or arrested. Embryos were then cultured in vitro for 3 days. Normal embryos grew normally, hatched from zona pellucida and flattened, whereas none of the abnormal embryos showed significant growth and they finally died (Fig. 2A). This observation suggested that the Aurora A null embryos were arrested before 16-cell stages of development.

As Aurora A is one of the key kinases during mitosis, we speculated that Aurora A null embryos might have problems during mitosis, which would lead to their growth arrest. We stained phospho-histone H3 serine 10, a mitotic marker, in these 3.5 day post coitum embryos. Normal embryos consistently showed strong staining in some cells that were in mitotic phase. However, none of these abnormal embryos displayed any staining (Fig. 2B). Although the numbers of cells are fewer in these abnormal embryos, which could decrease the chance of identifying mitotic cells among them, consistent absence of phospho-histone H3 serine 10 staining indicated that cells in these abnormal embryos may have a problem in proper cell cycle progression. To further dissect the problems in these abnormal embryos, we stained mitotic spindles using antibody against α-tubulin. Intact spindles could be found in some cells of normal embryos; however, none of the abnormal embryos contain any assembled spindles, indicating that these embryos do not have proper spindle assembly (Fig. 2C).

Aurora A+/− Mice Develop Spontaneous Tumors—Although Aurora A deficiency leads to early embryonic lethality, Aurora A+/− mice were born healthy and fertile with no obvious effects, albeit a slight decrease in Aurora A levels compared with Aurora A wild type mice (Fig. 3a). Given the requirement of Aurora A for early embryogenesis and normal cell cycle progression, it is possible that loss of one Aurora A allele might cause problems in proper cell cycle control. Such defects could lead to chromosomal instability and even promote tumorigenesis in these Aurora A+/− mice. To test whether this is the case, we established a cohort of Aurora A+/− and Aurora A−/− mice. We euthanized animals ranging from 50 to 70 weeks and performed necroscopies searching for tumors in these mice. The average age at euthanasia for these mice were 57.4 weeks for the Aurora A+/− mice and 56.7 weeks for the Aurora A−/− mice (supplemental Table S1). Quite dramatically, Aurora A+/− mice developed tumors at a higher frequency of 25.5% compared with 8.8% in Aurora A−/− mice (Fig. 3B). This 3-fold higher tumor incidence is statistically significant (p < 0.001).

The tumors found in Aurora A−/− mice were in various organs including thymus, lung, liver, and lymph node. For example, a lymphoma in the thymus and an adenocarcinoma in the lung were observed in one of the Aurora A−/− mice (Fig. 3C). Most tumors were lymphoma that invaded into the lung, with one squamous cell carcinoma, and one liver hepatoma, but surprisingly 5 lung carcinomas were also discovered (Table 1).

In an attempt to rescue the embryonic lethality of a complete loss of Aurora A we crossed these mice onto a p53−/− background. No double knock-out animals were generated when Aurora A+/− p53−/− animals were intercrossed suggesting that this lethality cannot be rescued by p53 loss (data not shown).

As we speculated, the observed increased tumor incidence in Aurora A+/− mice could potentially be caused by chromosomal instability because Aurora A is critically important for mitotic transitions. To directly document chromosomal instability in Aurora A+/− cells, we generated Aurora A+/− and Aurora A−/− MEFs from 13.5 day post coitum embryos. Analysis of DNA content using flow cytometry suggested that Aurora A−/− MEFs had 70% more cells containing >4N content. They also had a slightly but consistently higher percentage of cells with 4N content than their wild type counterparts, suggesting mild mitotic delay in these cells (Fig. 4a). We further determined chromosome numbers in these MEFs and discovered that Aurora A+/− MEFs consistently contain a higher percentage of cells with aneuploidy (Fig. 4, b and c). The chromosomal instability displayed in Aurora A+/− MEFs can
potentially explain the higher tumor incidence in Aurora A+/− mice, because aneuploidy or chromosomal instability is known to contribute to tumorigenesis. Collectively our results indicate that Aurora A is crucial for maintaining genomic stability and may function as a haploinsufficient tumor suppressor.

VX-680, an Aurora Kinase Inhibitor, Induces Aneuploidy in Wild Type MEFs—Because of the frequent Aurora A overexpression in tumors, many aurora kinase inhibitors have been generated and several of them effectively suppress tumor growth both in vitro and in vivo (25, 26, 29, 30). Because of the potency of these inhibitors in xenograft studies, some of these inhibitors are currently in clinical trials for cancer treatment (26). However, our study using Aurora A+/− cells and mice suggest that partial inactivation of Aurora A might lead to chromosomal instability and increased tumor incidence. Therefore, we decided to examine whether these Aurora kinase inhibitors would have any potentially side effects on normal cells.

VX-680 binds to the ATP binding region of the Aurora kinase, thereby trapping it in an inactive state (31). Cells treated with VX680 displayed mitotic defects, similar to those observed in our Aurora A+/− mice or cells. To directly address whether VX-680 can cause mitotic defects in normal cells, we examined chromosomal instability and cell cycle progression in cells treated with VX-680. MEFs were cultured with 100 nM VX-680 for 72 h and the DNA content of the cells was analyzed by flow cytometry. We found that treatment with 100 nM VX-680 led to a significant increase in mitotic cells, indicating a slower mitotic progression in the presence of this inhibitor. The percentage of cells carrying 4N DNA content was also dramatically increased, suggesting that these cells are aneuploid (Fig. 4d), which were further confirmed by metaphase spread showing a significant increase of cells with aneuploidy, particularly polyploidy, after VX-680 treatment (Fig. 4, e and f). Taken together, these data suggest that VX-680 exerts its inhibitory effect not only on tumor cells, but also on wild type cells, whereas loss or reduced Aurora kinase activity could lead to chromosomal instability and potentially tumorigenesis.

**DISCUSSION**

In this study, we demonstrate that Aurora A is crucial for embryonic development and cell cycle progression. Moreover, partial loss of Aurora A function promotes tumor progression.
as Aurora A\(^{+/−}\) mice have an almost 3-fold higher tumor incidence and cells from these mice harbor aneuploidy and chromosomal instability, hallmarks of genomic instability that would lead to tumorigenesis. Importantly inhibition of Aurora A in wild type MEFs by VX-680 results in high levels of aneuploidy and chromosomal instability, which should be a cause for concern in regard to long-term side effects of the use of these Aurora kinase inhibitors in clinical settings.

In the absence of Aurora A, embryos failed to survive after the 16-cell stage. This defect is likely to occur at mitotic onset, as no spindle could be assembled and no phospho-histone H3, which starts to be present at condensing chromosomes during prophase, could be observed. The initial 3 to 4 cycles of division from 1 cell to 16 cells in the null embryos may be driven by maternal Aurora A. Although Aurora A is known to be subjected to activated protein kinase C-mediated degradation and its protein level quickly goes down in G\(_1\) cells (32), the maternal Aurora A mRNA and protein may last for several cell cycles before finally being depleted. During the preparation of this article, there was another study reported that Aurora A knock-out mice are embryonic lethal (33). Consistent with our data, their knock-out embryos also die before implantation, but they seem to survive longer until the early blastocyst stage (33). Likewise, their knock-out embryos have a defect in proper spindle assembly and manifest disorganized spindles. Both of these Aurora A knock-out models support a critical role of Aurora A in early embryogenesis and mitosis.

It is not surprising that Aurora A null embryos have defects in spindle assembly. Besides its function in centrosome maturation and mitotic entry, Aurora A also has a direct role in spindle assembly and microtubule organization, probably through regulating one of its substrates TACC, which interacts with ch-TOG/SMAP215 and stabilizes microtubules (34, 35). Aurora A-dependent TACC phosphorylation is likely to be important for its localization to the centrosome as well as its function for stabilizing centrosome-nucleated microtubules (11, 34, 36).

Surprisingly, loss of one Aurora A allele causes an increased tumor...
incidence, suggesting that Aurora A may act as a haploinsufficient tumor suppressor. Combined with the overwhelming evidence that supports Aurora A as an oncogene, we propose that a balanced level of Aurora A is required for maintaining genomic stability. Too much Aurora A could cause aberrant mitoses with chromosome segregation defects and a defective spindle checkpoint. On the other hand, reduced Aurora A levels could also cause mitotic delay and improper chromosome segregation, all of which would lead to aneuploidy and finally promote tumorigenesis.

Because of the frequent Aurora A overexpression observed in human tumors, several Aurora kinase inhibitors are currently in clinical trials for cancer treatment (26). Ongoing clinical trials have thus far presented evidence that most of these Aurora inhibitors are well tolerated with only neutropenia as a dose-limiting toxicity, and early data shows that some patients have stable disease that do not progress (26). It appears that short-term treatment with these inhibitors does not cause any major side effects and are beneficial to the patients. However, the potential long-term effects of these inhibitors remain unknown. In this study, we used an Aurora inhibitor and showed that whereas they have great potency in inhibiting tumor cell growth, they also generate aneuploidy in normal mammary epithelial cells (37). The data from our Aurora A+/− mouse model would hint that chromosomal instability generated by Aurora inhibition may potentially cause secondary tumor formation later in life. Future efforts in designing a better means of delivery may significantly reduce these side effects and make these drugs more beneficial to cancer patients in the long run.

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