Aurora-A Overexpression in Mouse Liver Causes p53-Dependent Premitotic Arrest during Liver Regeneration

Chao-Chin Li,1,3 Hui-Yi Chu,1 Chu-Wen Yang,2 Chen-Kung Chou,4 and Ting-Fen Tsai1,3

1Department of Life Sciences and Institute of Genome Sciences, National Yang-Ming University; 2Department of Microbiology, Soochow University, Taipei, Taiwan; 3Division of Molecular and Genomic Medicine, National Health Research Institute, Zhunan, Taiwan; and 4Department of Life Science, Chang Gung University, Tao-Yuan, Taiwan

Abstract

Aurora-A, a serine-threonine kinase, is frequently overexpressed in human cancers, including hepatocellular carcinoma. To study the phenotypic effects of Aurora-A overexpression on liver regeneration and tumorigenesis, we generated transgenic mice overexpressing human Aurora-A in the liver. The overexpression of Aurora-A after hepatectomy caused an earlier entry into S phase, a sustaining of DNA synthesis, and premitotic arrest in the regenerating liver. These regenerating transgenic livers show a relative increase in binuclear hepatocytes compared with regenerating wild-type livers; in addition, multipolar segregation and trinucleation could be observed only in the transgenic hepatocytes after hepatectomy. These results together suggest that defects accumulated after first round of the hepatocyte cell cycle and that there was a failure to some degree of cytokinesis. Interestingly, the p53-dependent checkpoint was activated by these abnormalities, indicating that p53 plays a crucial role during liver regeneration. Indeed, the premitotic arrest and abnormal cell death, mainly necrosis, caused by Aurora-A overexpression were genetically rescued by p53 knockout. However, trinucleation of hepatocytes remained in the regenerating livers of the transgenic mice with a p53 knockout background, indicating that the abnormal mitotic segregation and cytokinesis failure were p53 independent. Moreover, overexpression of Aurora-A in transgenic liver led to a low incidence (3.8%) of hepatic tumor formation after a long latency period. This transgenic mouse model provides a useful system that allows the study of the physiologic effects of Aurora-A on liver regeneration and the genetic pathways of Aurora-A–mediated tumorigenesis in liver.


Introduction

Aurora-A is a serine-threonine kinase that plays a series of important roles in the regulation of mitotic progression in various organisms. Expression of Aurora-A is cell cycle dependent; it is initiated at late S phase and is at a maximum at G2-M phase (1, 2). The various functions of Aurora-A correlate with various different cell cycle events, including centrosome maturation and separation, bipolar spindle assembly, chromosome alignment, and the transition from prophase to metaphase as well as cytokinesis (1, 2). Aurora-A is activated by upstream activators and is further autophosphorylated during mitotic entry (3-5). After mitosis is finished, Aurora-A is rapidly degraded by the anaphase-promoting complex-ubiquitin-proteasome pathway (6, 7) as well as an ubiquitin-independent mechanism (8) and this is necessary for the cell to complete cytokinesis at telophase. Accordingly, the proper timing and the correct level of Aurora-A expression and activation are required for progression through mitosis. Previous studies have indicated that cytokinesis failure is one of the most significant defects induced by Aurora-A overexpression in a cell culture system and leads to centrosome amplification and polyplody after the first round of the cell cycle (9, 10). In addition, Aurora-A overexpression can override the checkpoint induced by defective spindle assembly and DNA damage, which leads to aneuploidy (11). These events may all contribute to the carcinogenic transforming activity of Aurora-A.

The effects of Aurora-A overexpression have been investigated intensively in cell culture; these studies suggested that additional genetic defects might be necessary and that they cooperate with Aurora-A to induce tumorigenicity (10, 12, 13). A previous study has shown that Aurora-A protein directly interacts with p53. The expression of p53 was found to suppress Aurora-A–induced centrosome amplification and cellular transformation (14). On the other hand, Katayama et al. (15) have shown that Aurora-A directly phosphorylates p53 at Ser88, leading to its ubiquitination by Mdm2 and proteolysis. In addition, Liu et al. (16) reported that Aurora-A–phosphorylated p53 at Ser205 abrogates p53 DNA binding and transactivation activity. Accordingly, disruption of this mutual
suppression mechanism between Aurora-A and p53 might trigger checkpoint abnormalities and chromosome instability.

When examined in an animal study, conditional expression of Aurora-A in a transgenic mouse model using the Cre-loxP system resulted in aberrant mitosis, increased binucleated cell formation, and apoptosis in the mammary epithelium. Moreover, hyperplasia but not malignant mammary tumor was found in the transgenic mice after a long latency (17). In another transgenic mouse model carrying a mouse mammary tumor virus-Aurora-A transgene, ~40% of the mice developed mammary tumors at 20 months of age. Specifically, Aurora-A overexpression led to genetic instability and activation of the AKT pathway at stages before tumor formation (18). These in vivo results from the mouse mammary tumor virus-Aurora-A transgenic mice established Aurora-A as an oncogene that causes malignant transformation of the mouse mammary gland.

The mRNA and protein of Aurora-A have been found to be frequently overexpressed in various human cancers, including hepatocellular carcinoma (1, 19). In addition, Aurora-A overexpression is significantly associated with higher-grade hepatocellular carcinoma and a poor prognosis (20). Given that aberrant expression/activity of Aurora-A has been found in hepatocellular carcinoma, the in vivo analysis of Aurora-A in a mouse model should provide insight into the mechanisms by which Aurora-A promotes liver tumorigenesis in a physiologic context. In addition, although Aurora-A is a potential oncoprotein, its effects on hepatocyte cell cycle progression are not known and its involvement in liver regeneration has not been explored. In this study, we have generated transgenic mice overexpressing human Aurora-A protein in the liver and for the first time studied the phenotypic effects of such overexpression on liver regeneration and tumorigenesis.

Results
Aurora-A Is Expressed in Fetal Liver and Regenerating Liver of Wild-Type Mice
To identify the potential regulatory roles of Aurora-A in liver, we examined the temporal expression pattern of Aurora-A mRNA by Northern blot hybridization. For fetal liver, high

![FIGURE 1. Expression patterns of mouse endogenous Aurora-A mRNA in fetal, postnatal, and adult livers as well as regenerating livers.
A. Northern blot analysis of Aurora-A and Pcsa mRNA expression for fetal livers at embryonic stages E11.5 to E18.5, postnatal day 1 (P1), and postnatal day 7 (P7) and adult mice at 10 wk of age. B. Northern blot analysis of Aurora-A mRNA expression during liver regeneration of wild-type mice after PH. The intensity of the 28S and 18S rRNA was used as internal control for total RNA loading. C. Western blot detection of Aurora-A protein during liver regeneration of wild-type mice after PH. The same blot was detected for Hsp70 antibody as a protein loading control. D. Representative photomicrographs of IHC staining of Aurora-A protein for liver sections prepared from 0, 2, 3, and 4 d (D) after PH. Arrow, mitotic hepatocyte undergoing chromosome segregation. Original magnification, ×200.](mcr.aacrjournals.org)
expression level of Aurora-A mRNA was sustained until embryonic day 15.5 (E15.5). The signals declined gradually thereafter in the embryonic livers and postnatal newborns until expression was barely detectable in the adult liver (Fig. 1A). Interestingly, the temporal expression pattern of Aurora-A coincided with the expression of the proliferation marker proliferating cell nuclear antigen (Pcna; Fig. 1A), implying that Aurora-A expression was correlated with ongoing hepatocyte proliferation in mouse liver. Although most hepatocytes are arrested at quiescent stage in adult liver, quiescent hepatocytes are able to reenter the cell cycle during liver regeneration, which is inducible by partial hepatectomy (PH; ref. 21). Our data indeed showed that expression of the Aurora-A mRNA was transiently induced at 2 days after hepatectomy, which coincides with G2-M phase during hepatocyte cell cycle progression, and then rapidly decreased to basal level 3 days after hepatectomy (Fig. 1B). A peak induction of the Aurora-A protein was also detected at 2 days after hepatectomy and gradually decreased thereafter when visualized by Western blot analysis (Fig. 1C). Immunohistochemical (IHC) staining revealed that Aurora-A protein was mainly localized to the nuclei of the hepatocytes at 2 days after hepatectomy, but Aurora-A did show additional staining in the cytoplasm at 3 to 4 days after hepatectomy (Fig. 1D).

**Generation of Aurora-A Transgenic Mice**

We successfully generated transgenic mice overexpressing human Aurora-A in the liver. The plasmid used to generate the transgenic mice is shown in Fig. 2A. Two founders of the PEPCK-Aurora-A transgenic mice were generated. The transgenic copy numbers of lines A79 and A80 are about 2 and 7, respectively. Southern blot analysis of transgene (Tg) copy number for transgenic lines A79 and A80. Genomic DNA was digested with EcoRI for Southern blot hybridization. Wt, wild-type. C, Northern blot analysis of the human Aurora-A mRNA expression in transgenic lines A79 and A80. The same blots were stripped and rehybridized with mouse Pepck cDNA probe to compare the mRNA expression of endogenous Pepck gene and the transgene that was driven by the human PEPCK promoter. The expression pattern of the PEPCK-Aurora-A transgene is slightly different from that of the mouse endogenous Pepck gene; this may be attributable to species difference in terms of the promoter activity. The intensity of the 28S and 18S rRNA was used as internal control for total RNA loading. D, Expression of Aurora-A protein detected by Western blot and IHC staining in transgenic mice. Liver extracts for Western blotting were prepared from transgenic mice (lines A79 and A80), wild-type mice, and wild-type mice at 2 d after hepatectomy. Liver sections for IHC staining were prepared from wild-type and transgenic mice (line A79) without any treatment. Original magnification, ×200. All of the liver samples were obtained from 2-mo-old male mice.
respectively, by Southern analysis (Fig. 2B). Strong expression of human Aurora-A mRNA was detected in the transgenic livers of lines A79 and A80, with a lower expression level detected in the other tissues (Fig. 2C). Elevated levels of the Aurora-A protein were shown by Western blot analysis and IHC staining (Fig. 2D). There was no observable change in liver development of the Aurora-A transgenic mice up to adulthood at around 8 weeks old. There was no significant difference in nuclear polyplody (Supplementary Fig. S1A) and the baseline proliferation of hepatocytes revealed by IHC staining of the Ki67 marker (Supplementary Fig. S1B) in the 9-week-old Aurora-A transgenic and wild-type mice before the PH surgery.

Overexpression of Aurora-A Caused an Earlier Entry into S Phase, a Sustaining of DNA Synthesis, and Premitotic Arrest in the Regenerating Livers

To investigate the effect of Aurora-A overexpression on liver regeneration, we analyzed hepatocyte cell cycle progression during liver regeneration induced by PH. To avoid possible artifacts attributed to the different integration sites of the transgene, both of the transgenic lines (A79 and A80) were analyzed. DNA synthesis was assessed by bromodeoxyuridine (BrdUrd) incorporation and detected by IHC staining. In the wild-type mice, the majority of the BrdUrd-positive hepatocytes were detected at 1.75 to 2 days; furthermore, there was a peak at 2 days and no or very few BrdUrd-positive cells could be detected at 3 days and thereafter after hepatectomy (Fig. 3A). In the Aurora-A transgenic mice, interestingly, there was a significantly increased percentage of the BrdUrd-positive hepatocytes detected at 1.75 days, which indicates an earlier entry into S phase. In addition, the duration of the DNA synthesis was extended to 4 days after hepatectomy (Fig. 3A). In the wild-type mice, the number of mitotic figures peaked at 2 days and decreased thereafter until 4 days after hepatectomy (Fig. 3B). Notably, a significant decrease in the mitotic figures was observed over the mitotic period from 2 to 4 days after hepatectomy in both transgenic lines (A79 and A80; Fig. 3B), which suggest that hepatocyte cell cycle progression was likely to be partly blocked before the mitotic phase in the regenerating livers of the Aurora-A transgenic mice.

To further study the abnormal hepatocyte cell cycle progression, we measured the mRNA expression of various cell cycle–associated markers, including Cyclin D1 (G1 phase), cyclin-dependent kinase (Cdk) 2 (G1-S phase), PcnA (S-G2-M phase), Cyclin B1 (G2-M phase), Cdk1 (G2-M phase), and mouse endogenous Aurora-A (G2-M phase). Abnormalities in the induction and temporal expression for both S-phase–associated and G2-M-phase–associated markers were observed in the regenerating livers of the Aurora-A transgenic mice. For the S-phase marker, a transient peak of induction was detected at 2 days and the mRNA had decreased to a basal level at 3 days after hepatectomy in the wild-type mice (Fig. 3C). In the Aurora-A transgenic mice, there was an earlier induction of the S-phase markers at 1 day and these peaked at 1.75 days after hepatectomy. In addition, there was a second wave of the S-phase marker induction at 3 to 4 days after hepatectomy in the Aurora-A transgenic mice.
but the cells showed an impaired progression across the G2-M transition and this resulted in premitotic arrest. Notably, in the Aurora-A transgenic mice, there was an earlier induction of the G2-M markers at 1.75 days and a significantly higher level of the G2-M gene expression was sustained until 3 to 4 days after hepatectomy (Fig. 3C). Because a significantly lower percentage of the mitotic hepatocytes was observed over the complete mitotic period from 2 to 4 days after hepatectomy in the transgenic mice (Fig. 3B), these results indicated that Aurora-A overexpression accelerated hepatocytes entering into G2 phase, these cells showed an impaired progression across the G2-M transition and this resulted in premitotic arrest.

**Cytokinesis Failure Seems to Follow G2-M Transition Impairment and the Latter Is Likely to Be Caused by the p53-Dependent Checkpoint in the Regenerating Livers**

To examine whether Aurora-A overexpression causes cytokinesis failure leading to binucleation in the hepatocytes of the regenerating liver, we counted the ratio of hepatocytes with double nuclei. Although binuclear hepatocytes are normally present in the nonregenerating liver, the percentage of binuclear hepatocytes was significantly increased at 4 days after hepatectomy in the regenerating livers of the Aurora-A transgenic mice when compared with the regenerating wild-type control (Fig. 4A). This indicates that cytokinesis failure may occur in the hepatocytes with Aurora-A overexpression. This conclusion is supported by the detection of tripolar segregation and trinucleation of hepatocytes in the Aurora-A transgenic mice after hepatectomy (Fig. 4B). A frequency of between 0.1% and 0.2% of hepatocytes showed trinucleation in the two Aurora-A transgenic lines and no such phenomenon was ever found in the wild-type mice (data not shown). Our results provide important clues and suggest that Aurora-A overexpression in hepatocytes seems to result in centrosome amplification, multipolar segregation, and multinucleation after cytokinesis failure under physiologic conditions.

To evaluate if the p53-dependent checkpoint is induced by Aurora-A overexpression during liver regeneration, we analyzed the expression levels of p53 protein by Western blot analysis. Our data showed that p53 protein was remarkably up-regulated at 2 and 4 days after hepatectomy (Fig. 4C). Quantification revealed that there was a 3.5- and 2-fold increase in the p53 protein level at 2 and 4 days after hepatectomy, respectively, in the transgenic mice (Fig. 4D). These results implied that p53 acts as the crucial checkpoint in hepatocyte cell cycle progression during liver regeneration and its expression would seem to be triggered by the abnormal events mediated by Aurora-A overexpression in the transgenic mice.

**Premitotic Arrest and Necrosis Caused by Aurora-A Overexpression Were Genetically Rescued by p53 Knockout**

To test the hypothesis that the p53 checkpoint plays a pivotal role in the regulation of hepatocyte cell cycle progression and is responsible for the premitotic arrest caused by Aurora-A overexpression, we generated Aurora-A transgenic mice (line A79) carrying two null alleles for the p53 gene (Aurora-A; p53−/− mice). Cell proliferation indices, including DNA synthesis (BrdUrd incorporation) and mitotic index, were analyzed...
Knockout of p53 genetically rescued the phenotypes of premitotic arrest and abnormal cell death in the regenerating livers of the Aurora-A transgenic mice. A. DNA replication as monitored by BrdUrd incorporation. The DNA synthesis level of Aurora-A;p53−/− (Y) has returned to the normal control level (X) at 4 d after hepatectomy. B. Mitotic index of the regenerating livers. Liver samples were prepared from wild-type mice, Aurora-A transgenic mice (Aurora-A), Aurora-A transgenic mice with p53 homozygous knockout (Aurora-A;p53−/−), and p53 homozygous knockout mice (p53−/−). The methods for quantification of BrdUrd incorporation and mitotic index are the same as in Fig. 3. The sum of the mitotic events (X) and the sum of the mitotic events (Y) are approximately equal. C. Liver/body weight for different genotypes of mice at different time points after hepatectomy. *, P < 0.05; **, P < 0.005. D. H&E staining for the wild-type mice, Aurora-A transgenic mice, Aurora-A;p53−/− mice, and p53−/− mice at 0, 3, and 4 d after hepatectomy. Blue arrows, necrotic area in the Aurora-A transgenic mice. Please note that the presence of trinuclear hepatocytes (black arrows; insets) in the Aurora-A;p53−/− mice is similar to the situation in the Aurora-A transgenic mice with p53 wild-type background (Fig. 4B). The transgenic line A79 was used for crossing with the p53 knockout mice. Original magnification, ×200.
during liver regeneration and these were compared across the different mouse genotypes (i.e., wild-type, *Aurora-A* transgenic, *Aurora-A;p53*/*−−*, and *p53*/*−−* mice). For DNA synthesis, notable change was detected at 4 days after hepatectomy (Fig. 5A). In the *Aurora-A;p53*/*−−* mice, the percentage of BrdUrd-positive hepatocytes was significantly decreased (*P* < 0.001) compared with the *Aurora-A* transgenic mice (*Aurora-A*, 8.28 ± 0.99%; *Aurora-A;p53*/*−−*, 2.38 ± 0.73%) and the results were comparable with those found for the wild-type mice at 4 days after hepatectomy (Fig. 5A). For mitotic index, the percentage of mitotic cells was remarkably increased (*P* < 0.001) at 3 days after hepatectomy in the *Aurora-A;p53*/*−−* mice compared with the *Aurora-A* transgenic mice (Fig. 5B). Interestingly, the total numbers of the mitotic hepatocytes at 2 and 3 days after hepatectomy in the *Aurora-A;p53*/*−−* mice (1.4 ± 0.65% at 2 days after hepatectomy; 2.59 ± 0.62% at 3 days after hepatectomy) are about equal to that of the wild-type mice (2.47 ± 1.08% at 2 days after hepatectomy; 1.27 ± 0.29% at 3 days after hepatectomy; Fig. 5B). Although the timing of mitosis was delayed slightly in the *Aurora-A;p53*/*−−* mice, these results indeed provide in vivo evidence that the impairment of G2-M transition and premitotic arrest of hepatocyte cell cycle caused by Aurora-A overexpression is genetically rescued by a *p53*/*−−* background. Furthermore, the observation that the premitotic arrest was rescued at 3 days after hepatectomy also provided an explanation as to why DNA synthesis returns to a normal level at 4 days after hepatectomy as shown in Fig. 5A. The overall ability of the liver to regenerate was determined by measuring the liver weight and the ratio of liver to body weight. There was no significant difference in the rate of mass reconstitution in the *Aurora-A* transgenic livers compared with the wild-type (Supplementary Fig. S2; Fig. 5C). It has been previously reported that regaining of liver mass is a general phenomenon in mouse models despite the fact that they are defective in liver regeneration; this mostly likely results from cell size increase (i.e., hypertrophy of hepatocytes; refs. 22-25). Interestingly, the ratios of regenerating liver to body weight for the *Aurora-A;p53*/*−−* mice were significantly higher than for the other genotypes of mice 5 days after hepatectomy (Fig. 5C). In addition, the proliferation indices, including DNA synthesis and mitosis, normally decrease to basal levels in the wild-type mice 4 days after hepatectomy. However, the proliferation indices were persistently higher and detectable in the *Aurora-A;p53*/*−−* mice 4 days after hepatectomy (Fig. 5A and B). These results clearly indicated that there is a synergistic effect between the two genetic events, *Aurora-A* overexpression and *p53* knockout, for promotion of cell cycle progression in the regenerating liver.

To rule out the possibility that the expression of human *Aurora-A* cDNA driven by the human *PEPCK* promoter was affected in the knockout background of *p53* gene because there was a regulatory role on the *PEPCK* promoter, we monitored the mRNA levels of the mouse endogenous *Pepck* gene. Our data revealed that there was no significant difference in mouse Pepck mRNA expression among different groups of mice during liver regeneration (Supplementary Fig. S3). Furthermore, the *Aurora-A* protein levels were directly examined by Western blot. Previously, a transgenic mouse study has revealed that transgenic *Aurora-A* protein was protected from degradation between G2-M but was rapidly degraded and barely detectable between G1-S despite an elevation of the *Aurora-A* mRNA levels (26). Our results were similar and showed a cell cycle–dependent turnover of the *Aurora-A* protein. Although the *Aurora-A* protein level is a little higher in the transgenic mice than wild-type control in the resting liver, there is a very significant increase in *Aurora-A* protein in the regenerating livers and much bigger differences compared with the transgenic and wild-type mice. Moreover, there was no significant change in the *Aurora-A* protein levels during liver regeneration when transgenic mice with and without the *p53* knockout were compared (Supplementary Fig. S4).

To examine whether there is apoptosis occurring in the regenerating livers of the *Aurora-A* transgenic mice, we have done a terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay. Our data revealed that there was no detectable TUNEL-positive cells in the liver sections prepared from transgenic and wild-type control mice after hepatectomy; this analysis included different time points of liver samples from day 1 to day 7 after hepatectomy in both groups of mice (data not shown). However, histopathologic analyses of liver sections revealed that abnormal cell death, mainly necrosis, was reproducibly detectable in the *Aurora-A* transgenic mice at 3 and 4 days after hepatectomy (Fig. 5D); there is between 5% and 10% of the liver parenchyma with necrotic hepatocytes in their liver sections. In addition, histologic examination revealed the presence of vacuoles at 4 days after hepatectomy, indicating hepatic fatty change (microsteatosis; Fig. 5D). Our findings indicate that overexpression of *Aurora-A* leads not only to impairment of the G2-M transition but also to necrosis during liver regeneration. Interestingly, the abnormal histopathology, including necrosis and fatty changes, was absent in the liver sections of the *Aurora-A;p53*/*−−* mice at 3 and 4 days after hepatectomy (Fig. 5D), showing that elimination of the *p53* checkpoint indeed rescues the premitotic arrest and histopathologic abnormalities caused by *Aurora-A* overexpression during liver regeneration.

**Overexpression of Aurora-A in Liver Led to a Low Incidence of Hepatic Tumor Formation after a Long Latency Period**

To study the potential effect of *Aurora-A* overexpression on liver tumorigenesis, we followed different ages of transgenic mice for tumor formation. Furthermore, to evaluate whether the genetic background made any difference to liver tumorigenesis, we bred the *Aurora-A* transgenic mice (line A79) into a series of different genetic background, including FVB/N inbred, FVB/N mixed with C57BL/6, and BALB/c background. After 25 months of follow-up, no hepatic tumors were found in 24 *Aurora-A* transgenic mice with the FVB/N inbred background. Two transgenic mice (AG11 and AH7) with the mixed genetic background were found to have developed hepatic tumor (Supplementary Table S1; Fig. 6A). Overall, there was an incidence of 3.8% (2 of 52) for hepatic tumor formation observed in *Aurora-A* transgenic mice with a variety of different genetic backgrounds (Supplementary Table S1). Histopathologic examination revealed that the tumors in specimens AG11 and AH7 were hyperplastic nodules resembling hepatocellular
adenoma (Fig. 6B; Supplementary Fig. S5). In specimen AH7, severe fatty changes were observed in multiple hyperplastic nodules (Supplementary Fig. S5). We also followed the tumor incidence among Aurora-A transgenic mice with a p53 heterozygous background (Aurora-A;p53+/−) to study their synergistic effect on liver tumorigenesis. However, no hepatic tumors were found in 16 Aurora-A;p53+/− mice with the FVB/N inbred background after 25 months of follow-up. Among Aurora-A transgenic mice with the p53 homozygous knockout (Aurora-A;p53−/−), because the p53−/− mice usually die with sarcoma or lymphoma at around 4 to 6 months of age before hepatic tumor formation, the issue will be answered in a separate study using mice carrying a liver-specific p53 knockout allele.

Discussion

The level of Aurora-A protein is tightly cell cycle regulated; it is degraded rapidly toward the end of mitosis and before cytokinesis in mammalian cells (6-8, 27). Previous studies have revealed that Aurora-A is a short-lived protein that has a rapid turnover rate with a half-life of ~2 hours (6). The periodic degradation of Aurora-A protein is crucial for modulating cell cycle progression and maintaining genomic stability (1, 2). Here, we show that there was a peak induction of Aurora-A protein detected at 2 days after hepatectomy, which coincides with G2-M phase during hepatocyte cell cycle progression, and the protein rapidly decreased to a basal level 3 days after hepatectomy in the wild-type mice. However, in the transgenic mice, the total amount of Aurora-A protein (transgenic plus endogenous Aurora-A) in the regenerating livers is significantly higher than that of wild-type livers from day 1 to day 7 after hepatectomy (Supplementary Fig. S4). Consequently, the enhanced levels of Aurora-A protein, which are also expressed at an inappropriate timing, seem to cause the phenotypic effects in the regenerating liver; these phenotypic abnormalities include earlier entry into S phase, premitotic arrest, and cytokinesis failure. In this regard, the Aurora-A transgenic mice indeed mimic the situation observed in human cancer cells, which frequently exhibit overexpression of Aurora-A protein regardless of the cell cycle stage (28).

Our results show for the first time that Aurora-A overexpression seems to cause cytokinesis failure and tetraploidization in the regenerating livers of transgenic mice. During liver regeneration induced by two-third PH in mice, the regenerating liver theoretically requires 1.66 proliferative cycles per residual hepatocyte to restore the original number of hepatocytes and complement the original liver mass. The first peak of the hepatocyte cell cycle is initiated around 2 days and the second one appears at 3 to 4 days after hepatectomy (21). In this study, several abnormal events, including cytokinesis failure, as indicated by a significant increase in binuclear hepatocytes, tripolar segregation, and trinucleation of hepatocytes, were observed at 4 days after hepatectomy. This suggests that there was an accumulation of defects after the first round of cell cycle and these may lead to pathologic changes, such as necrosis and cell death, in the regenerating livers of the Aurora-A transgenic mice. Notably, tripolar segregation and trinucleation of hepatocytes were unique phenomena that were only detectable in the Aurora-A transgenic mice. Centrosome amplification may result in multipolar segregation, which in turn gives rise to multinucleation. These abnormal events could be regarded as the precursors of aneuploidy and genomic instability (29, 30).

The p53-dependent checkpoint is likely to be an important method of preventing cells from transformation by Aurora-A overexpression (31-33). Our results revealed that the p53 checkpoint was indeed activated in the regenerating liver at 4 days after hepatectomy and this activation followed multipolar segregation and cytokinesis failure, which were induced by Aurora-A overexpression. Specifically, distinct multinucleation was still present in the Aurora-A transgenic mice with the p53 knockout background as shown in Fig. 5D, indicating that abnormal mitotic segregation and cytokinesis failure were not p53 dependent. Previously, it was shown in the mammary gland conditional Aurora-A transgenic mouse model that cytokinesis failure and multinucleation activated the p53-dependent checkpoint, which in turn acts in the postmitotic G1 phase to arrest tetraploid cells followed by cell death (17). Our observations show that, in a similar manner to the mammary gland, p53 plays a crucial role in cell cycle arrest in the regenerating livers of the Aurora-A transgenic mice.

Using linkage analysis and haplotype mapping of interspecific mouse crosses in a search for low-penetration susceptibility genes for cancer predisposition, Ewart-Toland et al. (34) identified Aurora-A gene as a candidate skin tumor oncogenic gene. The authors showed that a common genetic variant in Aurora-A, 91T→A (Phe31Ile), is preferentially amplified in tumor cells and is associated with aneuploidy and
chromosome instability. The Ile31 allele is a high-risk allele that induces more rapid cell growth of rat cells and increased xenograft tumorigenicity in nude mice than the Phe31 low-risk allele (34). In another Aurora-A association analysis for risk of cancer development in humans, Ewart-Toland et al. (35) showed that Aurora-A is a general cancer susceptibility gene for multiple tumor types with low penetrance. The results from the Aurora-A tumorigenesis study using the transgenic mouse approach showed that conditional expression of Aurora-A led to hyperplastic focus formation in the mammary glands but no malignant tumors were found over a long period of follow-up (17). In another transgenic study, the Aurora-A protein was directly expressed in the mammary gland using the mouse mammary tumor virus promoter. This study revealed that overexpression of Aurora-A could cause mammary tumor formation in ~40% of transgenic mice at ~20 months of age (18). In the present study, we overexpressed the human Aurora-A cDNA, which was obtained from HuH-7 hepatoma cells by reverse transcription-PCR (Phe31; Supplementary Fig. S6), in mouse liver and showed that Aurora-A overexpression could cause low incidence (3.8%) of hepatic tumor formation but not malignant hepatocellular carcinoma; this was also at long latency in the transgenic mice. Our study in liver is consistent with the previous studies and supports Aurora-A as a low-penetrance susceptibility gene in both human and mouse for tumor formation, which may modify cancer risk and thereby accelerate cancer formation by cooperation with additional factors, such as p53 deficiency.

Materials and Methods

Generation of Aurora-A Transgenic Mice

The PEPCKx vector contains the human PEPCK promoter, a 0.3-kb ApoAI intron, and polyadenylate signal of human growth hormone gene as previously described (36). The PEPCK-Aurora-A transgenic construct was generated by inserting the human Aurora-A cDNA into the PEPCKx plasmid. The human Aurora-A cDNA used in this study was cloned from HuH-7 hepatoma cell line by reverse transcription-PCR; the amino acid sequence is shown in Supplementary Fig. S6. The Aurora-A transgenic mice were generated by pronucleus microinjection of FVB/N fertilized eggs (37). The transgenic mice were bred in a specific pathogen-free facility. The genotypes of the mice were determined by PCR and/or Southern analysis of genomic DNA isolated from mouse tails (38). To detect the Aurora-A transgene, the 515-bp DNA fragment was amplified by PCR using the primers 5′-GGGTAAAGGAAAGTTGG-TAATG-3′ and 5′-CCACCTTCTCATCAGTATCC-3′. The transgenic copy numbers were estimated by comparing the relative intensity of the transgene with the copy number controls using Southern blot hybridization. We mixed different amounts of transgenic DNA equal to 1, 5, 10, 25, and 50 copies of the transgene per diploid mouse genome with liver genomic DNA isolated from wild-type mice to mimic the transgenic mouse genome. For example, to mimic that there is one copy of a 6-kb transgene present in the mouse genome, we mix 10 μg of liver genomic DNA with 20 μg of the 6-kb transgenic DNA fragment.

Collection of Fetal and Postnatal Livers

FVB/N wild-type females were bred with wild-type males for collection of embryos at different embryonic stages. Virginal plugs were checked in the morning before 10:00 a.m. Sampling of the embryos was carried out between 11:00 a.m. and 1:00 p.m. to obtain fetal livers at embryonic stage E11.5 to E18.5 (vaginal plug = embryonic stage E0.5). Postnatal day 1 and day 7 livers were also obtained from FVB/N inbred mice (37).

PH and Liver Regeneration

In this study, all PH experiments were done using male mice at 9 to 10 wk of age; age-matched transgenic and wild-type mice with the same inbred background (i.e., FVB/N) were used for comparison. They were treated under the regulations of the “Guide for the Care and Use of Laboratory Animals” (39). About 70% of the liver, including the median and left lateral lobes, was removed after they had been anesthetized with 50 mg/kg ketamine i.p. (40, 41). There was a mortality rate of 15% to 20% recorded during the 7 d of observation period after the surgery; this mortality rate is similar in the wild-type mice and Aurora-A transgenic mice with or without p53 knockout background. Regenerated liver tissues were harvested from five to nine surviving mice for each time point in each group of mice.

Trp53 (p53) Knockout Mice

The p53 knockout mice were obtained from JAX (BALB/c congenic strain). The p53 heterozygous mice were backcrossed to FVB/N inbred mice for five generations to obtain FVB/N congenic strain (~97% FVB/N background). Allele-specific PCR was used to distinguish the wild-type and knockout p53 alleles (42).

RNA Analysis

Total RNA was isolated using Trizol Reagent (Life Technologies). Northern blot and slot blot hybridization were done as described previously (38). The cDNA probe for mouse Aurora-A (NM_011497, from base 1208 to 1721) was generated by reverse transcription-PCR. The Cyclin B1 cDNA (RIKEN clone H3076D10, NCBI BG078426) was obtained from the NIA mouse 15K cDNA library. The cDNA probes for Cyclin D1, Pcna, CdK2, and CdK1 were prepared as previously described (36). The hybridization signal was standardized against the intensity of the 28S rRNA for all of the RNA samples.

Western Blot and IHC Analyses

Western blot was done as described previously (43) and detected using Visualizer kit (Upstate). The following antibodies were used: Aurora-A (recognizes both human and mouse Aurora-A proteins; BD Biosciences), p53 (BD Biosciences), Hsp70 (BD Biosciences), and β-tubulin (Upstate). IHC staining of Aurora-A protein was done using paraffin-embedded liver sections (3 μm). Liver sections were soaked in 10 mmol/L sodium citrate of antigen retrieval buffer (pH 6.0), placed in a microwave oven (650 W; Sunpentown), and irradiated for 5 min at superpower and 15 min at defrosting power. The sections were then incubated with primary antibody against Aurora-A...
(1:50; BD Biosciences) at 4°C for 18 to 24 h and visualized by the Chemicon IHC Select System.

**DNA Synthesis and Mitotic Index**

The number of S-phase cells was estimated by BrdUrd incorporation into nuclei. Mice were injected with BrdUrd (100 mg/kg; Sigma) i.p. 2 h before sacrifice. The IHC analysis was done on paraffin-embedded liver sections using anti-BrdUrd antibodies (1:100; DAKO) and LSAB kit (DakoCyto-mation). For the mitotic index, the numbers of hepatocytes with mitotic figures showing visibly condensed chromosomes were counted.

**Liver Histology**

The regenerating livers were collected, fixed with formalin, and embedded in paraffin. Serial liver sections were subjected to H&E and periodic acid-Schiff reaction staining (44). Fat accumulation was shown by Oil Red O staining of cryostat frozen sections.

**Statistical Analysis**

The results are presented as mean ± SD from at least three independent experiments. Differences among multiple groups were analyzed by one-way ANOVA (Statistical Package for the Social Sciences 14.0 statistical software). Comparisons between two groups were done using a Student’s t test. A P value of <0.05 was considered significant.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Acknowledgments**

We thank Yi-Ru Chen, Huen-Jane Chen, and Shu-Pei Wu for technical assistance.

**References**


Aurora-A Overexpression in Mouse Liver Causes p53-Dependent Premitotic Arrest during Liver Regeneration

Chao-Chin Li, Hui-Yi Chu, Chu-Wen Yang, et al.