marker (Collado *et al.*, 2005). Thus we performed immunofluorescent staining for HP1-γ (Supplementary Figure S6) and DcR2 in Aurora A-overexpressing mammary gland tissues and found that the numbers of positive cells were significantly high in the mammary glands of *p53−/−* mice only when Aurora A was overexpressed (Figures 3c and d). All these findings provide *in vivo* evidence that Aurora A overexpression induces cellular senescence in a p53-deficient background.

Increased p16 expression in Aurora A-overexpressing mammary glands

The most compelling link between cellular senescence and tumor suppression is their mutual dependence on tumor suppressor genes such as *p16*, *p21*, *p53* and retinoblastoma (*Rb*) (Bringold and Serrano, 2000). The Rb/p16 and p53/p21 tumor suppressor pathways in particular are important regulators of cellular senescence (Ferbeyre *et al.*, 2002). Therefore, we hypothesized that the Rb/p16 pathway may be involved in Aurora A-induced senescence in a p53-deficient background. To test this hypothesis, we performed immunofluorescence analysis of the DcR2 senescence marker and p16 by using serial tissue sections as shown in Figures 3c and 4a. The area having p16-positive cells (Figure 4a, lower panels) contained DcR2-positive cells (Figure 3c, right panels), suggesting that p16 positive cells have potential to become senescent. Immunohistochemical analysis also indicated upregulation of p16 expression in the ADH-like tumor lesions of *CAG-CAT-Aurora A;Wap-Cre;p53−/−* mice (Figure 4b).

Aurora A overexpression induces DNA damage in p53-deficient MEFs

Senescence is considered a cellular response to stress that limits the proliferation of damaged cells (Campisi, 2001). It can be provoked by DNA damage, oxidative stress and activated oncogenic ras (Lundberg *et al.*, 2000). To investigate the mechanism that induces cellular senescence after Aurora A overexpression, we assessed DNA damage by monitoring the presence of phosphorylated histone H2AX (γ-H2AX). γ-H2AX-positive foci were detected in primary MEFs after Aurora A overexpression (Figure 5a); substantially more of those foci were present in *CAG-CAT-Aurora A;p53−/−* MEFs after Aurora A overexpression than in *CAG-CAT-Aurora A;p53+/+* MEFs (Figure 5b). These findings indicate that Aurora A overexpression elicits DNA damage when p53 functions are impaired, which is one potential mechanism for induction of cellular senescence.

Discussion

We discovered several new findings in *p53−/−* mice after Aurora A overexpression in the present study in comparison with *p53+/+* mice in our previous study. Differences in the response of *p53+/+* and *p53−/−* mice are (1) significantly different induction of cellular senescence, as judged by the staining of three senescence markers, SA-β-gal activity, HP1-γ and DcR2. The proportion of SA-β-gal-positive cells in *p53−/−* mice showed a sevenfold increase compared with cells in *p53+/+* mice, indicating that cellular senescence of the mammary epithelium is hypothesized to be an important contributor to prevent the development of malignant tumors by Aurora A in *p53−/−* mice; (2) different pathological changes between *p53+/+* and *p53−/−* mice. Induction of Aurora A overexpression in *p53−/−* mice resulted in development of precancerous lesions that were histologically similar to ADH, a precancerous

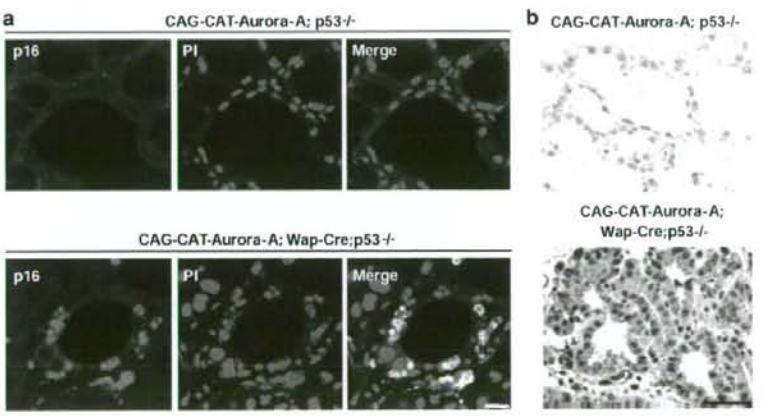

Figure 4 Expression of p16 is upregulated in p53-deficient Aurora A-overexpressing tissues. (**a**) Immunofluorescent staining of p16 expression. Mammary glands from the *CAG-CAT-Aurora A;p53−/−* (upper panels) and *CAG-CAT-Aurora A;Wap-Cre;p53−/−* mice (lower panels) shown in Figure 3c were immunostained with anti-p16 antibody (green, left); DNA was visualized with propidium iodide (PI) (red, middle). Scale bar, 20 μm. (**b**) Immunohistochemical analysis of p16 in the mammary gland of *CAG-CAT-Aurora A;p53−/−* and *CAG-CAT-Aurora A;Wap-Cre;p53−/−* mice. p16 expression was high in hyperplastic lesions of the *CAG-CAT-Aurora A;Wap-Cre;p53−/−* mouse but not in the mammary gland cells of the *CAG-CAT-Aurora A;p53−/−* mouse. Scale bar, 50 μm.

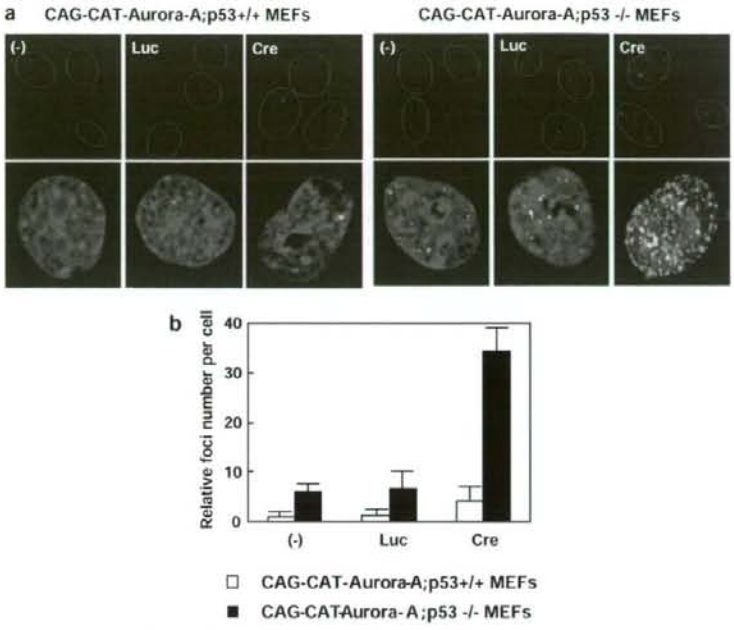

Figure 5 Aurora A overexpression induces DNA damage in p53-deficient mouse embryonic fibroblasts (MEFs). (**a**) Immunofluorescent analysis of primary MEFs from *CAG-CAT-Aurora A;p53+/+* and *CAG-CAT-Aurora A;p53−/−* mice with an anti-γ-H2AX antibody at 48 h after infection with adenoviral luciferase (Luc) or AxCANCre (Cre) virus. (−), uninfected. Nuclei are outlined in blue (upper panels). Double staining with anti-γ-H2AX and propidium iodide (PI) in representative cells from the upper panels are shown in the lower panels. (**b**) Quantification of γ-H2AX-positive foci per cell after infection with adenoviral Luc or Cre from (**a**). Values shown are the means from three independent experiments in which 50 interphase cells were counted.

lesion in human mammary tissue. In our previous study, Aurora A overexpression only induced small papillary hyperplasia without ADH in *p53+/+* mice and (3) upregulated p16 expression in *p53−/−* mice after Aurora A overexpression, indicating that p16 is hypothesized to be critical in preventing mammary gland malignancy by inducing senescence.

The presence of Aurora A overexpression in various human cancers suggests that Aurora A may function as an oncogene; however, even though considerable evidence indicates that Aurora A overexpression provokes mitotic abnormalities and chromosome instability, to date no evidence has been presented proving that it leads to malignancy *in vivo*. Indeed, several groups have reported that Aurora A alone can not induce malignancy. Anand *et al.* (2003) reported that overexpression of Aurora A could not transform primary cells. Fukuda *et al.* (2005) demonstrated that a conditional transgenic system for mouse Aurora A showed no abnormalities during mouse development, possibly because the Aurora A protein is unstable and readily degraded in normal mouse tissues. In our present study, Aurora A was stably expressed but did not lead to the development of malignant tumors because of the induction of cellular senescence. In contrast, Wang *et al.* (2006) reported recently that overexpression of Aurora A could cause mammary tumor formation through inducing genetic instability and activating AKT. The discrepancy between their results and ours may stem from the use of different mouse strains. The FVB/N mice used by Wang *et al.* (2006) are highly susceptible to chemically induced squamous cell carcinomas, with a high rate of malignant conversion from papilloma to carcinoma. The different genetic backgrounds of this mouse strain, perhaps, impairment of factors associated with senescence and apoptosis, could result in different outcome of tumorigenesis.

Tumorigenesis in humans is a multistep process, and genetic alterations including inactivation of tumor suppressor genes and activation of oncogenes lead to the progressive conversion of normal cells into cancer cells (Hanahan and Weinberg, 2000). The p53 tumor suppressor gene acts to reduce the incidence of cancer by regulating genes involved in cell cycle arrest, apoptosis, and other pathways (Levine, 1997). p53-mediated apoptosis is an important part of the tumor suppressor phenotype (Symonds *et al.*, 1994). Loss of p53 function not only facilitates resistance to apoptosis but also induces genomic instability (Hanahan and Weinberg, 2000). In tetraploid cells resulting from mitotic abnormalities such as failure of mitotic spindle assembly or cytokinesis, the p53-dependent postmitotic checkpoint is

activated to induce G_1 arrest and apoptosis. However, in p53-deficient cells, tetraploidy caused by mitotic failures leads to the propagation of errors of late mitosis and to the generation of aneuploidy (Andreassen *et al.*, 2001). Tetraploidy enhances the occurrence of chromosomal alterations and promotes tumor development in p53-null cells (Fujiwara *et al.*, 2005). In this study, we found that Aurora A overexpression led to mitotic aberrations, including increased numbers of centrosomes and hyperploidy, which consequently generate aneuploid cells, in p53-deficient MEFs. However, in our p53-null model, Aurora A overexpression induced benign lesions but not malignant ones. These findings provide important evidence that overexpression of Aurora A can promote hyperproliferative precancerous lesions when p53 functions are impaired but that additional factors are required for malignant transformation.

Cellular senescence is a state of permanent growth arrest provoked by a variety of stresses (Lundberg *et al.*, 2000). Expression of oncogenic ras in primary human or rodent cells results in permanent G_1 arrest and provokes premature cell senescence (Serrano *et al.*, 1997). A new study using a mouse model of cancer initiation showed that senescent cells exist in premalignant tumors but not in malignant ones, providing circumstantial evidence that oncogene-induced senescence acts to counter tumorigenesis (Collado *et al.*, 2005). Our mouse model suggests that both senescence and apoptosis seem to act as tumor suppression mechanisms that prevent the further evolution of preneoplastic cells in response to Aurora A dysregulation. Some signaling pathways have been shown to promote apoptosis in one cell type but to induce senescence in others; for example, the transcription factor E2F-1 cooperates with p53 to mediate apoptosis in murine cells but induces senescence when overexpressed in normal human fibroblasts (Wu and Levine, 1994; Dimri *et al.*, 2000). We illustrate here that oncogenic Aurora A can activate both the p53/p21 and the Rb/p16 pathways to induce both apoptosis and senescence programs and that the two programs seem to serve as backups for each other.

Cellular senescence can be also triggered by various types of DNA damage or chromatin remodeling in addition to oncogenic forms of ras or raf (Chen *et al.*, 1995; Serrano *et al.*, 1997; Zhu *et al.*, 1998). Much evidence exists supporting a link between DNA damage, p16 expression and cellular senescence. For example, in normal human fibroblasts and tumor cells, DNA double-strand breaks lead to increased p16 expression and senescence (Robles and Adami, 1998; te Poele *et al.*, 2002). To examine whether p16 expression can be a major factor to induce senescence in Aurora A overexpressing cells, we infected the immortalized mouse fibroblasts derived from *CAG-CAT-Aurora A* mice, which do not express p16, with p16-expressing adenovirus and/or AxCANCre virus, which induces Aurora A expression and tested senescence of those cells with an SA-β-gal assay. While no SA-β-gal-positive cells were detected among immortalized mouse fibroblasts infected with either the p16-expressing adenovirus or the AxCANCre virus, >20% of the fibroblasts co-expressing p16 and Aurora A underwent senescence (data not shown). These findings suggest that deregulated Aurora A promotes cellular senescence in a p16-dependent manner.

We speculate that induction of DNA damage by Aurora A could be the mechanism by which Aurora A induces cellular senescence. Possible mechanisms of DNA damage induction might involve the aberrant cell cycle progression mediated by Aurora A overexpression. Further, given that Aurora A is overexpressed not only in mitotic cells but also in interphase cells, it might activate aberrant signaling pathways involved in DNA synthesis and repair. Another possible mechanism is the induction of oxidative stress, a major cause of DNA damage and genetic instability. Recent studies have proposed that activation of oncogenes such as c-Myc and ras alters biochemical pathways to produce reactive oxygen species that lead to DNA damage (Lee *et al.*, 1999; Vafa *et al.*, 2002). Overexpressed Aurora A may have a similar effect. p53 was recently reported to protect the genome from oxidation by reactive oxygen species; downregulation of p53 resulted in excessive oxidation of the DNA (Sablina *et al.*, 2005). We found that the number of γ-H2AX-positive foci was significantly higher in p53-deficient MEFs than in p53-wild-type MEFs after Aurora A overexpression. Our results provide new evidence that Aurora A overexpression induces massive DNA damage in p53-deficient cells that can override postmitotic G_1 arrest, indicating that p53 might protect the genome from the oxidative stress induced by Aurora A overexpression. Our future studies will focus on illustrating the mechanism by which Aurora A overexpression induces DNA damage in p53-deficient cells. One can speculate that p53 prevents Aurora A-mediated oxidative stress-induced DNA damage that would otherwise trigger p16 expression and cellular senescence. Alternatively, disruption of both p53/p21 and p16/Rb pathways may be necessary for neoplastic transformation by Aurora A.

We still do not know by which pathway p16 induces cellular senescence in our model. p16 specifically inhibits cyclin-dependent kinase 4 (cdk4) and cdk6-mediated G_1/S progression and therefore limiting cdk4/6-Cyclin D complex formation and phosphorylation of pRb. Narita *et al.* (2003) reported that the formation of heterochromatic foci depends on the Rb pathway in senescent cells. Given that we found HP1-γ expression in p53-deficient tissue in our model, the Rb pathway may be involved in the ability of p16 to promote senescence. The Rb2/p130 protein, which is a member of the Rb family, is also reported to mediate the senescent growth arrest (Helmbold *et al.*, 2006). Similar to pRb, Rb2/p130 is hyperphosphorylated by cyclin D/cdk4 or 6 and cyclin E or A/cdk2 complexes and considered acting as a master regulator of pRb in a sustained senescent arrest. We are working on identifying the status of phosphorylated pRb and Rb2 in our model to confirm whether these factors are responsible for the induction of senescence.

Other future studies will include our overexpressing Aurora A in the mammary glands of INK4a/Arf-null mice reported by Serrano *et al.* (1996). These mice,

which carry a targeted deletion of the *INK4a* locus that eliminates both $p16^{INK4a}$ and $p19^{ARF}$, are viable but prone to spontaneous and carcinogen-induced tumors (Serrano *et al.*, 1996); moreover, the introduction of oncogenic ras into INK4a-deficient fibroblasts resulted in neoplastic transformation. We speculate that polyploidy associated with disruption of both p53/p21 and p16/Rb pathways may be necessary for neoplastic transformation by Aurora A.

Materials and methods

Generation of transgenic mice

We previously generated *CAG-CAT-Aurora A* transgenic mice (strain C57BL/6) carrying a *pCAG-loxP-CAT-loxP-Aurora A* construct designed to induce human Aurora A overexpression by Cre-mediated recombination; we also generated *CAG-CAT-Aurora A;Wap-Cre* double-transgenic mice by mating *CAG-CAT-Aurora A* mice with *Wap* (whey acidic protein)-*Cre* mice (Zhang *et al.*, 2004). *CAG-CAT-Aurora A;Wap-Cre;p53+/−* transgenic mice, which carry one allele of p53, and *CAG-CAT-Aurora A;Wap-Cre;p53−/−* transgenic mice, which carry neither p53 allele, were typically generated from crosses of *CAG-CAT-Aurora A;p53+/−* mice and *Wap-Cre;p53+/−* mice. For the study reported here, *CAG-CAT-Aurora A;Wap-Cre;p53+/−*, *CAG-CAT-Aurora A;Wap-Cre;p53−/−* and littermate control female mice were mated at 8 weeks of age and housed with male mice thereafter to induce Aurora A expression. Animals were examined weekly for evidence of tumor development.

Generation and culture of MEFs and immunofluorescence and flow cytometry analyses

Primary MEFs were generated from mouse embryos at E13.5 days and cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. Early passage MEFs (p2–3) were used at the initiation of all experiments. Immunofluoresence staining was performed as follows: cells were fixed with 4% paraformaldehyde for 20 min, permeabilized with 0.2% Triton X-100 in Tris-buffered saline (TBS) for 5 min, blocked for 1 h with 5% bovine serum albumin in TBS, and incubated with primary antibodies in 0.3% bovine serum albumin in TBS. Primary antibodies were mouse monoclonal anti-α-tubulin antibody (B-5-1-2; 1:1000, Sigma-Aldrich, St Louis, MO, USA); rabbit polyclonal anti-phosphorylated histone H2AX antibody (1:250; Trevigen, Gaithersburg, MD, USA); and rabbit polyclonal anti-pericentrin antibody (1:1000; Covance, Denver, PA, USA). Fluorescein isothiocyanate (FITC)-conjugated antibodies (Biosource, Carlsbad, CA, USA) were used as secondary antibodies. Cells were counterstained with propidium iodide (PI) before being mounted under glass coverslips and analysed by confocal microscopy (FV300, Olympus). For flow cytometric analysis, MEFs were infected by adenovirus for 48 h, after which BrdU (10 ng ml^{-1}) was added in culture medium and the MEFs incubated for further 4 h. Cells were then fixed with 70% ethanol, stained with anti-BrdU-FITC and PI, and subjected to flow cytometry according to the manufacturer's instructions (Becton Dickinson, Franklin Lakes, NJ, USA).

Histologic and immunohistochemical, and immunofluoresence analyses

For conventional histologic analysis, mammary glands were fixed in 10% phosphate-buffered formaldehyde for 24 h and embedded in paraffin. Sections (3 μm thick) were stained with H&E using standard techniques. For cryosections, tissues were fixed with 4% paraformaldehyde for 6 h, and then transferred to a 30% sucrose solution for 24 h before 10-μm-thick sections were prepared. Immunohistochemical and immunofluoresent analyses were performed as previously described (Zhang *et al.*, 2004). Primary antibodies were rabbit polyclonal anti-Aurora A (1:100; TransGenic, Kumamoto, Japan); rat monoclonal anti-E-cadherin (ECCD2, 1:1000; Takara, Otsu, Japan); rabbit monoclonal anti-Ki67 (SP6, 1:200; NeoMarkers, Fremont, CA, USA); rabbit polyclonal anti-active caspase 3 (1:100; Chemicon, Temecula, CA, USA); mouse monoclonal anti-HP1-γ (1:1000; Chemicon); rabbit polyclonal anti-DcR2 (1:250; Stressgen, Ann Arbor, MI, USA) and rabbit polyclonal anti-p16 (M-156, 1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA).

SA-β-gal and TUNEL assays

Cryosections (10 μm thick) were subjected to SA-β-gal and TUNEL assays as follows. SA-β-gal activity was detected with a Senescence β-Galactosidase Staining Kit according to the manufacturer's instructions (Cell Signaling). Apoptotic cells in mammary tissues were detected by TUNEL with an *In situ* Apoptosis Detection Kit (TaKaRa).

Adenovirus infection

Primary MEFs were infected with adenovirus at multiplicities of infection of at least 20 for 1 h, after which the virus solution was exchanged for fresh medium. Infection efficiency under these conditions is normally >95%.

Statistical analysis

Statistical analyses were performed using commercially available software (Statview, version 5.0, SAS Institute, Cary, NC, USA). The data obtained were analysed by *t*-test. Values represent the mean ± s.e. Statistical significance was defined as $P<0.05$.

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