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Establishment of Ku70-Deficient Lung Epithelial Cell Lines and Their Hypersensitivity to Low-Dose X-Irradiation

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ABSTRACT. In clinical situations, cellular resistance to chemotherapy and radiotherapy is a significant component of tumor treatment failure. The DNA repair protein Ku70 is a key contributor to chemoresistance to anticancer agents, e.g., etoposide and bleomycin, or radioresistance. Ku70 plays a key role as a sensor of DNA double-strand breaks (DSBs) induced following exposure to ionizing radiation as well as treatment with some chemotherapeutic drugs. The responses of different organs to radiation vary widely and likely depend on the cell population in the organs. However, it is not clear whether Ku70 plays a role in the low-dose radioresistance of lung epithelial cells. In this study, we established Ku70-deficient epithelial cell lines from murine lungs lacking Ku70. Ku70–/– lung epithelial cells exhibited reduced Ku80 expression. Moreover, Ku70–/– lung epithelial cells were more sensitive than controls (Ku70+/– lung epithelial cells) to low-dose X-irradiation (< 0.5 Gy). We also found that consistent with the Ku70 function as a sensor of DSBs, Ku70 mainly localized in the nuclei of murine lung epithelial cells. These findings clearly indicate that Ku70 plays a key role in regulation of the Ku80 expression level in and the radioresistance of lung epithelial cells. Our data also suggest that these cell lines might be useful not only for study of Ku70 functions and the DSB repair pathway, but also for study of the molecular mechanism underlying the sensitivity to chemotherapeutic drugs and radiation in lung epithelial cells.
KEY WORDS: anticancer treatment, epithelial cell, Ku70, Ku80, radiation.

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Various chemotherapeutic drugs, e.g., etoposide and bleomycin, and ionizing radiation have been clinically applied to the treatment of many types of animal and human malignancy. On the other hand, the responses of different organs to clinical radiation or chemotherapeutic drugs vary widely and likely depend on the intrinsic chemosensitivity or radiosensitivity of the different cell populations in the organs. It is important to clarify the mechanisms underlying the chemosensitivity or radiosensitivity of each normal tissue type and each cancer cell type in order to deliver much higher doses to cancer cells than is possible with standard techniques without severe side effects.

A DNA double-strand break (DSB) is the most serious DNA damage [9]. Unrepaired or improperly repaired DSBs can lead to chromosomal truncations and translocations, which can contribute to the development of cancer in higher eukaryotic organisms. DSBs are induced following exposure to ionizing radiation as well as treatment with etoposide or bleomycin [19, 24, 28]. Two major pathways exist in mammalian cells for repair of DNA DSBs: nonhomologous DNA-end-joining (NHEJ) repair and homologous recombination (HR) [9, 25]. The NHEJ repair process, which is responsible for repairing a major fraction of DNA DSBs in somatic cells of all multicellular eukaryotes, is considered to begin with the binding of Ku, i.e., a heterodimer of Ku70 and Ku80 [6, 21]. However, it was remains unclear which pathway, NHEJ, HR or both, functions in the low-dose radiation response.

In general, Ku70 is ubiquitously expressed in mammalian cells and plays the key role as a sensor of DNA DSBs induced following exposure to ionizing radiation as well as treatment with some chemotherapeutic drugs [11, 15, 21]. On the other hand, Ku70 has a function limited to some specific cells, e.g., V(D)J recombination in the immune system and retroviral integration into a host genome [1]. Therefore, it is important to clarify the role and regulation mechanism of Ku70 in each of the cell and tissue types.

The control mechanisms underlying the heterodimerization and subcellular localization of Ku70 play a key role in regulating the physiological function of Ku [15]. It was reported that Ku70 is observed mainly in the cytoplasm of mouse and rat fibroblasts [2, 29]. It was also reported that Ku70 is translocated from the cytoplasm to the nucleus in rat and mouse fibroblasts after X-irradiation [2, 29]. On the other hand, we have demonstrated that the nuclear translocation of Ku70 is, at least in part, controlled at a nuclear localization signal (NLS)-recognition step by NLS receptors and regulated by heterodimerization with Ku80 [12–15, 17].

Mice containing targeted disruption in Ku70 show an increased sensitivity to IR. Cells from Ku70–/– mice, e.g., embryonic stem (ES) cells and cells from bone marrow, show an increased sensitivity to IR [4, 23]. However, there are few reports about the epithelial cells from Ku70–/– mice. On the other hand, the cellular response in terms of DSBs is substantially different between low- (< 0.5 Gy) and high-radiation doses [7]. Therefore, it is necessary to understand the role of Ku70 and the NHEJ pathway in the response to low-dose radiation as well as to high-dose radiation.

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Many of the studies of the radiosensitivity of cells have been performed using cultured cells or mice exposed to high-dose radiation typically used for analysis. In contrast to high-dose radiation, hypersensitivity to low-dose radiation is less well understood in mammalian cells [7], particularly in epithelial cells. More than 90% of cancers are of epithelial cell origin. Lung cancer is classified into non-small cell lung cancer (NSCLC), which includes adenocarcinoma and squamous cell carcinoma (SCC), and small cell lung cancer (SCLC). Recently, it has been reported that BRCA1, BRCA2 and Ku80 are tumor suppressors in lung adenocarcinoma and SCC [20]. In this study, we established and characterized Ku70-deficient epithelial cell lines from murine lungs lacking Ku70.

MATERIALS AND METHODS

Mice, cell lines, cultures and transfection: Heterozygotes carrying the Ku70 mutation were interbred, and offspring were genotyped by PCR analysis of tail DNA using specific primers, as has been described previously [5]. All the mice were reared and handled in accordance with the guidelines on the care and use of laboratory animals of the National Institute of Radiological Sciences. Three lung epithelial cell lines were established from the lungs of 7-day-old Ku70–/– or Ku70+/– mice by standard procedures and SV40 immortalization. Briefly, the SV40 T antigen expression vector (pLNCLT) was transfected using Effectene (Qiagen Inc., Chatsworth, CA, U.S.A.), and stable cell lines were established using G418 (0.3 mg/mL), as described previously [16]. A cell line of a human tumor, HeLa, was cultured as described in previous studies [10, 12]. The cells were grown in a monolayer culture in DMEM (Nissui Seiyaku Co., Tokyo, Japan) supplemented with 10% fetal bovine serum and were maintained in a humidified incubator at 37°C under 5% CO_2. Transient transfections were performed in Ku70–/– cells using FuGene6 (Roche Diagnostics K.K., Indianapolis, IN, U.S.A.) as described previously, and the cells were cultured for 2 days and then monitored using an FV300 confocal laser scanning microscope (Olympus, Tokyo, Japan) as previously described [18, 19].

X-irradiation: Cells were exposed to X-rays at a dose rate of 0.86–0.91 Gy/min at room temperature [16]. X-rays were generated at 200 kVp/20 mA and filtered through 0.5-mm Cu and Al filters, as described previously [16].

Immunoblotting: Total lysates were extracted as previously described [10, 11]. The supernatants were electrophoresed on 5–20% SDS-polyacrylamide gels (Wako, Osaka, Japan). The fractionated products were electrotransferred onto Hybond-P membranes (GE Healthcare Bio-Sci. Corp., Piscataway, NJ, U.S.A.). After blocking nonspecific binding sites with an Advance blocking agent (GE Healthcare Bio-Sci. Corp.), the membranes were incubated with a goat anti-Ku70 polyclonal antibody (C-19; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), a goat anti-Ku80 polyclonal antibody (M-20; Santa Cruz Biotechnology), a mouse anti-E-cadherin monoclonal antibody (BD Biosciences, Bedford, MA, U.S.A.), a mouse anti-SV40 T antigen monoclonal antibody (Ab-2; Merck, Darmstadt, Germany) or a mouse anti-β-actin monoclonal antibody (Sigma, St. Louis, MO, U.S.A.). The binding to corresponding proteins was visualized using an Advance Western blotting detection system (GE Healthcare Bio-Sci. Corp.), in accordance with the manufacturer's instructions.

Immunofluorescence staining: Immunofluorescence staining was performed as previously described [19]. Briefly, cells were grown on culture slides, washed with PBS, and fixed at room temperature. The fixed cells were first blocked for 30 min using a blocking solution and then incubated for 30 min at room temperature with a goat anti-Ku70 polyclonal antibody (C-19) or a goat anti-Ku80 polyclonal antibody (M-20). After washing with PBS three times, antibody binding was detected by incubation with Alexa Fluor 488-conjugated secondary antibodies (Molecular Probes, OR, U.S.A.). DNA was stained with DAPI fluorescent dye. The distribution of fluorescent signals was monitored using an FV300 confocal laser scanning microscope (Olympus) as previously described [18, 19].

Clonogenic survival assay: Cells were seeded at a density of 200 cells per dish in 60-mm dishes. The cells were then exposed to X-rays (0.125, 0.25, 0.5, 1, 2 Gy) and were further incubated for 7 days postirradiation. The cells were subsequently washed with PBS, fixed in 2% methylene blue (containing 50% methanol) and visualized using an Olympus CKX41 inverted phase-contrast microscope. Colonies containing more than 50 cells were scored as survivors. The colonies were counted, and survival was calculated by dividing the number of colonies of treated cells by that of the control. All exposures were carried out in triplicate and were repeated three times.

RESULTS

Ku70 is a key contributor to chemoresistance to anticancer agents, e.g., etoposide, or radioresistance [4, 8, 23]. To clarify whether Ku70 plays a role in low-dose radioresistance in lung epithelial cells, we tried to generate Ku70-deficient lung epithelial cell lines. Gu *et al.* demonstrated that somatic cells from Ku70–/– mice, i.e., MEFs, displayed severe growth retardation and an early onset of replicative senescence [5]. Mice heterozygous for the Ku70 mutation (Ku70+/–) were indistinguishable from wild-type (Ku70+/+) mice in growth (body weight); however, Ku70–/– mice exhibited pronounced growth retardation [5]. In addition, Ku70–/– ES cells were markedly sensitive to γ-irradiation as compared with Ku70+/– cells, whose radiation sensitivity was indistinguishable from that of Ku70+/+ ES cells [4]. On the other hand, it was reported that the well-established immortalization method using the SV40 T antigen or Abelson murine leukemia retrovirus does not affect the radiosensitivity of Ku80–/– cells [22, 27]. In this study, on the basis of this information, we tried to establish lung epithelial cell lines from Ku70–/– mice and control mice (Ku70+/–) by transfection of primary cultures with the SV40 T antigen to

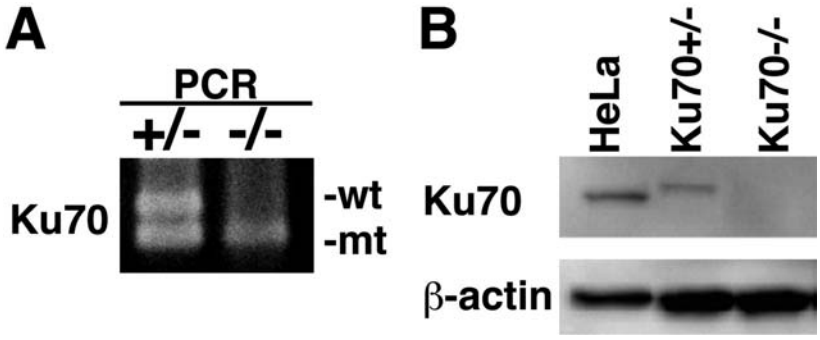

Fig. 1. Establishment of Ku70–/– lung epithelial cell lines. (A) PCR analysis of genomic DNA from control (Ku70+/–) and Ku70(–/–) mice. The wild-type and mutant fragments are about 0.4 kbp (wt) and 0.3 kbp (mt), respectively. (B) Expression of Ku70 in total cell lysates from control (Ku70+/–) and Ku70(–/–) lung epithelial cells. Total cell lysates from two lung epithelial cell lines and a HeLa cell (positive control for Ku70) line were analyzed by Western blotting using an anti-Ku70 antibody or an anti-β-actin antibody.

overcome replicative senescence. Each of the three independent clones was obtained at 0.3 mg/m*l* G418, and all clones were established. As shown in Fig. 1A, the donor mice, i.e., Ku70–/– and Ku70+/– mice, were confirmed by genomic PCR analysis. Moreover, the Ku70–/– or Ku70+/– cell lines established were identified by Western blotting analysis (Figs. 1B and 2). At passage 13, the established Ku70–/– lung epithelial cells, as well as control cells, exhibited not only active growth, but also no sign of a premature termination of cell divisions (data not shown).

To assess Ku70, Ku80, SV40 T antigen and E-cadherin expressions, we performed Western blotting analyses of Ku70–/– and Ku70+/– lung epithelial cells and HeLa cells (as the control). As shown in Fig. 2, expectedly, the expressions of E-cadherin, which is an epithelial cell marker, and SV40 T antigen were detected in both Ku70–/– and Ku70+/– cells, but not in HeLa cells. These observations confirmed that the established cell lines were of epithelial cell origin and expressed the T antigen stably. Moreover, although Ku70 was clearly detected in Ku70+/– cells, we did not detect any Ku70 in Ku70–/– cells. Previous studies have shown that Ku80 expression is reduced in Ku70-deficient cells, e.g., ES and MEF cells, and extracts from the lungs of Ku70–/– mice [4, 5, 23]. As shown in Fig. 2, Ku80 expression was markedly reduced in Ku70–/– lung epithelial cells as compared with that in Ku70+/– cells. These findings strongly support the idea that the stability of Ku80 depends on Ku70. We surmised that the Ku80 expression level is regulated by Ku70 in lung epithelial cells.

To clarify whether Ku70 plays a role in low-dose radioresistance in lung epithelial cells, we tested the radiosensitivity of Ku70–/– cells and Ku70+/– cells by a colony survival assay. As shown in Fig. 3, Ku70–/– cells were markedly sensitive to X-irradiation as compared with Ku70+/– cells. In addition, Ku70–/– cells were sensitive to X-irradiation at low doses, from 0.125 to 0.5 Gy. These findings suggest that Ku70 plays a key role in radioresistance to not only high-dose X-irradiation, but also low-dose X-irradiation

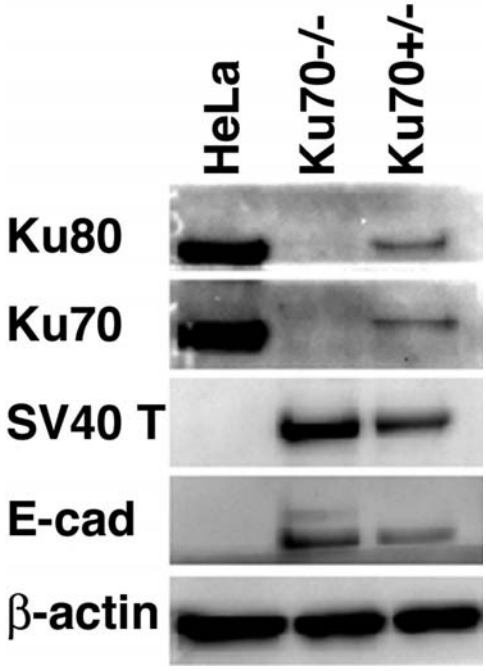

Fig. 2. Western blot analysis of control (Ku70+/–) and Ku70–/– lung epithelial cells. Total cell lysates from two lung epithelial cell lines and a HeLa cell (negative control for E-cadherin and SV40 T antigen) line were analyzed by Western blotting using an anti-Ku70 antibody (Ku70), anti-Ku80 antibody (Ku80), anti-E-cadherin antibody (E-cad), anti-SV40 T antigen antibody (SV40 T) or anti-β-actin antibody (β-actin).

(< 0.5 Gy), in lung epithelial cells.

To examine the subcellular localization of Ku70 in lung epithelial cells, we investigated the distribution of Ku70 and Ku80 by confocal laser microscopy. As shown in Fig. 4A, indirect immunofluorescence staining using the anti-Ku70

or anti-Ku80 antibody showed that fluorescence was mainly detected in the nucleoplasm of Ku70+/– lung epithelial cells during interphase. These findings suggest that Ku70 and its heterodimer partner Ku80 mainly localized in the nuclei of murine lung epithelial cells. To determine the subcellular localization of Ku70 in living cells, we transfected the EGFP-tagged Ku70- or EGFP-expression vector and examined the localization of EGFP-Ku70 in living Ku70–/– lung epithelial cells. As shown in Fig. 4B, we found that in the EGFP-Ku70 transfectants, EGFP-Ku70 mainly localized in the nuclei of the living cells, whereas in the EGFP transfectants, EGFP localized throughout the nuclei and cytoplasm. These observations clearly demonstrated that in murine lung epithelial cells, Ku70 mainly localizes in the nuclei of interphase cells.

DISCUSSION

We established and characterized Ku70-deficient epithelial cell lines from murine lungs lacking Ku70. Our data showed that the Ku70–/– lung epithelial cells produce no detectable Ku70 and very little Ku80, suggesting that Ku70 plays a key role in regulation of Ku80 expression. The Ku70–/– cells showed an increased sensitivity to not only high-dose, but also low-dose X-irradiation compared with Ku70+/– cells. Moreover, our findings demonstrated that Ku70 is mainly localized in the nuclei of murine lung epithelial cells. These findings suggest that Ku70 plays a key role in the repair of DSBs caused by not only high-dose X-irradiation, but also low-dose X-irradiation, in murine lung epithelial cells.

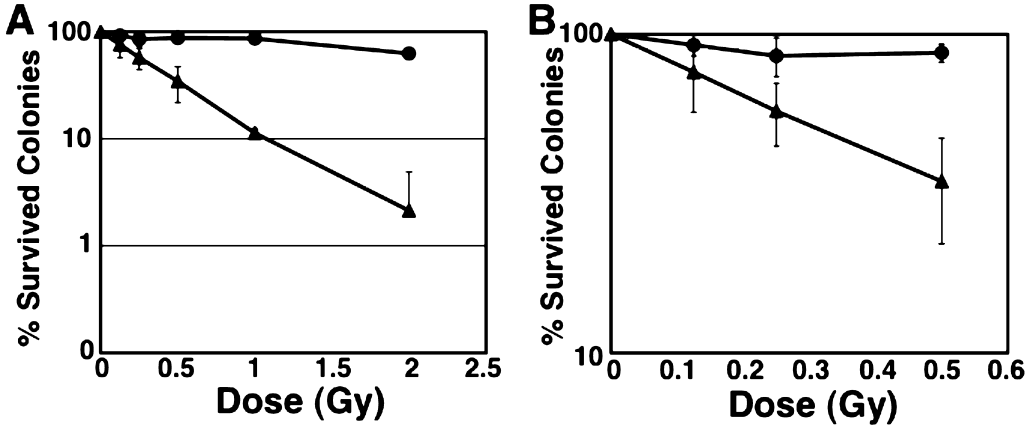

Fig. 3. Ku70 –/– lung epithelial cells showed an increased sensitivity to X-irradiation. Ku70–/– and control (Ku70+/–) cells were exposed to X-irradiation at a dose of 0.125, 0.25, 0.5, 1 or 2 Gy (A). (B) High magnification of corresponding results for dose ranges under 0.5 Gy in A. Each point in this plot is the average of results of triplicate experiments and is a percentage of the colony counts from each unirradiated cell line. Error bars represent the SD.

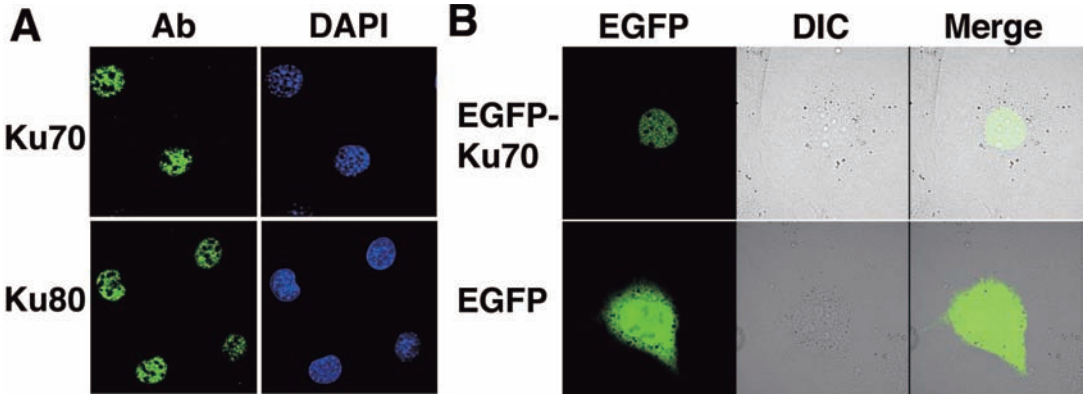

Fig. 4. Subcellular localization of Ku70 in murine lung epithelial cells during interphase. (A) Immunostaining of Ku70+/– lung epithelial cells with anti-Ku70 antibody and anti-Ku80 antibody. The cells were fixed and stained with an anti-Ku70 antibody or an anti-Ku80 antibody and DAPI. The stained cells were analyzed by confocal laser microscopy. (B) Intracellular distribution of Ku70 in living cells during interphase. Ku70–/– living cells transiently expressing EGFP-Ku70 or EGFP were analyzed by confocal laser microscopy. For the same cells, EGFP images (left) and differential interference contrast images (DIC; center) are shown alone or merged (right) as indicated. Recorded using a 60X objective.

In this study, we confirmed and are the first to note that Ku80 expression is reduced in Ku70–/– lung epithelial cells, indicating that Ku70 stabilizes Ku80 in murine lung epithelial cells. We speculate that Ku80 was rescued from intracellular degradation by binding with Ku70 in Ku70+/– lung epithelial cells, although further studies need to clarify this. On the other hand, it is known that Ku70 stabilizes Ku80 in other cell types. Previous studies showed that Ku80 protein expression levels substantially decrease in Ku70–/– ES cells and Ku70–/– fibroblasts [4, 23]. Furthermore, it was reported that Ku80 is detectable only in the kidneys of Ku70–/– mice, but at a reduced level compared with that of Ku70+/– mice, whereas Ku80 is undetectable in extracts from the brain, lungs and liver [5]. The heterodimerization of Ku70/Ku80 is essential for Ku80 accumulation at the DNA damage sites and Ku70-dependent DSB repair, i.e., the classical NHEJ pathway [8, 18]. We speculate that Ku70 plays a key role in regulation of the Ku80 expression level in order to control the activity of Ku70-dependent NHEJ at the DSB recognition step.

The control mechanisms underlying the subcellular localization of Ku70 and Ku80 play a key role in regulating the physiological function of Ku [15]. The nuclear translocation of Ku70 is, at least in part, controlled at a nuclear localization signal (NLS)-recognition step by NLS receptors and regulated by the heterodimerization with Ku80 [12–15, 17]. In hamster cells, Ku70/Ku80 heterodimer accumulation at DSBs produced using a 405-nm laser starts immediately after irradiation [18], strongly supporting the idea that the heterodimer of Ku70 and Ku80 is a sensor of DSBs in the nuclei. Most recently, we have reported that Ku80, in contrast to H2AX, is highly mobile in the nuclei of hamster cells and that the mobility of a major portion of Ku80 is not affected by DNA DSBs produced by treatment with antitumor drugs [19]. Ku70 is mainly detected in the nuclei of normal human diploid lung fibroblasts, e.g., TIG-3 and MRC-5, and human cancer cells, e.g., HeLa and MCF-7 cells [11, 13, 15]. Interestingly, it was reported that Ku70 is observed mainly in the cytoplasm of mouse and rat fibroblasts [2, 29], although where Ku70 is mainly localized in murine lung epithelial cells has not yet been clarified. In addition, it was reported that Ku70 and Ku80 are translocated from the cytoplasm to the nucleus in rat and mouse fibroblasts after X-irradiation [2, 29]. In this study, our findings clearly demonstrate that consistent with the Ku70 function as a sensor of DSBs, Ku70 is mainly detected in the nuclei of murine lung epithelial cells, although the discrepancy remains unclarified. We are now interested in whether the difference in the subcellular localization of Ku70 between murine lung epithelial cells and other cells is dependent on the difference in some culture conditions.

Presently, human lung cancer causes many deaths worldwide. Therefore, it is important to elucidate the molecular mechanism underlying the radioresistance and chemoresistance of lung cancer cells. It was reported that BRCA1, BRCA2, and Ku80 are tumor suppressors in lung adenocarcinoma and SCC [20]. Recently, it has been reported that lung cancer susceptibility and prognosis are associated with polymorphisms in the NHEJ pathway genes, e.g., the Ku70 and Ku80 genes [26]. There are no reports on the sensitivities of murine lung epithelial cells, although the importance of Ku70 in DNA DSB repair after ionizing irradiation is well demonstrated by the profound enhancement of the radiosensitivity of Ku70-deficient cells e.g., ES cells and cells from the bone marrow [4, 23]. On the other hand, it was demonstrated that using gene-targeting methodologies, targeted disruption of Ku70 in a human colon cancer cell line, HCT116, is lethal [3], suggesting that using the conventional methods, it is not possible to generate human cells deficient in Ku70 because Ku70 is essential in human somatic cells. The cell lines established in this study might be useful for analysis of the role of Ku70 in relation to lung cancer susceptibility and prognosis, as well as for analysis of the molecular mechanism underlying cellular resistance to chemotherapy and radiotherapy, although further studies are required to demonstrate this.

In conclusion, we established lung epithelial cell lines from Ku70–/– mice. The findings suggest that Ku70 plays an important role in the mechanism underlying the radioresistance of lung epithelial cells exposed to not only high-dose X-irradiation, but also low-dose X-irradiation. On the other hand, it was reported that the Ku70–/– ES cell lines exhibit hypersensitivities to the antitumor drug etoposide [8]. Further studies to elucidate the molecular mechanism underlying the radioresistance and chemoresistance of the established lung epithelial cell lines will lead to a better understanding of not only the molecular mechanisms of Ku70 function in lung epithelial cells, but also the development of new gene therapies, radiotherapies and chemotherapies for many types of malignancies originating from epithelial cells.

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