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Lack of *Rev7* function results in development of tubulostromal adenomas in mouse ovary

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ABSTRACT

Rev7 is a subunit of Polζ, one of the translesion DNA synthesis (TLS) polymerases involved in DNA damage repair. We recently found that *Rev7* is also essential for germ cell development in mouse. In the present study, we found the development of ovarian tumors in *Rev7* mutant mouse, suggesting the involvement of TLS deficiency in the etiology of ovarian tumor. The *Rev7* mutant mice showed complete lack of oocytes and follicles in the ovary. The lack of follicles causes a significant increase of gonadotropin level and an increase in the proliferation of ovarian cells. As a result, the weight of the ovaries of *Rev7* mutant mice increased with age and they developed tubulostromal adenomas. However, the remarkable overgrowth of ovaries occurred after gonadotropin level decreases at older ages, suggesting gonadotropin-independent progression of the ovarian tumors. In addition, the *Rev7* mutant fibroblasts and ovarian cells showed significant accumulation of DNA damage. These findings suggest that not only increased gonadotropin levels but also lack of DNA damage repair function could be responsible for the development of ovarian tumors in the *Rev7* mutant mouse.

1. Introduction

Rev7 encodes a subunit of Polζ, one of the error-prone DNA polymerases involved in translesion DNA synthesis (TLS) to repair DNA damage during DNA replication (Lange et al., 2011; Loeb and Monnat, 2008). When the replication fork is encountered at DNA damage sites during DNA replication, the replication fork stalls and this arrest of DNA replication causes genome instability and cell death; however, cells can relieve this arrest of DNA replication by bypassing DNA damage sites using the TLS polymerases. Therefore, the TLS polymerases play an essential role in maintaining genome integrity and tolerance to DNA damage in mammalian cells, and most of the reported targeted mutations of the TLS polymerases show defects in DNA integrity and stability. For example, conditional knockout mouse of *Rev3L*, another subunit of Polζ, in skin epithelial cells leads to hypersensitivity to UV radiation, chromosomal instability, and defects in wound healing caused by proliferation deficiency. Furthermore, these mice show age-dependent lymphomas and skin carcinomas (Lange et al., 2013; Wittschieben et al., 2010), indicating an essential role for Polζ in preventing tumor development caused by genome instability.

Recently, we and others reported unique functions of *Rev7* in mouse development and fertility (Khalaj et al., 2014; Pirouz et al., 2013; Watanabe et al., 2013). We found that *repro22* mouse, an ENU-induced mutant mouse with missense mutation of the *Rev7* gene, exhibits severe germ cell depletion leading to male and female infertility, partial embryonic lethality, and growth retardation (Khalaj et al., 2014). Interestingly, the *Rev7* mutant male mouse showed hyperplasia of Leydig cells at adult stages, presumably caused by secondary effects of germ cell depletion (Khalaj et al., 2008). In the present study, therefore, we investigated the ovarian phenotype of *Rev7* mutant female mice and found hyperproliferation of ovarian cells and the development of ovarian tumors. Although the etiology of ovarian tumors is not well understood, functional oocyte/follicle

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absence and genetic factors were considered as two main risk factors in ovarian tumors. Several lines of evidence show that an increased circulating gonadotropin level caused by the absence of functional follicles is involved in a predisposition to ovarian tumors (Auersperg et al., 2008; Choi et al., 2007; Parrott et al., 2001; Syed et al., 2001). For example, increased incidences of ovarian cancer in the perimenopausal period in humans are associated with elevated gonadotropin level (Choi et al., 2007). On the other hand, mutations in particular genes have been reported to be causes of ovarian tumors (Campbell et al., 2004; Carpten et al., 2007; Hennessy and Mills, 2006; Hennessy et al., 2006). In particular, higher risks for ovarian cancer have been reported in human patients with mutations in genes associated with DNA damage repair and genome integrity, including BRCA1 and BRCA2 (Zweemer et al., 1999), indicating that genome integrity also plays an important role in ovarian tumorigenesis.

In the present study, we show that an increased gonadotropin level caused by the absence of functional oocytes and the accumulation of DNA damage in cells caused by the lack of DNA damage repair function could be responsible for the development and progression of ovarian tumors in the *Rev7* mutant mouse.

2. Materials and methods

2.1. Mice

The *repro22* mouse bearing a missense mutation in the *Rev7* gene (designated *Rev7*^{C70R}) (Khalaj et al., 2014) was produced and provided by the ReproGenomics Program at The Jackson Laboratory (Lessard et al., 2007). The homozygous mice were obtained by mating between *Rev7*$^{C70R/+}$ heterozygous mice. The genotypes of the *Rev7* gene were determined by the length difference of PCR products after digestion with the *Bst*UI restriction enzyme, as described previously (Khalaj et al., 2014). Mice were euthanized with CO_2, body and ovary weights were recorded, and the ovaries were collected. Blood and ovarian samples were collected from 3-, 6-, and 10-month-old *Rev7*$^{C70R/\ C70R}$ and wild-type mice. The blood samples for hormone assay were collected between 10:30 a.m. and 11:30 a.m to avoid collection of blood at LH surge period. Experimental procedures in the present study were conducted according to the guidelines of the Animal Care and Use Committee of Okayama University.

2.2. BrdU incorporation assay

Mice were injected intraperitoneally with 5-bromodeoxyuridine (BrdU, Sigma) at a dose of 75 μg/g body weight, and euthanized with CO_2 2 hours after injection. The ovaries were removed, fixed in 4% paraformaldehyde solution, embedded, and sectioned at 4 μm thickness. BrdU incorporated into the nuclei of proliferating cells was detected using immunohistochemistry. The sections were treated with 1 M HCl for 20 min at 37 °C for DNA denaturation, followed by 10 μg/ml proteinase K for 15 min at room temperature for antigen retrieval. BrdU-incorporating cells were detected using a BrdU In-Situ Detection Kit (BD Bioscience, San Diego, CA, USA) and diaminobenzidine (DAB) chromogen (DAKO, Kyoto, Japan) according to the manufacturers' instructions. Counterstaining was performed with hematoxylin.

2.3. Histology, immunohistochemistry, and immunofluorescent staining

Mouse ovaries were fixed in 4% paraformaldehyde (PFA) in PBS or Zamboni's fixative overnight, embedded in paraffin, sectioned at 5 μm thickness, and mounted on siliconized slides. The sections were stained with hematoxylin and eosin and observed by light microscopy. Immunohistochemical assay was performed as described previously (Abbasi et al., 2009). Briefly, the sections were deparaffinized and rehydrated using standard procedures. The sections were incubated with PBS (-) containing 0.3% H_2O_2 for the suppression of endogenous peroxidase activity for 20 minutes at room temperature. The sections were blocked with 3% BSA in PBS (-) for 1 hour at room temperature and incubated with anti-cytokeratin8 (CK-8) rabbit monoclonal antibody (1:500) (ab53280; Abcam) diluted with 3% BSA in PBS (-) for 24 hours at 4 °C or 3 hours at 37 °C. After incubation with the CK-8 antibody, sections were incubated with horseradish peroxidase-conjugated anti-rabbit IgG (1:500) diluted with 3% BSA in PBS (-) for 1 hour at room temperature. The sections were visualized by incubation with 3,3'-diaminobenzidine tetrahydrochloride (DAB) chromogen (Dako, Kyoto, Japan), and counterstained with Mayer's hematoxylin after dehydration. For Immunofluorescent staining, mouse monoclonal anti-phospho-Histone H2A.X antibody (1:500 dilution, 05-636, Upstate Biotechnology Inc., Lake Placid, NY) was used in phosphate buffer saline (PBS) containing 5% skim milk for ON at 4 °C. Goat anti-mouse IgG-Alexa Fluor 594 secondary antibody (1:500 dilution, A-11005, Thermo Fisher Scientific, Waltham, MA) was applied with the same buffer as the primary antibody and incubated for 1 h at RT. Nuclei were counterstained with DAPI, mounted using VECTASHIELD® Mounting Medium (H-1200, Vector Laboratories Inc., CA, USA), and observed using a fluorescent microscopy.

2.4. Hormone assays

Plasma concentrations of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) were measured using an enzyme-linked immunosorbent assay (ELISA) kit for FSH and LH (ERKR7007 and ERK7010; Endocrine Technologies, Inc., USA), according to the manufacturer's instructions with some modifications. Basically, all samples within each experiment were assayed at the same time for either LH or FSH. Plasma samples were assayed in duplicate, and values differing by 10% or more were discarded. Plasma samples isolated from heparinized blood were stored at –80 °C until hormone measurement. Plasma and standard samples were thawed to room temperature 30 min before the assay and 30 μl of the samples was dispensed in 96-well plates in duplicate. Optical absorbance of the reaction was determined using BioRad Model 680 Microplate Reader at 450 nm. The FSH standard curve ranged from 0.5 to 100 ng/ml, and the LH standard curve ranged from 1 to 50 ng/ml. Assay sensitivity for FSH and LH was 0.5 ng/ml. The intra-assay coefficients of variation were on average 4.3% and 3.2% for FSH and LH, respectively.

2.5. Culture of Rev7$^{C70R/C70R}$, Rev7$^{C70R/+}$, and normal MEF and cytometric assessment

Rev7$^{C70R/C70R}$, *Rev7*$^{C70R/+}$, and normal MEF were isolated from embryonic day (E) 14 embryos as described previously (Khalaj et al., 2014), and cultured for 3, 6, 9, and 12 days. The cells were harvested and fixed in 70% ethanol (–20 °C). The cells were then permeabilized with 1% BSA and 0.1% Triton X-100 in PBS and stained with Alexa Fluor 488-conjugated anti-γH2AX (pS139) (BD Biosciences, San Jose, CA) according to the manufacturer's instructions. After incubation, the cells were washed with PBS and stained with 50 μg/ml propidium iodide (PI) in the presence of 0.1 mg/ml RNase. The cells were analyzed using FACS Canto II (Becton Dickinson, San Jose, CA) with FACS Diva software. Flow Jo software (Tree Star, Ashland, OR) was used for data analysis and display. The γH2AX mean fluorescence intensity (MFI) was evaluated in G1, S, and G2/M phases of the cell cycle and the ratio of the MFI of γH2AX in non-S phase to that in the S phase was calculated.