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Original Article

Conditional knockout of *brca1/2* and *p53* in mouse ovarian surface epithelium: Do they play a role in ovarian carcinogenesis?

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Alterations of genes are known to be critical for the induction of tumorigenesis, but the mechanism of ovarian carcinogenesis is little understood and remains to be elucidated. In this study, we investigated the roles of *brca1, brca2* and *p53* genes in the development of ovarian cancer using conditional knockout mice generated by a Cre-loxP recombinant system. Following the application of recombinant adenovirus expressing Cre *in vitro*, the proliferation of ovarian surface epithelium (OSE) was increased. For instance, a significant increase in cell growth was observed in OSE cells *in vitro* by conditional knockout isolated from the mice bearing concurrent floxed copies of *brca1* and *brca2/p53*. However, the proliferative effect of the ovarian cells was not observed in concurrent *brca1/brca2* or *p53* knockout mice *in vivo*, indicating that we could not observe the direct evidence of the involvement of *brca1, brca2*, and *p53* in ovarian carcinogenesis. Since morphological changes including tumor formation were not observed in mice bearing floxed copies of concurrent *brca1/brca2* or *p53*, the inactivation of *brca1/2* or *p53* is not sufficient for the induction of tumor formation. Taken together, these results suggest that the deficiency of these genes may not be involved directly in the mechanism of ovarian carcinogenesis.

Keywords: *brca*, *p53*, conditional knockout, ovarian cancer, tumor suppressor genes

Introduction

Ovarian cancer is the most lethal cause of cancer-related death from gynecological malignancies in Western women [1,28]. Approximately 90% of ovarian cancer is believed to arise from a single layer of flat to cuboidal cells forming the outer layer of ovarian surface epithelium (OSE) [1,2,17,26] and OSE has received much attention in the last few years. However, the study of the etiology and cellular mechanisms of human ovarian cancer was limited because there has been no appropriate animal model to explain the occurrence of ovarian cancer. Although it has been reported that concurrent inactivation of *Rb* and *p53* genes contributes to the initiation of ovarian cancer development [12], the cause of ovarian carcinogenesis is poorly understood. *Brca1/2* and *p53* are known to play important roles in DNA repair and the cell cycle. Recent evidence has shown that the mutation of the *brca1* gene increases the incidence of preneoplastic changes in murine OSE and the inactivation of *p53* reverses the increase of apoptosis induced by the loss of function of *brca1 in vivo* [9]. This indicates that concurrent inactivation of *brca1* and *p53* pathways may be involved in critical changes responsible for the induction of ovarian carcinogenesis.

Brca1 and *brca2* are the DNA damage repair genes and are important in maintaining genomic integrity [7,30,32]. The mutation of these genes increases susceptibility to ovarian cancer [13] and the inactivation of *brca1* induces the activation of a number of oncogenes involved in tumorigenesis [4,11,35]. The *p53* protein is well known as a transcriptional factor and functions as a tumor suppressor gene. *p53* also plays an important role in apoptosis and the cell cycle and its mutation is known to be involved in the incidence of 50% of human tumors [24]. Furthermore, there is accumulating evidence that the loss of *brca1* induces the inactivation of *p53*, which is involved in *brca1*-associated tumorigenesis [5,10]. Although there have been many studies of the role of these genes in ovarian carcinogenesis, a definitive elucidation has not been achieved because an animal model of ovarian cancer in

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mouse OSE cells has not been established. The inactivation of *brca1* induced developmental delay and resulted in embryonic lethality in mice [11,14,23]. Furthermore, deficiency of *brca2* also induced embryonic lethality by the retardation of embryonic development [23,33]. To overcome these problems, the Cre-loxP system was employed to elucidate the roles of *brca1* in tumorigenesis through the targeted deletion of genes. Using the Cre-loxP system, early studies showed that the conditional knockout of *brca1* induces mammary tumor and also that concurrent inactivation of *p53* and *Rb1* is sufficient for the induction of ovarian carcinogenesis in conditional *p53* and *Rb1* knockout mice [12,36]. In this study, we investigated the role of *brca1* or *brca2* with *p53* in ovarian carcinogenesis using the Cre-loxP system in floxed mice.

Materials and Methods

Cell culture

Ovaries were dissected and washed with phosphate-buffered saline (PBS; Invitrogen Life Technologies, USA). The ovaries were placed in DMEM/F12 medium containing Collagenase (100 U/mL) and Dispase II (0.6 U/mL) for 25 min at 37°C in a humidified atmosphere. OSE cells were cultured in DMEM/F12 (Sigma-Aldrich, USA) supplemented with 10% FBS (Hyclone, USA), 100 U/mL penicillin G and 100 μg/mL streptomycin (Life Technologies, USA) at 37°C in a humidified atmosphere of 5% CO_2.

Mice maintenance

Homozygote mouse strains with *brca1*$^{loxP/loxP}$, *brca2*$^{loxP/loxP}$, and *p53*$^{loxP/loxP}$ were purchased from the Mouse Repository of Mouse Models of Human Cancers Consortium program (US National Cancer Institute, USA). FVB inbred mice (Charles River, USA) were used as a control group. The mice were maintained at a constant temperature (23 ± 2°C), relative humidity of 50 ~ 60%, and a 12 h light/dark cycle. Standard rodent chow and purified water were available *ad libitum*. All animal procedures were approved and carried out in accordance with the Animal Care Committee of the University of British Columbia.

Genotyping and DNA preparation

The mice carrying a *brca1, brca2*, and *p53* conditional allele were genotyped by PCR (58°C, 1 min; 72°C, 1 min; 94°C, 1 min; 30 cycles). Total RNA extracts were prepared from the mouse cells using TRIZOL Reagent (Invitrogen Life Technologies, USA) and reverse transcribed from 2.5 ng of total RNA using the First-Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech, USA). The sense/antisense primers for the floxed sequence and the wild-type sequence and the predicted sizes of PCR products of *brca1, brca2, p53*, and glyceraldehyde-3-phosphate dehydrogenase are described in Table 1.

Adenovirus administration

Mouse strains with wild type, *brca1*$^{loxP/loxP}$, *brca2*$^{loxP/loxP}$, and *p53*$^{loxP/loxP}$ were injected with adeno-Cre virus (AdCMVCre) and adeno-EGFP (AdCMVEGFP) (University of Iowa Gene Transfer Vector Core) as previously described [12]. Briefly, the adenovirus was injected using a 1 mL syringe with a 30-gauge beveled needle after 0.5 ~ 2% isofurane anesthesia. Ovaries were individually accessed via dorsal incision and the right ovaries were injected with AdCMVCre and the left ovaries were injected with AdCMVEGFP.

For *in vitro* infection, 2×10^5 OSE cells were incubated with 4×10^7 AdCMVCre, and AdCMVEGFP (MOI 200, respectively) in 500 μL of serum-free medium for 1.5 h at 37°C. Then the cells were washed twice with PBS and

Table 1. The oligo-sequences of the primers and the predicted sizes of PCR products for *brca1, brca2*, and *p53* floxed mice

mRNA		Gene sequences (5′-3′)	Expected size
brca1 (5′ floxed)	Sense	TATCACCACTGAATCTCTACC	461-bp
	Antisense	GACCTCAAACTCTGAGATCCA	
brca1 (3′ floxed)	Sense	TATTCTTACTTCGTGGCACAT	562-bp
	Antisense	TCCATAGCATCTCCTTCTAAA	
brca2 (5′ floxed)	Sense	GGCTGTCTTAGAACTTAGGCT	376-bp
	Antisense	CTCCACACATACATCATGTGT	
brca2 (3′ floxed)	Sense	CTCATCATTTGTTGCCTCACTTC	529-bp
	Antisense	TGTTGGATACAAGGCATGTACAC	
p53	Sense	AGCTGAAGACAGAAAAGGGGAGGG	585-bp (floxed)
	Antisense	ACAGAAAAGGGGAGGGATGAAGTGA	432-bp (wild)
*GAPDH**	Sense	ACACATTGGGGGTAGGAACA	223-bp
	Antisense	AACTTTGGCATTGTGGAAGG	

**GAPDH*: glyceraldehyde-3-phosphate dehydrogenase.

incubated in DMEM/F12 with 10% FBS. Cells isolated from *brca1*$^{loxP/loxP}$ and *brca2*$^{loxP/loxP}$ mice were infected with AdCMVCre to conditionally delete *brca1* and *brca2* genes. A multiplicity of infection (MOI) value was calculated as the number of infectious viruses divided by the number of cells.

Proliferation assay

The proliferation assay was performed using [^{3}H]-thymidine incorporation assay as previously described [8]. Briefly, cells were placed in 24-well plates with 0.5 mL DMEM/F12 with 10% FBS. The cells were infected with the adenovirus as described above. Following incubation for 2 days, 1 μCi [^{3}H]-thymidine (0.5 Ci/mmol; Amersham Pharmacia Biotech, USA) was added. The cells were incubated for 6 h and precipitated with 0.5 mL 10% trichloroacetic acid for 20 min at 4°C. The precipitate was washed in methanol twice and solubilized in 0.5 mL 0.1 N sodium hydroxide. The radioactivity was measured in the Tri-Carb Liquid Scintillation Analyzer (Model 2100TR; Packard Instrument, USA).

Histopathological analysis

Brca1, brca2, brca1/2, and *brca2/p53* floxed mice were injected intrabursally with AdCMVCre and AdCMVEGFP into the right and left ovaries, respectively. *brca1*$^{loxP/loxP}$, *brca2*$^{loxP/loxP}$, or *p53*$^{loxP/loxP}$ mice infected with AdCMVCre and AdCMVEGFP adenovirus were sacrificed by carbon dioxide inhalation according to the standard protocol. Ovaries were removed and fixed in 10% formalin, and paraffin sections were prepared for microscopic evaluation. Serial sections were prepared for hematoxylin and eosin staining, and histopathological examination then performed under a light microscope.

Statistical analysis

Statistical analysis was performed by one-way ANOVA followed by Tukey's multiple comparison test. $p < 0.05$ was considered statistically significant. The results of *in vitro* proliferation assays are presented as the mean ± SD following three independent experiments.

Results

Detection of the insertion of loxP sites in *brca1* and *brca2*

The insertions of loxP sites in mice carrying a *brca1*, *brca2*, and *p53* conditional allele were confirmed by RT-PCR. As demonstrated in Fig. 1A, predicted PCR products of 5′ floxed *brca1* and 3′ floxed *brca1* were obtained at 461 and 562-bp, respectively, and 5′ and 3′ floxed *brca2* PCR products were observed at 376 and 529-bp, respectively (Fig. 1B). PCR products from the wild type of *brca1* and *brca2* mice were obtained with the same primer sequences of *brca1* and *brca2*. The mice bearing floxed *p53* gene as confirmed by RT-PCR were used for further experiments (Fig. 1C. Lanes 3 and 5).

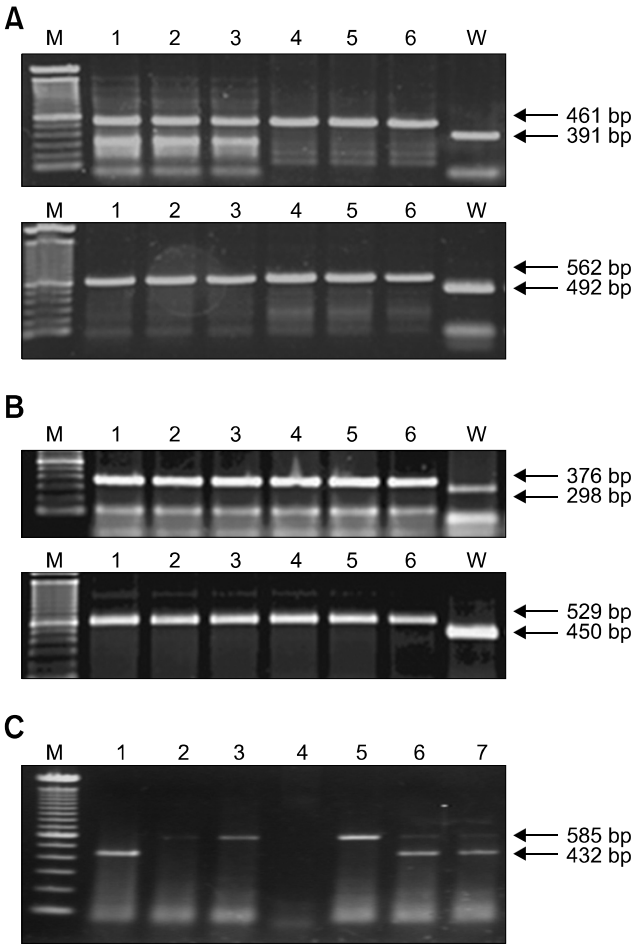

Fig. 1. Genotyping of floxed mice for *brca1* and *brca2*. (A) The predicted 461-bp (5′ floxed) and 562-bp (3′ floxed) PCR products of were observed in *brca1*$^{loxP/loxP}$ mice (Lanes 1-6). (B) The predicted 376-bp (5′ floxed) and 529-bp (3′ floxed) PCR products of *brca2* were confirmed in *brca2*$^{loxP/loxP}$ mice (Lanes 1-6). (C) Confirmation of PCR products of homozygous *p53*$^{loxP/loxP}$ mice (Lanes 3 and 5). Lanes 6 and 7, heterozygous *p53*$^{loxP/loxP}$ mice. Lane M: Molecular weight marker, Lane W: wild-type.

Determination of MOI to delete the loxP sites of *brca1* and *brca2* following AdCMVCre infection

Cre-loxP recombination was performed to inactivate *brca1* and *brca2* genes. For experiments, 3×10^5 cells were seeded in 0.5 mL of serum-free medium with serial dilution of AdCMVCre. After 1.5 h incubation at 37°C, the cells were washed twice with PBS and covered with DMEM/F12 containing 10% FBS. Infection of AdCMVCre at MOI 20 ~400 determined by the formula resulted in a decrease of *brca1* and *brca2* expression (Fig. 2A), indicating that AdCMVCre administration activated the loxP sequence,

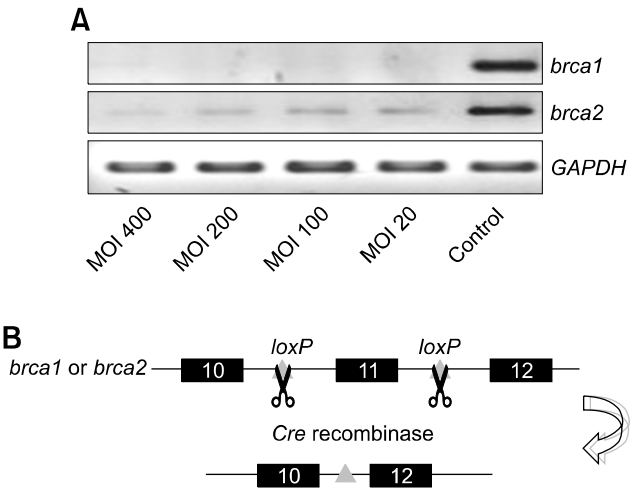

Fig. 2. Evaluation of multiplicity of infection (MOI) by adeno-Cre virus (AdCMVCre) infection for deleting the genes in ovarian surface epithelium. (A) The expression of *brca1* and *brca2* following treatment with serial dilutions of AdCMVCre. Mouse glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used to confirm the same amount of loading. (B) Schematic explanation of Cre-loxP recombination.

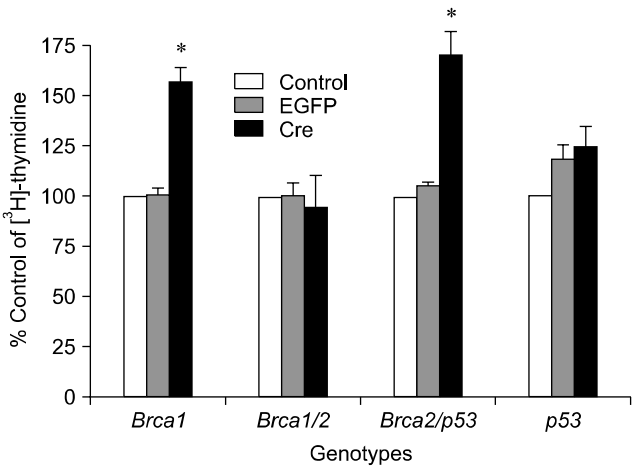

Fig. 3. Effect of inactivation of *brca1, brca2*, and *p53* on ovarian surface epithelium (OSE) cell proliferation. OSE cells from diverse genotypes were infected with AdCMVCre at MOI 200 for 1.5 h. Then, cell proliferation was measured by thymidine incorporation assay in the OSE cells with $brca1^{loxP/loxP}$, $brca1/2^{loxP/loxP}$, $brca2/p53^{loxP/loxP}$ and $p53^{loxP/loxP}$ after infection. Adeno-EGFP (AdCMVEGFP) was used as a control. Proliferation levels are expressed as a relative fold change. Values are the mean ± SD. $*p < 0.05$ *vs*. AdCMVEGFP infection.

which successfully excised the *brca1* and *brca2* genes as described in a schematic diagram (Fig. 2B).

Effect of the inactivation of *brca1, brca2*, and *p53* and on the proliferation of OSE

OSE cells were obtained from $brca1^{loxP/loxP}$, $p53^{loxP/loxP}$, $brca1/2^{loxP/loxP}$, and $brca2/p53^{loxP/loxP}$ mice and infected with AdCMVCre at MOI 200 to investigate whether the inactivation of *brca1, brca2*, and *p53* affected the proliferation of mouse OSE cells. Data from the infection group of AdCMVCre were compared to those of the vehicle group or the AdCMVEGFP infected group. Infection with AdCMVCre in OSE cells resulted in a significant increase of proliferation in $brca1^{loxP/loxP}$ and $brca2/p53^{loxP/loxP}$ groups (Fig. 3), but no difference was observed in *brca1/brca2* floxed OSE cells (Fig. 3). Although the infection of $p53^{loxP/loxP}$ cells with AdCMVCre seemed to increase proliferation of OSE, the increase was not significant (Fig. 3).

Effect of the inactivation of *brca1* and *brca2* on ovarian carcinogenesis

To evaluate the effect of the deletion of *brca1* and *brca2* genes, wild type, $brca1^{loxP/loxP}$ and $brca2^{loxP/loxP}$ mice were injected with AdCMVCre intrabursally. Following 3 months of AdCMVCre infection, ovaries were collected and examined under a light microscope following staining. No morphological changes were observed in the OSE cells of *brca1* and *brca2* floxed mice (data not shown).

Since the effect of concurrent inactivation of *brca1* and *brca2* in ovarian carcinogenesis is not known, double knockout mice of these genes were obtained by cross breeding. There were no observable differences between AdCMVCre and AdCMVEGFP infected groups. In addition, no morphological changes were observed in $brca2/p53^{loxP/loxP}$ mice following AdCMVCre administration (data not shown).

Discussion

The carriers of *brca1* and *brca2* mutations have increased susceptibility to breast, prostate, and ovarian cancer [13,35]. The unique signatures of gene expression profiling of *brca1* and *brca2* have been revealed in hereditary and sporadic ovarian cancers [16]. In addition, a significantly higher proportion of advanced serous adenocarcinoma in ovaries has been observed in the cases of *brca1* and *brca2* mutations [31], indicating that the loss of function of these genes leads to the outgrowth of ovarian cells. Somatic mutations also seem to be involved in ovarian cancer development [6,19]. The germline mutations of *brca1* and *brca2* genes in ovarian carcinomas demonstrate a higher growth percentage than sporadic cancers do [20,22]. It has been reported that *brca1* dysfunction is often involved in simultaneous *brca2* dysfunction in ovarian cancers [18]. Although many studies have been performed to elucidate the precise role of *brca1* and *brca2* on ovarian carcinogenesis, the studies have struggled to define the exact contribution because the knockout of these genes in mice induced developmental delay and resulted in embryonic lethality

[11,14,23,33].

Previous study has shown that when using a conditional knockout method, the Cre-loxP system, mutation of *brca1* resulted in mammary tumor formation in the mouse [36]. Also, a single intrabursal administration of recombinant Cre-expressing adenovirus in double-floxed mice of *p53* and *Rb1* was sufficient for reproducible induction of ovarian epithelial carcinogenesis [12]. In this study, we investigated whether the concurrent mutation of *brca1*, *brca2*, and *p53* genes is involved in ovarian carcinogenesis. Following the confirmation of genomic phenotype of floxed *brca1, brca2* and *p53* in $brca1^{loxP/loxP}$, $brca2^{loxP/loxP}$ and $p53^{loxP/loxP}$ mice, double homozygous mice of $brca1/2^{loxP/loxP}$ or $brca2/p53^{loxP/loxP}$ were obtained by cross breeding of the single-floxed mice with $brca1^{loxP/loxP}$, $brca2^{loxP/loxP}$, and $p53^{loxP/loxP}$ mice. We confirmed the loss of copies of *brca1/2* and *brca2/p53* in $brca1/2^{loxP/loxP}$ or $brca2/p53^{loxP/lox}$ mice by genotyping using PCR. To explore the function of *brca1, brca2*, and *p53* in OSE *in vivo*, AdCMVCre was injected intrabursally to induce Cre-*loxP* recombination to delete the exons of *brca1* and *brca2*. We examined tumor formation after 3 months of AdCMVCre infection. No morphological changes were observed in AdCMVCre injected ovaries. A previous report has shown that the inactivation of *brca1* is sufficient for preneoplastic changes such as hyperplasia and inclusion cysts following adeno-viral Cre infection but no tumor formation was observed [9]. There is also controversy over the issue of the effect of inactivation of *brca1* on the capacity of proliferation of OSE cells [3,9,29]. Discrepancies might be due to the different viral constructs or the period of infection. In addition, no susceptibility to mammary tumors was observed in heterozygous *brca1* mutant mice [37], suggesting that the existence of the normal functioning *brca1* gene is possible.

P53 is well known to be absent or mutated in several cancer types and the dysfunction of *p53* is commonly related to ovarian cancer, particularly with germline *brca1* muation [21,27]. *brca1/2* are known to have similar phenotypic consequences of disruptions [34]. Since the precise nature of *brca1/2* and *p53* interaction remains to be defined, we evaluated the effect of the mutation of these genes on OSE proliferation. OSE cells obtained from the ovaries of conditional knockout mice were infected with AdCMVCre to delete the exons of *brca1, brca2*, and *p53 in vitro*. Infection with AdCMVCre in $brca1^{loxP/loxP}$, $brca2/p53^{loxP/loxP}$ OSE cells resulted in an increase of cell proliferation, but no proliferative increase was found in the AdCMVCre infected $brca1/2^{loxP/loxP}$ group and $p53^{loxP/loxP}$ group. In addition, no morphological changes were observed in $brca2/p53^{loxP/loxP}$ mice following AdCMVCre administration. However, it has been reported that the individual inactivation of *p53* and *Rb1* increases OSE proliferation *in vivo* and the concurrent inactivation of those genes demonstrates a synergistic effect on cell proliferation [12]. These discrepancies may be partially due to the experimental methods used to measure the proliferative index, since the level of proliferation is similar among these results. However, our results are consistent with the finding that the concurrent mutation of these genes was not necessary in breast cancer development [15]. In addition, a previous report showed that *brca2* interacts with *p53* gene and inhibits *p53*'s transcriptional activity in cancer cells [25], suggesting that the concurrent inactivation of these genes may not be involved in ovarian cancer development and that it is more likely that other genes may play a role in ovarian carcinogenesis. It is possible that a single intrabursal administration of AdCMVCre was not sufficient to inactivate these genes and induce ovarian cancer *in vivo* and further studies using more frequent administration or long term maintenance following AdCMVCre infection are needed before a conclusion can be reached.

In conclusion, we demonstrated in this study that the proliferation of OSE was significantly increased in mice bearing concurrent floxed copies of *brca1* and *brca2/p53 in vitro*. However, we did not observe direct evidence of the involvement of *brca1, brca2*, and *p53* in ovarian carcinogenesis. Also, morphological changes including tumor formation were not observed in mice having floxed copies of concurrent *brca1/brca2* or *p53* following an intrabursal administration of recombinant Cre-expressing adenovirus *in vivo*. Taken together, these results suggest that the inactivation of *brca1, brca2* or *p53* is unlikely to be sufficient for the induction of ovarian epithelial carcinogenesis.

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