Volume 15, No 2 | International Journal of Radiation Research, April 2017

Effects of low dose radiation on the expression of proteins related to DNA repair requiring Caveolin-1 in human mammary epithelial cells

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► ***Original article***

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Revised: June 2016
Accepted: Aug. 2016

Int. J. Radiat. Res., April 2017; 15(2): 141-148

DOI: 10.18869/acadpub.ijrr.15.2.141

ABSTRACT

Background: Radiotherapy is an effective and important therapeutic method for breast cancer, but at the same time it has a radiation-induced bystander effect on normal tissue around the tumor. Repair of double-strand breaks (DSBs) by normal cells can reduce the extent of damage caused by this effect. Caveolin-1 (Cav-1) is an important regulatory molecule in cell signal transduction. However, the response of normal human mammary epithelial cells following low dose radiation (LDR)- induced DSBs and the role of Cav-1 in the repair of the DSBs are not clear. The present study examined the DNA damage repair mechanism triggered by LDR in human mammary epithelial cells. ***Materials and Methods***: Human mammary epithelial (MCF10A) and Cav-1 haplo-insufficiency (MCF10A-ST1) cells were irradiated with LDR gamma rays and the effect of this radiation on cell proliferation was determined by cytometric method. Western blot analysis was then used to measure the expression levels of different proteins associated with cell proliferation and DNA repair. ***Results:*** LDR enhanced the radiation responsiveness of MCF10A cells in a dose- and time-dependent manner. At a dose of 100 cGy, LDR increased the expression levels of several proteins involved in DNA repair pathways, such as ATM, p53, DNA-PKcs and also activated Cav-1-mediated cell proliferation and survival pathways, such as the MAPK and AKT pathways. The expression of the various DNA repair related proteins was changed after down-regulating the Cav-1 expression. ***Conclusion:*** LDR could increase the radiation responsiveness of human mammary epithelial cells through activating the DNA repair pathways, including both HR and NHEJ pathways, as well as triggering the cell proliferation and survival pathways, both of which required Cav-1.

Keywords: *LDR, DNA repair, Caveolin-1, human mammary epithelial cells, NHEJ, HR.*

INTRODUCTION

Breast cancer is a malignant tumor located in the human mammary epithelial tissue. Every year, more than 1.2 million women from all over the world are suffering from breast cancer and this incidence is rising by 0.2% to 8% per year. In USA, breast cancer is the most prevalent cancer for women. In China, the incidence of breast cancer is increasing, trailing just behind metrocarcinoma, which is the most serious and common malignant tumor that affects the health of women.

At present, the treatment used for breast cancer is a comprehensive treatment system that integrates surgical treatment, chemo-therapy and radiotherapy. Radiotherapy is considered to be the most effective breast

[DOI: 10.18869/acadpub.ijrr.15.2.141]

cancer treatment method because it can reduce both recurrence rate and mortality. However, ensuring the maximum effect of radiotherapy in controlling tumor can also result in radiation-induced bystander effect on normal tissue around the tumor. Radiation-induced bystander effect is mainly the damaging effect exerted by low dose radiation (LDR) on the DNA of the normal cells around the tumor, causing double-strand breaks (DSBs). The best way for the cells to deal with the radiation-induced bystander effect that causes DSBs is to repair the DSBs. Thus, it is important to study the mechanism of DNA repair involved in the repair of DNA damage inflicted by LDR.

So far, three kinds of DNA damage repair pathways are known to participate in the repair of DSBs, and these are single strand annealing (SSA), homologous recombination (HR) and non-homologous end-joining (NHEJ) pathways, with the last two being the main repair pathways [1]. HR is mediated by ataxia-telangiectasia mutated (ATM) whereas NHEJ is mediated by DNA-dependent protein kinase (DNA-PK), and they are the two pathways used by cells to repair DSBs damage [1, 2]. ATM protein kinase, the key protein of the HR repair pathway, is a phosphorylated enzyme with a molecular weight of 350 kD, encoded by the ataxia-telangiectasia mutant gene. ATM is also an important regulatory protein of p53, and it participates in the regulation of the cell cycle progress, as well as in DNA damage recognition and repair [3, 4]. Although the HR repair pathway mediated by ATM can repair DSBs, it is the NHEJ pathway that plays a key role in the progress of DSBs repair in mammalian cells [5, 6]. The NHEJ repair pathway mainly depends on four core factors: DNA-PK, X-ray cross-complementing group 4 (XRCC4), DNA joining enzyme IV and Artemis. DNA-PK consists of a catalytic subunit DNA-PKcs with serine/threonine kinases activity and two Ku subunits, which can initiate NHEJ repair [7]. DNA-PKcs is an important DNA damage repair protein, which is not only involved in NHEJ and VDJ recombinant and the maintenance of telomere structure, but can also phosphorylate many transcription factors and DNA repair proteins [8]. Recently, many studies have reported that DNA-PK deficiency or its down-regulation can lead to loss of DSBs repair capacity and increased sensitivity to ionizing radiation (IR), indicating that DNA-PK is a necessary component of the response of the cell to LDR and is involved in tumorigenesis and tumor progression [9]. More and more studies have focused on the functional mechanism of DNA-PK.

Caveolae is a depression caveolae of cytoplasmic membrane and an important passageway across which every signal enter the cell. The protein marker Caveolin-1 (Cav-1) is often used as a tumor suppressor as it possesses several functions, not only those participating in multiple signal transduction pathways, but also those that are closely related to cell transformation and tumor formation, especially the transformation of breast epithelial cell and the formation of breast cancer [10]. Previous studies have found that Cav-1 is involved in the repair of DNA damage through both the HR and NHEJ pathways in cancer development and progression [11, 12]. However, these studies have mainly focused on the DNA damage repair mechanism of tumor cells, whereas the response of normal human mammary epithelial cells to LDR-induced DSBs, the expressions of the DNA repair related proteins and the involvement of Cav-1 in the DSBs repair induced by LDR have been largely ignored.

In the present study, we sought to understand the DNA repair mechanism of normal human mammary epithelial cells following LDR-induced DSBs. Radiation-induced bystander effect was simulated in the human mammary epithelial cells MCF10A. First, we observed the effects of LDR on cell growth and proliferation. We then used western blot to detect the expression of the main proteins in the DNA repair pathways (e.g HR and NHEJ pathways) and the proliferation [e.g Mitogen-activated protein kinase (MAPK)/ Extracellular regulated protein kinases (ERK)] and survival [e.g Phosphatidylinositol 3-kinase (PI3K)/AKT] pathways mediated by Cav-1. Finally, we used Cav-1 haplo-insufficiency mammary cell line MCF10A-ST1 (which was established in our lab [13]) to detect the changes

[DOI: 10.18869/acadpub.ijrr.15.2.141]

in expression levels of DNA repair related proteins, and to determine the role of Cav-1 in DSBs repair induced by LDR.

MATERIALS AND METHODS

Materials and reagents

Human mammary epithelial cell line MCF10A was purchased from ATCC (CRL-10317™), A Cav -1 haploinsufficiency cell line MCF10A-ST1 was established in our laboratory. Dulbecco's modified eagle's medium-F12 (DMEM/F12) and horse serum were obtained from Hyclone Biotechnology. ECLTM reagent was purchased from Amersham Pharmacia Biotechnology. All antibodies used in this study are listed in table 1. Other reagents were obtained locally.

Table 1. List of antibodies used in this study and their sources

Antibodies	Sources
Anti-Caveolin-1 polyclonal antibody, anti-ATM monoclonal antibody, anti-Ku80 (DNA protein kinase regulatory subunit) polyclonal antibody and anti-p53 monoclonal antibody	Santa Cruz Biotechnology Inc.
Anti-DNA-PKcs monoclonal antibody	Abcam Biotechnology
Anti-Nuclear factor kappa B (NF-κB) monoclonal antibody, anti-AKT polyclonal antibody, anti-p-AKT polyclonal antibody, anti-MAPK polyclonal antibody, anti-p-MAPK monoclonal mouse antibody and anti-PI3K polyclonal antibody	Cell Signaling Biotechnology
Anti-b-Actin monoclonal antibody	Wuhan boster Biological Engineering Co., Ltd.
Anti-Rabbit IgG-HRP antibody produced in goat and anti-Mouse IgG-HRP antibody produced in goat	Beijing Zhongshan Jinqiao Biological Engineering Co.

Cell culture

MCF10A and MCF10A-ST1 cell lines were cultured as previously described [9].

Cell LDR treatment

Cells were plated in 24-well or 96-well plate, and after adherence, the cells were bombarded with 50 cGy or 100 cGy LDR, and incubated at 37°C in a 5% CO_2 incubator for the following experiments.

Growth curves and doubling times for the cells were determined by the cytometric method. In brief, first digested by 0.25% trypsin and prepared into single cell suspension in new medium, then plated in a 96-well plates at a density of 5×10^5 (100 μL) cells/well and incubated at 37 °C in the presence of 5% CO_2. 3 parallel wells were arranged for every well, complete medium was regarded as the control. The cells of 3 parallel holes were digested every 24 h, the number of the cells were counted under the microscope after trypan blue staining. The numbers of the cells were counted for 4 d, the data were used to plot the growth curves. From the growth curves obtained, the cell doubling time for each treatment was calculated according to the formula: TD = T×log2/(logN-logN0) (Td: cell doubling time, T: time interval, N: the final cell number, N0: the initial cell number).

Western blot

Western blot was performed as previously described [9], and β-actin was used as a loading control. The density of each target band was quantitated using Image Quant analysis.

Statistical analysis

Data analysis was performed using the *t-test* and analysis of variance. Data were expressed with means ± SDs, and significant differences were considered at the $^*P<0.05$ or $^{**}P<0.01$ level.

RESULTS

Effect of LDR on the growth of humanmammary epithelial cells

In order to observe whether LDR can induce

[DOI: 10.18869/acadpub.ijrr.15.2.141]

DNA damage in human mammary epithelial cells, we treated MCF10A cells with two different doses of radiation, 50 cGy and 100 cGy, and then measured their growth rates. The result showed that cell proliferation was clearly inhibited by both doses of radiation compared to control cells after 3 d of incubation (figure 1A). Although the higher dose (100 cGy) resulted in stronger inhibition than the lower dose (50 cGy) after 4 d, the difference between them was not statistically significant. Treatment of MCF10A cells with LDR increased the doubling time of the cells (figure 1B). However, the increase in doubling time over control was not significant in the case of the 50 cGy treatment. On the other hand, the 100 cGy dose increased the doubling time of the cell by about 17%, and it was statistically significant. Furthermore, treatment with LDR also caused some cells to detach from the vessel surface, resulting in reduced confluency of the culture, an indication of cell death.

Effect of LDR on the expression of DNA repair proteins in human mammary epithelial cells

ATM is an important member of the homologous recombination of DNA repair pathways, and its expression level directly reflects the repair capacity of cells. To obtain further insight into the effect of LDR on the cells, changes in the expression level of ATM in LDR-treated MCF10A cells were measured over time. The level of ATM in MCF10A cells treated with 100 cGy increased significantly with time, reaching about 3.5 fold the level present immediately after treatment (at 0 h) (figure 2A). In addition to ATM, LDR treatment also caused the level of p53 to increase over time, and by 24 h post LDR treatment, the level of p53 was about 3 fold the level at 0 h (figure 2B).

The effect of LDR treatment on the level of Ku80, an 80-kD subunit of the DNA-PK complex, was also determined. Except for some increase at 2 h post LDR treatment, the level of Ku80 beyond the 2 h time point remained somewhat similar to that at 0 h (figure 2C). On the other hand, the level of DNA-PKcs, the catalytic subunit of DNA-PK, appeared to undergo a drop 2 h after treatment, but then rose again after 12 and 24 h (Figure 2D). DNA-PK is the main component of the NHEJ repair pathway. Taken together, the results suggested that exposure of MCF10A cells to LDR boosted the level of enzymes associated with DNA repair system in the cells.

Effect of LDR on the expression of the proteins of proliferation and survival pathways mediated by Cav-1 in human mammary epithelial cells

Research has shown that IR can induce activation of the Ras-MAPK/ERK pathway, which is mediated by many cancer cell growth factors.

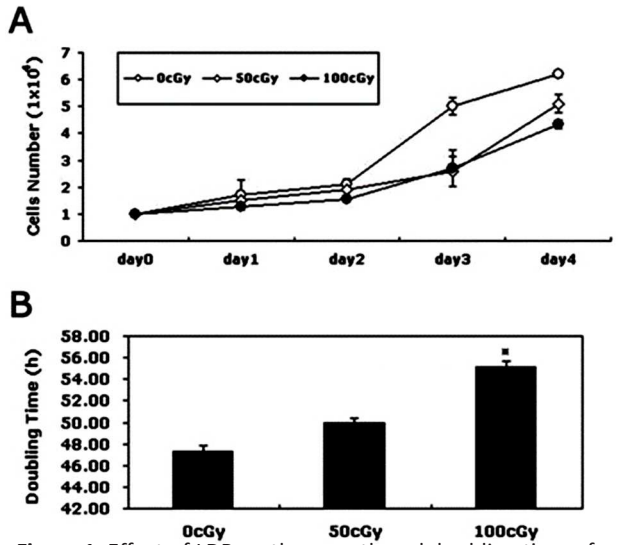

Figure 1. Effect of LDR on the growth and doubling time of MCF10A cells. (A) Growth curve. (B) Doubling time. *P<0.05.

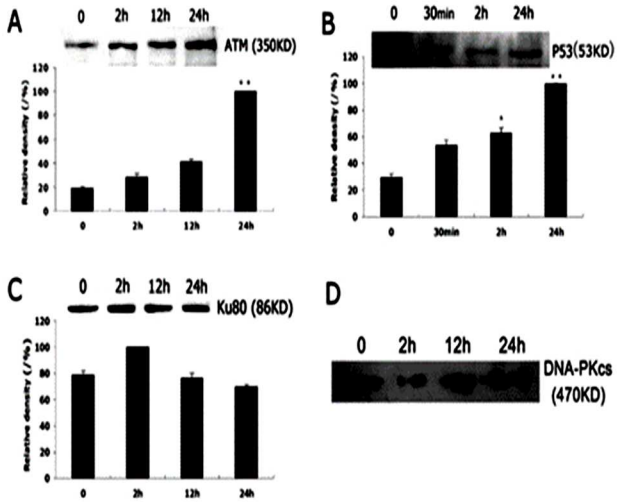

Figure 2. Effect of LDR on the expression levels of DNA repair related proteins in MCF10A cells following treatment with 100 cGy LDR. (A) ATM. (B) p53. (C) Ku80. (D) DNA-PKcs. **P<0.01.

[DOI: 10.18869/acadpub.ijrr.15.2.141]

However, the relationship between LDR and the proliferation pathway of normal epithelial cells has not been studied before. In our study, The expression of total MAPK in MCF10A cells did not change after treatment with LDR, but the overall level of phosphorylated MAPK (p-MAPK) increased, reaching a maximum level at 24 h after the treatment, being significantly higher than the level at 0 h (figure 3A). This indicated that LDR could cause the activation of MAPK, and therefore partly activated the proliferation pathway of mammary epithelial cells.

Another relevant pathway that was examined was the PI3K/AKT pathway, which plays an important role in cell survival and anti-apoptotic signal transduction. A recent study has shown that signaling mediated by PI3K/AKT pathway is involved in DNA damage repair induced by IR, as PI3K and AKT are essential proteins for the activation of DNA-PKcs [12]. To see whether the PI3K/AKT pathway is involved in LDR-induced DNA damage repair, the effect of LDR on the expression of AKT and PI3K was investigated. The level of PI3K in MCF10A cells exposed to LDR at 100 cGy dose remained unchanged across the different times after exposure (figure 3B). However, the level of AKT and phosphorylated AKT (p-AKT) increased significantly after 24 h of exposure, reaching at least twice the level at 0 h (figure 3C). This suggested that LDR exposure could activate the AKT-signaling pathway.

Cav-1 usually as an inhibitor of breast tumor, as it can negatively regulate the MAPK/ERK and PI3K/AKT signaling pathways, and is closely involved in the transformation of mammary epithelial cells and the occurrence of breast cancer. Normal human mammary epithelial cells such as MCF10A have a high expression level of Cav-1. However, after treatment with 100 cGy, the expression of Cav-1 increased slightly after 2 h where it then leveled off, before dropping sharply to about half the level at 0 h after exposure for 24 h (figure 3D). The trend in Cav-1 expression level in response to LDR exposure was therefore different to those observed for MAPK and AKT, indicating that Cav-1 negatively regulated the MAPK/ERK and PI3K/AKT pathways in DNA repair induced by LDR.

Effect of LDR on the expression of DNA repair related proteins in Cav-1 haploinsufficiency cells of human mammary epithelial cells

In order to further explore the involvement of Cav-1 in the repair of LDR-induced DNA damage carried out by other DNA repair related proteins, we used the Cav-1 haploinsufficiency cell line MCF10A-ST1 to detect the changes in the expression levels of DNA repair related proteins. The results showed that the expression of Cav-1 was slowly increased by 2.5 fold 2 h after LDR exposure, followed by a slight decrease and then a sharp decrease after 4 h to just about one third the level at 0 h (figure 4A). The expression of Ku80 increased throughout the 24 h after exposure to LDR to about 3.5 fold the level at 0 h (figure 4B). The expression level of p53 also showed an increasing trend and

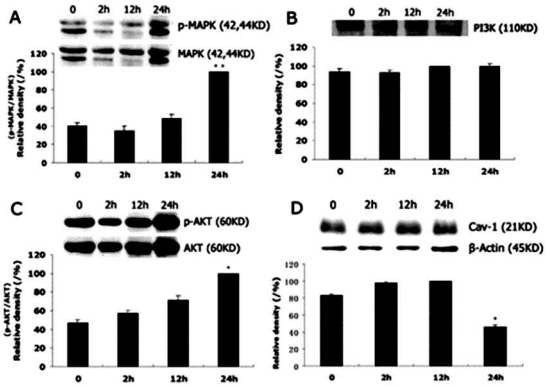

Figure 3. Effect of LDR on the expression levels of proliferation- and survival-related proteins in MCF10A cells following treatment with 100 cGy LDR. (A) p-MAPK and MAPK. (B) PI3K. (C) p-AKT and AKT. (D) Cav-1. **P*<0.05, ***P*<0.01.

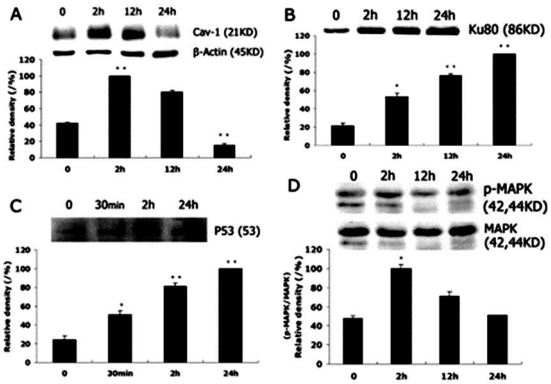

Figure 4. Effect of LDR on the expression levels of DNA repair -related proteins in MCF10A-ST1 cells following treatment with 100cGy LDR. (A) Cav-1. (B) Ku80. (C) P53. (D) p-MAPK and MAPK. **P*<0.05, ***P*<0.01.

[DOI: 10.18869/acadpub.ijrr.15.2.141]

reached the highest 24 h after LDR exposure (figure 4C). The level of phosphorylated MAPK obviously increased 2 h after LDR exposure and then gradually decreased, reaching a similar level as that observed at 0 h after 24 h (figure 4D). The expression levels of DNA repair-related proteins changed after Cav-1 expression was down-regulated, indicating that Cav-1 was required in DNA damage repair induced by LDR in human mammary epithelial cells.

DISCUSSION

Research has shown that LDR can significantly inhibit the growth and proliferation of breast cells. Normally, LDR would damage the DNA of the cells (especially in phase G0/G1), and then caused DNA replication to stop at the G2 checkpoint so that the HR and NHEJ pathways can repair the damaged DNA. Meanwhile, the cell-cycle cannot enter the G2 and S phases until DNA-repair is completed. This is consistent with the fact that LDR radiation would induce cell cycle arrest, and then inhibits cell growth and proliferation [14].

We have found in this study that 100 cGy LDR significantly inhibited the growth of MCF10A cells, leading to increased doubling time. This suggested that the cells were sensitive to the radiation dose of 100 cGy. If the radiation did not kill the cells, but only induced certain DNA damage, cell proliferation would return to the normal state after the DNA repair process is completed [15].

So far, there have been three kinds of DNA damage repair pathways that are used by the cell to repair DSBs. These are the SSA, HR and NHEJ repair pathways, with the last two being the main repair pathways [1]. ATM is a key protein in the HR repair pathway. It regulates the expression of p53 and thereby participates in the progress of cell cycle regulation, DNA damage recognition and repair [3]. Once ATM is activated, it usually inhibits the cell cycle through its downstream target p53, causing the cell cycle to arrest at the G1 phase, so that the damaged DNA can be repaired. LDR treatment increased the level of ATM and p53, indicating that ATM and p53 are important repair factors in the HR repair pathway. The fact that the level of ATM started to increase 2 h following LDR treatment and right up to 24 h (figure 2A) may suggest that ATM played an important role in the early stage of DSB repair. The increased level of p53 observed following LDR treatment was consistent with the fact that p53 is a downstream target of ATM, and thus an increasing level of ATM was expected to up-regulate the level of p53. The increasing level of p53 would effectively block cell replication, allowing the radiation-affected cell to repair the damaged DNA via the HR pathway and NHEJ pathway, therefore maintaining the integrity of the cell genome before cell replication could continue [16, 17].

The NHEJ pathway plays a vital role in the progress of DSBs repair in mammalian cells. Normal NHEJ finishes the DNA repair process by combining and connecting the two free ends of the homologous or non-homologous DNA. DNA-PK is a serine/threonine protein kinase and a key component of the NHEJ repair pathway. It is composed of the catalytic subunit DNA-PKcs and the regulatory subunit Ku protein. One function of the DNA-PKcs is to protect and connect broken ends of DNA, either alone or as a part of protein complex that binds Ku protein, allowing it to further recruit other DNA-repair proteins to the site of DNA damage. In addition, like Ku protein, DNA-PKcs is found at the damaged ends of the DNA where it plays a role in maintaining telomere stability and preventing the fusion of chromosome ends [18]. It is thought that through phosphorylation of its downstream target proteins that DNA-PKcs improves the molecular sensors involved in DNA damage repair. Recently, DNA-PKcs has also been found to play a vital role in regulating gene amplification and apoptosis via p53 [19]. It has been known that mutation in the DNA-PKcs gene or changes in its expression can partly lead to DSBs repair defects in malignant transforming cells, thereby increasing genomic instability [20]. Khanna *et al.* has shown that DNA-PKcs deficient embryonic stem cells are very sensitive to radiation, and exhibit growth delay, radiation hypersensitivity and occurrence of lymphoma. It

[DOI: 10.18869/acadpub.ijrr.15.2.141]

was proved that DNA-PKcs plays a very important role in maintaining normal immunity function, regulating DNA repair and preventing the malignant transformation of cells in human [9].

In our study, treatment of MCF10A cells with LDR did not alter the level of Ku80 while resulting in enhanced level of DNA-PKcs, indicating that DNA-PKcs was a crucial repair factor in the NHEJ repair pathway and that it exhibited strong sensitivity to radiation.

The PI3K/AKT pathway plays an important role in the anti-apoptotic mechanism. It also launches a series of biological processes related to cell cycle regulation, telomere activity, angiogenesis and cell migration and invasion [21]. Toulany *et al.* has shown that in human tumor cells, ionizing radiation can alter the phosphorylation status of DNA-PKcs through the PI3K/AKT signaling pathway, DNA-PKcs directly acted on the X-ray repair protein XRCC1 in cut repair (BER) [22]. However, our result showed that the expression of PI3K did not change significantly after exposure to LDR, but the activity of AKT was significantly enhanced, accompanied by increased expression of DNA-PKcs. This was consistent with the notion that PI3K/AKT signaling induced by LDR promoted the activation of DNA-PKcs, ultimately resulting in the repair of DSBs via the NHEJ pathway.

The MAPK/ERK signal transduction pathway in mammals is well-known. There is high expression of p-MAPK or MAPK in many malignant tumor tissues such as breast cancer, lung cancer, prostate cancer, melanoma, etc. [23]. Previous studies have shown that IR can induce the activation of MAPK/ERK pathway mediated by cancer cell growth factor, but the relationship between LDR and the proliferation pathway of normal epithelial cells has not been reported. Our results showed that activation of MAPK in MCF10A cells was significantly enhanced after treatment with LDR while the total level of MAPK appeared to remain unchanged, suggesting that LDR partly activated the proliferation pathway of mammary epithelial cells. MAPK acts as a key regulator of cell differentiation, the MAPK/ERK signal transduction pathway is closely related to cell growth and proliferation [23]. The mechanism underlying the response of the cell to DSBs induced by LDR requires further investigation.

Cav-1 is a major structural protein of caveolae, and it is involved in many physiological and patho-physiological processes, such as cardiovascular diseases, eurological disorders and cancers. Previous studies have suggested that in most tumor cells, Cav-1 is either not expressed or expressed in low level, and a close relationship has been observed between Cav-1 and the proliferation, differentiation, invasion, metastasis and apoptosis of many tumor cells [24]. In recent years, more and more evidence have shown that Cav-1 is also a key protein in DNA damage repair and that it is involved in DNA damage repair during cancer development and progression [11, 12]. A high level of Cav-1 was detected in normal human mammary epithelial cells such as MCF10A, but following exposure to LDR, the level of Cav-1 slowly increased (2 h and 12 h) and then sharply decreased (24 h), suggesting that Cav-1 might be an important protein involved in LDR-induced DNA damage repair. To confirm this result, we further analyzed the level of Cav-1 in the haploinsufficiency cell line MCF10A-ST1 to establish its connection with the expression of DNA repair-related proteins. The expression levels of DNA repair-related proteins changed following the down-regulation of Cav-1 expression, indicating the requirement of Cav-1 in DNA damage repair triggered by LDR in human mammary epithelial cells, and this was consistent with the conclusion drawn from studies conducted with cancer cells.

CONCLUSION

LDR could increase the radiation sensitivity of the human mammary epithelial cells, triggering the repair mechanism that involved the activation of DNA repair pathways including both HR and NHEJ and also the proliferation and survival pathways, in which Cav-1 may play an important role.

[DOI: 10.18869/acadpub.ijrr.15.2.141]

ACKNOWLEDGEMENTS

This work was supported by the National Natural Science Foundation of China [30970353] and the Natural Science Foundation of Liaoning Province [2015020568].

***Conflicts of interest*:** *Declared none.*
The authors contributed equally to this work.

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[DOI: 10.18869/acadpub.ijrr.15.2.141]