

Original Article



## Effect of silencing SSB1 gene on the expression of NBS1 in irradiated rat submandibular gland cells

Linjing Gao<sup>1, #</sup>, Qiuli Lv<sup>2, #</sup>, Yude Huang<sup>1</sup>, Yiyang Fan<sup>1</sup>, Lixiang Zhao<sup>1</sup>, Yanfei Zhao<sup>1</sup>, Xian Wang<sup>1</sup>, Dongqin Mo<sup>1</sup>, Haoyu Lu<sup>1</sup>, Daiyou Wang<sup>1, \*</sup>

<sup>1</sup> Department of Oral and Maxillofacial Surgery, Hospital of Stomatology, Guangxi Medical University, Nanning, China

<sup>2</sup> Affiliated Stomatology Hospital of Guilin Medical University, Guilin, China

### Article Info

### Abstract



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The salivary gland (SG) is a kind of organ vulnerable to ionizing radiation (IR). Radiotherapy is an important treatment for head and neck cancer, but in the process of radiotherapy, salivary gland cells will be injured by radiation to a certain extent. Ionising radiation-induced DNA double-strand break (IR-DSBs) is one of the most serious DNA damage. DNA repair proteins such as Nijmegen breakage syndrome protein 1 (NBS1) play a key role in the identification and repair of DNA damage, but the interaction between single-stranded DNA-binding protein 1 (SSB1) and NBS1 has not been elucidated. In this study, we irradiated rat submandibular gland (SMG) cells, which were either infected with a rAdE5-SSB1-1p2-shRNA recombinant adenovirus to silence SSB or a control virus, to explore the effect of IR on the expression of NBS1 in the absence of SSB. Our results showed that the SSB1 mRNA transcripts and protein expression of SSB1 and NBS1 initially increased and decreased later with increased doses. The relative expression reached the highest levels when the SMG cells were irradiated with 2Gy of IR. Silencing the SSB1 gene suppressed the expression of both SSB1 and NBS1 regardless of irradiation. The expression of NBS1 decreased when the SSB1 gene was silenced. We concluded that IR affected the expression of both SSB1 and NBS1 and there is a synergistic effect on IR-induced NBS1 suppression and DSBs repair in SMG cells. These observations shed light on further investigation and elucidation of IR-caused DNA repair mechanisms.

**Keywords:** Irradiation, <sup>60</sup>Co gamma-ray, Submandibular gland cell, SSB1, NBS1, replication-defective adenoviral vector, DNA damage, DNA double-strand breaks

## 1. Introduction

Selective radiotherapy (RT) is the preferred treatment for patients with early oropharyngeal squamous cell carcinoma [1]. However, ionizing radiation (IR) will cause varying degrees of injury and side effects to a variety of organs in the head and neck [2]. Although RT has achieved great success, it is still a difficult problem to enhance the effect of RT on tumor tissue and reduce its adverse reaction to normal tissue [3]. IR for head and neck cancer (HNC) inevitably causes radiation damage to salivary glands (SGs) of patients [4], because SGs are markedly susceptible to IR, and radiation-induced SG injury (RIS-GI) is a serious adverse event [5], salivary gland function is severely disrupted by RT [6,7], patients often experience moderate to severe acute mucosal toxicity, resulting in progressive, painful mucositis, nausea, thick saliva [8], xerostomia [9,10]. Permanent xerostomia can also occur due to SGs losing their secretory acinar cells [11]. About two-thirds of the volume of unstimulated saliva is contributed by the submandibular glands (SMGs) [12], IR-induced damage in SMGs is common [13].

The most significant IR target for cell death is DNA, causing a variety of DNA damages including oxidative da-

mage, base modifications, interstrand crosslinks, ring-opened bases, single-strand breaks (SSBs), and double-strand breaks (DSBs) [14,15]. DSBs induced by IR are the most harmful form of DNA damage [16]. About 10% of DSBs is preserved by cells after IR [17]. After the DNA fragment was destroyed, DNA repair proteins were recruited to the DNA double-stranded junction and triggered the DNA damage response (DDR) [18,19], thus realizing the removal and repair of the destroyed DNA fragment. The role of DDR is to identify damage, inhibit the cell cycle, and repair damage [20]. DDR is a complex protein regulatory network that participates in the regulation of DNA damage and repair [21]. DNA damage checkpoint (DDC) response can prevent the occurrence of chromosomes that can not be repaired or destroyed by terminating cell cycle, re-coding genes, and mobilizing DNA repair factors [22].

Mismatch repair (MMR), base excision repair (BER), nucleotide excision repair (NER), homologous recombination (HR) and non-homologous end connection (NHEJ) are the most important repair methods [23], typically, HR and NHEJ intervene in DSBs repair [24]. HR requires a DNA template of the same origin as the injured site, while NHEJ does not need to splice the end of the DNA double-

\* Corresponding author.

E-mail address: [wangdaiyou@sina.com](mailto:wangdaiyou@sina.com) (D. Wang).

# These authors contributed equally

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strand and does not need a sequence of the same source [25]. HR involves DNA double-strand end cleavage, homologous search, homologous gene invasion and DNA damage repair. NHEJ is an error correction method that can reassemble the ends of two DSBs without using the same template [26]. NHEJ plays an important role in the regulation of DNA DSBs [27]. Defects in NHEJ make cells more sensitive to IR [28]. About 70% of all DSBs caused by IR are repaired by NHEJ pathway [29].

SSB1 (single-stranded DNA-binding protein 1), also known as hSSB1, OBFC2B [23], is a key protein involved in DDR [19]. Studies have shown that SSB1 is an important part of the DNA damage recognition and repair pathway, and can participate in DSBs repair through HR and NHEJ [30]. NBS1 (Nijmegen breakage syndrome protein 1) is a critical component of the MRN (MRE11/RAD50/NBS1) complex. This complex is a crucial orchestrator of the DDR [31], and is one of the primary vehicles for repairing DSBs and maintaining genomic stability [32]. MRN is essential for detecting and repairing DNA damage [33]. NBS1 C terminal combines Mre11, enabling NHEJ and HR to play important roles in repairing DSBs [34]. Although they are involved in DNA repair, the quantitative relationship between SSB1 and MRN complex has not been elucidated. A deeper understanding of the mechanisms of radiation-induced DNA damage is key to improving cancer outcomes [24].

In this study, the recombinant adenovirus vector of rat SSB1-shRNA was constructed and transfected into SMG cells to explore the effect of silencing SSB1 gene on the expression of NBS1 protein, as well as the effect of irradiation on the expression of SSB1 and NBS1 protein, revealing the relationship between SSB1 and NBS1, and providing new ideas for the study on the repair mechanisms of DSBs.

## 2. Materials and methods

### 2.1. Primary culture of SMG cells in rats

Two SPF grade SD rats at 3 days of age (purchased from the Animal Test Center of Guangxi Medical University, License number for Experimental Animal use: SYXK GUI 2014-0003, Production license number: SCXK GUI 2014-0002) were sacrificed by cervical dislocation and sterilized in 75% ethanol solution for 10 min. The rats were transferred into a sterile petri dish on a superclean bench, their neck area was disinfected with iodophor, and an inverted T-shaped incision was made in the center of the neck. The bilateral SMGs were completely removed by blunt dissection and transferred to a sterile petri dish placed on ice. Appropriate amount of PBS solution was added, and the envelope, blood vessels and fibrous connective tissue were carefully removed with the help of a magnifying glass. Then the SMGs were cut into small pieces, blown and rinsed three times with PBS solution. The small pieces of SMGs were transferred to a 2 mL centrifuge tube, and 1 mL 0.25% pancreatic enzyme (HyClone, South Logan, UT, USA) with 0.01% EDTA was added, blown and mixed, and placed in an incubator for digestion at 37°C for 30 min, shaking every 5 min. After digestion, the pieces were transferred to a 15 mL centrifuge tube, and 3 mL high-sugar DMEM culture solution (HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (GIBCO, Rockville, MD, USA) was added to terminate digestion, and centrifuged at 500 rpm for 5

min. The precipitate was retained. Then 6 mL complete culture solution -10% fetal bovine serum high-glucose DMEM culture solution, which contains insulin (5 µg/ml), epidermal growth factor (10 ng/ml), transferrin (5 µg/ml), penicillin (100 U/ml), streptomycin (0.1 mg/ml) and hydrocortisone (100 ng/ml), was added to re-suspend cells and tissue blocks, and then inoculated evenly into two 25 cm<sup>2</sup> culture bottles on average, with cross shaking to disperse evenly. After 3 days, the culture solution was discarded, 2 mL PBS solution was added, culture bottles were gently shaken and washed twice, then 3 mL complete culture solution was added, and the culture solution was changed about once every 3 days. About 7 days later, the SMG cells crawling out of the edges of the tissue blocks increased and began to grow in layers, like paving stones.

### 2.2. Subculture of the SMG cells in rats

Trypsin digestion combined with the differential adhesion method was used. Two mL of PBS solution was added into a culture flask covered with primary SMG cells, gently shaken and washed twice, then 1 mL of 0.25% pancreatic enzyme with 0.02% EDTA was added. The cells were evenly dispersed by cross shaking and digested at room temperature for 2 min. Alternatively, when most of the cells had shrunk and became round and bright, beat the culture bottle to make the cells fall off, and immediately added 3 mL of high-sugar 10% fetal bovine serum in DMEM culturing medium terminate digestion, filtered the tissue blocks with a 200-mesh screen, centrifuged in a 15 mL centrifuge tube at 800 rpm for 5 min, discarded the supernatant, added 3 mL of complete culture solution to re-suspend the cells, and inoculated them into a 25 cm<sup>2</sup> culture flask. After the culture flasks were incubated for 1 h, the supernatant was sucked out and inoculated into two new 25 cm<sup>2</sup> culture flasks and cultured at 37°C in 5% CO<sub>2</sub> incubator. The culture solution was changed every 3 days thereafter.

### 2.3. Construction of rAdE5-SSB1-1p2-shRNA recombinant adenovirus vector

The cDNA sequence of rat SSB1 was found from GenBank, and according to the design principle of short hairpin RNA (shRNA), the online design software of American Ambion Inc. was used to select two target sequences of RNA joint interference against SSB1. They are SSB1 (844): 5'-CCAGAACGGGAATGGACTGAG-3', SSB1 (539): 5'-GGCAGCATCAACATCTCAGTGT-3'. DNA template primers were designed and synthesized: Oligo SSB1 (844)A: TTGGTCTCCTGCAGCTCAGTC-CATCCCGTTCTGGTTTTTTAAGCTTCC; SSB1 (844)C: TTGGTCTCCTGCACTCAGTCCATTCC-CGTTCTGGCGGTGTTTCGTCCTT; SSB1 (539)D: TTGGTCTCGTCCCACACTGAGATGTTTTGAAG-GTTCGAATCCTTCTGCTGCCCTAGTATGACCGCC; SSB1 (539)B: TTGGTCTCTGGGACGATGTCTGCA-GACATCGTCCCACACTGAGAGCTAGCGGATC-TGAC. The primers were divided into A+B and C+D templates, and PCR amplification was performed at the same time. After purification, the amplified products of the two groups were digested by BsaI enzyme to obtain the binding product None-1p2. None-1p2 was transformed into receptor cell DH5a and plasmid was extracted and identified by PstI. The SSB1-1p2-shRNA expression frame was obtained and inserted into plasmid vector pGenesilM. The

SSB1-1p2-shRNA expression frame was transferred to the pGSadeno adenovirus expression vector by LR homologous recombination *in vitro*, and the extracted plasmids were further identified by digestion with the restriction enzyme XbaI and 1% Agarose gel electrophoresis. HEK 293 (human embryonic kidney 293) cells with 50-70% fusion degree were transfected according to the instructions using METAFECTENE™ (Biontix, Guangzhou, China), and recombinant adenovirus was confirmed by fluorescent microscopy. rAdE5-SSB1-1p2-shRNA recombinant adenovirus vector solution and empty adenovirus solution with the concentration of  $1 \times 10^9$  PFU/mL were obtained. Recombinant plasmid construction, adenovirus vector construction and packaging services were provided by Wuhan Xima Biotechnology Co., LTD. (Wuhan, China).

#### 2.4. SMG cells were transfected with rAdE5-SSB1-1p2-shRNA recombinant adenovirus

The second generation of logarithmic growth SMG cells was taken, after digestion, cell suspensions with a density of  $2 \times 10^4$  cells /mL were inoculated into 24-well culture plates containing complete culture solution, randomly divided into rAd-shRNA group in which cells were transfected with rAdE5-SSB1-1p2-shRNA recombinant adenovirus vector, rAd-HK group in which cells were transfected with empty adenovirus vector, and normal control group, and cultured overnight. After 24 h, the culture solutions of rAd-shRNA group and rAd-HK group were replaced with recombinant adenovirus vector solution with a multiplicity of infection (MOI) of 50 and empty adenovirus vector solution, respectively, and 500  $\mu$ L was added per well. The control group did not add a virus. The plates were shaken evenly every half hour. After 2 h culture, the culture solutions were replaced with 10% fetal bovine serum-containing complete culture solution, and the culture was continued for 72 h, Each group's total protein content was extracted and chilled to  $-80^\circ\text{C}$  in the refrigerator. Each sample was concurrently infected, and three copies of each test were run.

#### 2.5. Irradiation delivery methods

Rat SMG cell cultures were IR treated in five groups with doses of 1, 2, 4, 6 and 8Gy, respectively. With GWXJ80 cobalt-60 teletherapy unit (China Nuclear Power Research and Design Institute) as the radiation source, and each group was irradiated with the corresponding single dose. The control group was not irradiated. Cells in each group had their total RNA extracted within 1 hour after irradiation according to the instructions of the total RNA extraction kit. After the purity and concentration were determined, the cDNA was synthesized according to the reverse transcription kit procedure and stored in the refrigerator at  $-80^\circ\text{C}$  for instantaneous fluorescence detection using the quantitative polymerase chain reaction (RT-qPCR). Each sample was inoculated in parallel and each test was per-

formed in triplicate. In addition, the total protein of each group was extracted from the radioimmunoprecipitation assay (RIPA) (PMSF, Solarbio, Beijing, China) lysosomal buffer (PMSF) with 1% PMSF within 1 h after irradiation, and placed in the refrigerator at  $-80^\circ\text{C}$  for Western blot analysis. According to the method in 2.4, the SMG cells transfected by adenovirus were randomly divided into 4 groups. The radiation doses in the rAd-shRNA group and the rAd-shRNA+IR group were 0Gy and 6Gy, respectively. The other two groups were the control group and the 6Gy irradiated (IR) group. The extraction of total protein in each group was carried out according to the method in 2.4.

#### 2.6. The expression levels of SSB1 and NBS1 in transfected and irradiated SMG cells were detected by Western blot

Bicinchoninic acid (BCA) protein concentration assay Kit (Beyotime, Shanghai, China) was used to detect protein concentrations extracted from transfected and irradiated SMG cells. RIPA (PMSF, Solarbio, Beijing, China) lysosomal buffer (PMSF) with 1% PMSF, transferred onto polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA), and blocked with 5% skim milk for 1 h. Primary antibody incubation: the membrane is separately placed into the diluted SSB1 polyclonal antibody (1:3000, Novus Biologicals, Littleton, CO, USA), NBS1 rabbit monoclonal antibody (1:500, Cell Signaling Technology, Inc. Danvers, MA, USA) and  $\alpha/\beta$ -Tubulin antibody (1: 1000, Cell Signaling Technology, Inc. Danvers, MA, USA), overnight in shaker at  $4^\circ\text{C}$ . The membrane was washed in TBST for 3 times, 10 min each time. Secondary antibody incubation: The membrane was placed in goat anti-Rabbit IgG/FITC (1:15000, KPL, USA) and incubated at room temperature for 1-2 h in darkness. The membrane was washed in TBST for 3 times, 10 min each time. Odyssey infrared two-color fluorescence system (LICOR Biosciences, Atlanta, GA, USA) was used to analyze protein bands and calculate gray values. The ratio of the gray values of the target proteins SSB1, NBS1 and the reference protein  $\beta$ -Tubulin was used as the relative expression of the target proteins. The experiment was repeated three times at different times.

#### 2.7. Determination of expression of SSB1 and NRB1 mRNA by QPCR method

Total RNA was extracted from irradiated and non-irradiated rat SMG cells and reverse-transcribed into cDNA (PrimeScript™ RT reagent kit purchased from TaKaRa Bio, Dalian, China). The relative expression level of SSB1 was detected by ABI StepOnePlus™ Real-Time PCR System (Life Technologies, Gaithersburg, MD, USA), and the sequences of primers are shown in Table 1. Reaction conditions: predenaturation at  $95^\circ\text{C}$  for 30 S;  $95^\circ\text{C}$  for 5S,  $60^\circ\text{C}$  for 30 S, with a total of 40 cycles. After the reac-

**Table 1.** Primer sequences of qRT-PCR.

	primer sequence	amplification fragment length
SSB1	forward primer 5'-AGCTTTGGGATTAGAGGGTGAAG-3' reverse primer 5'-ACAGACAGCATCAGCAGGACA-3'	122 bp
GAPDH	forward primer 5'-GGCACAGTCAAGGCTGAGAATG-3' reverse primer 5'-ATGGTGGTGAAGACGCCAGTA-3'	143 bp

tion, the Ct values of each sample were recorded, and the relative expression of SSB1 was calculated by the  $2^{-\Delta\Delta Ct}$  method.

### 2.8. Statistical analysis

Statistic Package for Social Science (SPSS) 22.0 statistical software (SPSS, Chicago, IL, USA) was used to process and analyze the data. The measurement data were expressed as mean  $\pm$  standard deviation ( $\bar{x} \pm s$ ), analysis of variance was used for comparison among multiple groups, and LSD-t test was used for comparison between the two groups.  $P < 0.05$  was considered statistically significant.

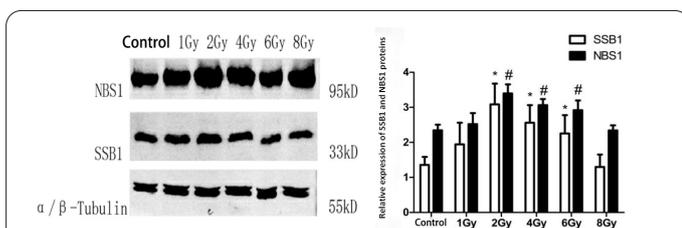
## 3. Results

### 3.1. Changes of SSB1 mRNA expression in rat SMG cells after irradiation with different doses of 60Co rays

As shown in Table 2, the relative expression of SSB1 mRNA first increased and then decreased with the increase in dose, and the relative expression of SSB1 mRNA reached the highest when the radiation dose was 2Gy. One-way analysis of variance was performed, and the difference was statistically significant ( $P < 0.05$ ). Pairwise comparison between 2, 4 and 6Gy radiation groups and the control group, the difference was also statistically significant ( $P < 0.05$ ).

### 3.2. Changes in SSB1 and NBS1 protein expressions in SMG cells of rats after irradiation with different doses of 60Co $\gamma$ rays

As can be seen from Figure 1, the relative expression levels of SSB1 and NBS1 proteins increased first and then decreased with the increase of dose, and the relative expression levels reached the highest when the radiation dose was 2Gy. One-way analysis of variance found that the differences in SSB1 and NBS1 among 6 groups were statistically significant (all  $P < 0.05$ ). Pairwise comparison between groups showed that the protein expressions of SSB1 ( $P < 0.05$ ) and NBS1 ( $P < 0.05$ ) in 2, 4 and 6Gy groups were significantly different from those in the control group. Pearson correlation analysis showed that the relative ex-



**Fig. 1.** The relative expressions of SSB1 and NBS1 proteins in SMG cells of rats after different doses of 60Co  $\gamma$ -ray irradiation (n=6). \*.# Compared with control group,  $P < 0.05$

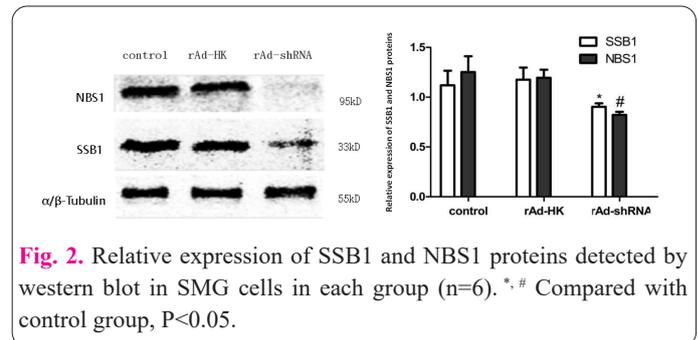
pression level of SSB1 protein was positively correlated with that of NBS1 protein ( $r=0.584$ ,  $P < 0.05$ ).

### 3.3. The relative expression levels of SSB1 and NBS1 proteins in rat SMG cells transfected with rAdE5-SSB1-1p2shRNA recombinant adenovirus

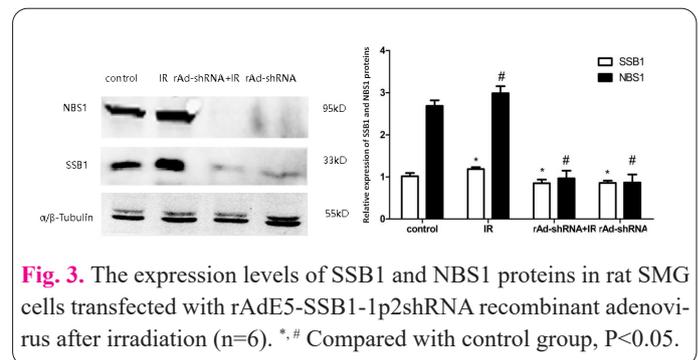
One-way analysis of variance found that there were statistically significant differences in SSB1 and NBS1 protein relative expression levels among 3 groups (all  $P < 0.05$ ). Pairwise comparison between groups showed that compared with control group and rAd-HK group, the protein expression levels of SSB1 and NBS1 in rAd-shRNA group were significantly decreased (all  $P < 0.05$ ), and the differences were statistically significant. There were no significant differences in SSB1 and NBS1 between rAd-HK group and control group ( $P > 0.05$ ). The results are shown in Figure 2. According to Pearson correlation analysis, the relative expression levels of SSB1 and NBS1 proteins were positively correlated ( $r=0.511$ ,  $P < 0.05$ ). These results indicated that silencing SSB1 gene could reduce the relative expression of SSB1 and NBS1 proteins.

### 3.4. The expression levels of SSB1 and NBS1 proteins in rat SMG cells transfected with rAdE5-SSB1-1p2shRNA recombinant adenovirus after irradiation

One-way analysis of variance found that there were statistically significant differences in SSB1 and NBS1 protein relative expression levels among 4 groups (all  $P < 0.05$ ). As can be seen in Figure 3, compared with control



**Fig. 2.** Relative expression of SSB1 and NBS1 proteins detected by western blot in SMG cells in each group (n=6). \*.# Compared with control group,  $P < 0.05$ .



**Fig. 3.** The expression levels of SSB1 and NBS1 proteins in rat SMG cells transfected with rAdE5-SSB1-1p2shRNA recombinant adenovirus after irradiation (n=6). \*.# Compared with control group,  $P < 0.05$ .

**Table 2.** The relative expression of SSB1 mRNA in rat SMG cells after irradiation with different doses of 60Co rays (n=6).

Groups	Relative expression of SSB1 mRNA ( $\bar{x} \pm s$ )
control	1.010 $\pm$ 0.012
1Gy	1.410 $\pm$ 0.390
2Gy	1.909 $\pm$ 0.439*
4Gy	1.721 $\pm$ 0.311*
6Gy	1.770 $\pm$ 0.287*
8Gy	1.305 $\pm$ 0.255

\* Compared with control group,  $P < 0.05$

group: the protein expression levels of SSB1 and NBS1 in IR group were significantly increased (all  $P < 0.05$ ), while those in rAd-shRNA+IR and rAd-shRNA groups were significantly decreased (all  $P < 0.05$ ). However, there were no statistically significant differences in these indexes between rAd-shRNA+IR and rAd-shRNA groups (all  $P > 0.05$ ). According to Pearson correlation analysis, the relative expression of SSB1 protein was positively correlated with that of NBS1 protein ( $r = 0.860$ ,  $P < 0.05$ ). These results indicated that silencing SSB1 gene could reduce the relative expression of SSB1 and NBS1 proteins.

#### 4. Discussion

DNA is the carrier of genetic information but is inherently unstable [30] due to the influences of endogenous and exogenous factors. To provide genomic information required for DNA replication, recombination and repair, double-stranded(ds)DNA must be unwound to form single-stranded (ss) intermediates [35]. The genes of cells are continuously destroyed due to DNA damage [36]. In the lifetime of a cell, naturally occurring DNA damage continues, resulting in SSBs or DSBs [37]. Exogenous factors include IR, UV, and environmental chemicals, and endogenous factors include reactive oxygen species, incorrect DNA replication, accidental ribozyme cleavage, metabolic intermediates [28], erroneous DNA replication, and collapse. Unrepaired or misrepaired DSBs will hinder chromosome replication and transcription [38]. DSBs can cause abnormal chromosome translocation [39], which leads to cell instability, and then leads to a variety of diseases. If gene modification is not carried out timely and effectively, it will lead to gene mutation and genomic instability, which will lead to tumorigenesis [40]. Correct repair of damaged DNA is the key to maintaining genome stability. There is a huge and complex DSB repair supervision system [41]. The stability of DNA is affected by many mechanisms of DNA damage repair [23]. SSB1 and MRN play a key role in the process of DNA damage repair.

SSB1 is a DNA-binding protein. The single stranded DNA binding protein family (SSB) consists of several highly conserved OB regions connected by oligomers [23], consisting primarily of replicating proteins A (three subunits: RPA70, RPA32, and RPA14 [42,43]) and SSB(SSB1 and SSB2 [30]). SSB have two seemingly independent but closely related functions: one is to rapidly cooperate with the DNA sequence, and the other is bind to protein that binds to the DNA sequence [44]. SSB are essential to protect ssDNA [23], are involved in all aspects of DNA metabolism [45], and are indispensable for cell viability [35]. The N-terminal domain of SSB contains the OB-fold required for ssDNA binding, and its C-terminal tail is required for protein-protein interactions [46,47]. During DNA metabolic processes, enzymes such as helicase and nuclease act on DNA duplex, briefly exposing ssDNA and producing ssDNA intermediates. SSB binds quickly and tightly to ssDNA to protect it from the breakdown of various nucleases, and at the same time, as a molecular medium, SSB regulates its activity by using/recruiting many enzymes (chaperone proteins) that play a role in DNA metabolism [48]. These chaperone proteins contain more than 20 kinds of DNA binding proteins, which constitute a DNA protection system called SSB interactome, and play a key role in maintaining the stability of DNA [45]. Both SSB2 deletion and SSB1/2 conditional double dele-

tion (CDKO) can cause B cell differentiation disorder. The knockout of SSB1/2 in B lymphocytes makes DNA more exposed, which causes the breakage of DNA vulnerable points, slows down the process of the cell cycle, and aggravates DNA damage. SSB1 deficient cells showed compensatory expression of homologous protein SSB2-[30].

DDR is mainly composed of three key kinases of Pikk family: ATM, ATR and DNA-PKcs. When DNA is damaged, they are activated and then many target proteins downstream of them are activated, thus promoting DNA repair [15]. SSB1 plays important roles in the activation of the DDR, the initiation of DNA damage checkpoints, and sphosphorylation of ATM and its downstream targets, such as tumor suppressor protein P53 (P53, also known as TP53), CHK1 (checkpoint kinase 1) and CHK2 (checkpoint kinase 2), the repair of stalled replication forks and oxidativestress-induced DNA damage, the repair of DNA damage by HR and NHEJ, bone formation during normal embryogenesis [30]. SSB1 (SOSSB1), together with SOS-SA (INTS3) and SOSSC (C9orf80), is a kind of single-stranded DNA complex 1 (SOSS1), which can bind to single-stranded DNA and mediate the recruitment of the complex to DNA damage sites [30], facilitates signaling of DSBs, participates in DSBs repair through mismatch repair (MMR) and HR, and it is critical for the regulation of DNA damage checkpoint activation as well as for the resection of DSBs prior to HR [49]. When SSB1 is absent in a cell, it increases radiosensitivity, resulting in defects in DNA double-strand break repair, DNA oxidative damage, and replication bifurcations that cannot be restarted [19].

MRN regulates ATM- and ATR-mediated DDR [34]. MRN complex is the center of DNA damage repair, which can identify, process and transmit DNA damage, and regulate the process of DNA damage repair. MRN initiates patching of DSBs. MRN senses DNA damage and begins DNA damage repair [50]. MRNs can quickly identify DNA fragments, which is the key to triggering DNA damage and clear DNA fragments [51]. The ends of DSBs are recognized both by the MRN and by KU70-KU80 proteins [22]. MRN can recruit Mre11, Rad50 and other proteins to the damaged DNA and participate in the activation of ATM. ATM can regulate its activity by activating p53, Chk2, mdc1 and so on [52], and H2AX(the variant of histone family 2A) [34,53], start a series of signals, form a transcriptional process, and stop cell division, play a crucial role in genome stability and integrity [54]. Recently, it has been found that MRN complex interacting protein (MRNIP) can accumulate in liquid form after DNA injury and participate in DNA damage repair. MRNIP can be used as a liquid aggregate to enhance the ability of MRNIP to repair DSBs damage by enhancing the sensitivity of MRNIP to DSBs and terminal cutting. When DNA double-strand breaks, MRNIP proteins transfer to damaged DNA, accelerating the fusion of DNA double-strand breaks and MRN protein complex, which leads to autophosphorylation of ATM; DNA damage response signals are activated, and can inhibit cell division through multiple activated molecular pathways [51], and repair damaged DNA. There are genetic variations in the three MRN components, which are related to ataxia telangiectasia-like disorder (ATLD), Nijmegen breakage syndrome (NBS), NBS-like disorder (NBSLD) and many kinds of tumors respectively [32]. MRN complex has the function of inhibiting and promoting tumor growth. Mre11, Rad50,

NBS1 and other protein components in the MRN complex are often highly expressed, which is very important for the occurrence and development of tumors [55]. SSB1 existed at the site of DNA double-strand break in the early stage after DNA injury and could combine with MRN complex, suggesting that it was involved in the initial process of DNA double-strand break [56]. When exposed to radiation, SSB1 is rapidly recruited to the location of DSBs, and the appearance of SSBs is very important for the efficient recruitment of MRN complex and its downstream pairs, while when SSBs is inhibited, the location of MRN recruitment to DSBs will be blocked, which suggests the role of SSB1 and MRN in the process of DNA damage repair [18].

In this study, it was found that there was also a certain amount of SSB1 and NBS1 expression in normal rat SMG cells without radiation, which may be caused by endogenous factors as mentioned above. The changing trend of NBS1 protein expression in SMG cells of rats after different radiation doses was consistent with that of SSB1, indicating that there is a dose relationship between NBS1 and SSB1 in DSBs repair of rat SMGs. In order to investigate the potential interaction between SSB1 and NBS1, recombinant adenovirus transfection technique was used in this study to silence the expression of SSB1 in SMG cells of rats, and to detect the expression of SSB1 and NBS1 proteins at the same time, so as to clarify the influence of SSB1 on NBS1.

Recombinant adenovirus vectors (AdVs), including replication-defective adenoviral vectors [57], with the characteristics of good genetic stability, high gene transduction efficiency, large amount of transformation and high transformation rate, it has been widely used in the fields of gene therapy and gene function [58,59]. Ad5 vector propagated in HEK 293 cells is widely used. Homologous recombination is a technique for generating AdV vectors, it is related to the recombination of two kinds of DNA in one cell line. Using the right end of the linearly purified ADV DNA and ADV genome, the transporters containing the target gene and the left end of the ADV genome were recombined with HEK293 cells to obtain the desired viral vector [60]. In this study, recombinant adenovirus vectors carrying enhanced 1plus2 shRNA were selected to greatly improve the ability to down-regulate the expression of target genes. Fluorescent confirmation recombinant adenovirus assay revealed that recombinant adenovirus carried a eukaryotic expression frame of fluorescent protein, and obvious expression of fluorescent protein was found in HEK293 cells after transfection, which proved that the recombinant adenovirus rAdE5-SSB1-1p2-shRNA with infectivity was successfully packaged. In the previous experiment, our research group tested the transfection efficiency of the virus in rat SMG cells, and found that the transfection efficiency was close to 90% when the MOI was 50. rAdE5-SSB1-1p2-shRNA is a replication-deficient recombinant adenovirus (rAd) vector that can cause a decrease in SSB1 expression. In this study, expression of reference protein  $\alpha/\beta$ -Tubulin was not affected in each group after transfection with this vector, while there was no significant change in the rAd-HK group and the control group, indicating that the recombinant adenovirus of rAdE5-SSB1-1p2-shRNA transfected into SMG cells could down-regulate the expression of SSB1 protein, and adenovirus vector itself had no effect on the expression of

SSB1 protein, adenovirus expression vector rAdE5-SSB1 1p2-shRNA which silenced rat SSB1 gene was successfully constructed. At the same time, with the decrease of SSB1 protein expression, the expression of NBS1 protein decreased, and there was a positive correlation between the two protein expressions. The results of viral transfection experiment further found that SSB1 protein expression was silenced and NBS1 protein expression was significantly decreased regardless of radiation, indicating that SSB1 loss could cause NBS1 expression impairment, and there may be a synergistic relationship between the two in DSBs repair mechanism of rat SMG cells. It may be because SSB1 and NBS1 in DSBs repair regulatory signals are activated in different order, SSB1 starts earlier, which is the "key" to start DSBs repair signal pathway. It may also be because the regulatory sites of SSB1 and NBS1 are different in the process of DSBs damage repair, and SSB1 is located upstream of NBS1.

Our results show that SSB1 may be an upstream molecule of MRN, which contributes to the recruitment of MRN in DSB and is necessary for the efficient cutting of DSB. In this study, the relative expression levels of SSB1 mRNA and protein in SMG cells of rats showed the same change trend, increasing first and then decreasing with the increase of irradiation dose. The relative expression levels of SSB1 mRNA and protein in SMG cells reached the maximum when the radiation dose was 2Gy. When the radiation dose is increased to a certain extent, the expression of SSB1 is not increased but decreased, which may be caused by partial apoptosis and necrosis of cells, weakened repair ability and reduced SSB1 expression due to high dose radiation. The relative expression levels of SSB1 mRNA, SSB1 and NBS1 proteins in rat SMG cells were the highest at 1 h after a single dose of 2Gy irradiation, the dose was lower than those (10 Gy X-rays, HUVECs [61]; 15.0 Gy X rays, SMG-C6 cells [4]) reported in the literatures. Another study with human GBM cell line U87MG as the research object and DNA DSBs as the observation index found that the amount of DSBs in the cells irradiated with 2 Gy of iodine-131 was greater than the cells irradiated with 2 Gy of 6 MV X-ray. Radiation hazard refers to the risk of chromosome instability and corresponding mutation when wild-type mouse fetal fibroblasts are irradiated by 0.25-2 Gy gamma-rays, but not upon high-dose irradiation, which caused permanent cell-cycle arrest [62]. However, the above studies differ in study purpose, cell type used, observation time point, and selected indicators. X-ray and gamma-ray result in similar, although not always identical, physiological effects [63].

In this study, the expression of SSB1 in DSBs repair of rat SMG cells was analyzed from mRNA and protein levels. In vitro culture of rat SMG cells and transfection of adenovirus expression vector rAdE5-SSB1 1p2-shRNA to silence the SSB1 gene of rat SMG cells, which initially revealed that there may be a synergistic relationship between SSB1 and NBS1 protein expression in DSBs repair of rat SMG cells, and silencing SSB1 can down-regulate the expression of NBS1 protein. On this basis, we will further carry out viral transfection experiments in vivo in the later stage to deeply reveal the dynamic changes of SSB1 expression after silencing SSB1 gene in rat SGs and the relationship between SSB1 expression and MRN complex in DSBs repair signaling pathway, which will provide a perfect theoretical basis for radiation damage gene therapy.

## 5. Conclusion

The expression level of SSB1 was consistent with that of DSBs in vitro. It is suggested that SSB1 plays an important role in DSBs damage repair. The change trend of NBS1 expression was consistent with that of SSB1, and SSB1 silencing could down-regulate NBS1. There is a synergistic relationship between SSB1 and NBS1 in DSBs damage repair of SMG cells in rats.

## Conflict of Interests

The author has no conflicts with any step of the article preparation.

## Consent for publications

The author read and approved the final manuscript for publication.

## Ethics approval and consent to participate

This study was approved by the Animal Ethics Committee of Guangxi Medical University Animal Center.

## Informed Consent

The authors declare not used any patients in this research.

## Availability of data and material

The data that support the findings of this study are available from the corresponding author upon reasonable request.

## Authors' contributions

Linjing Gao, Qiuli Lv and Daiyou Wang designed the study and performed the experiments, Yude Huang, Yiyang Fan and Lixiang Zhao collected the data, Yanfei Zhao, Xian Wang, Dongqin Mo and Haoyu Lu analyzed the data, Linjing Gao, Qiuli Lv and Daiyou Wang prepared the manuscript. All authors read and approved the final manuscript.

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