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HHR23A, a human homolog of *Saccharomyces cerevisiae* Rad23, regulates xeroderma pigmentosum C protein and is required for nucleotide excision repair ☆,☆☆

Hui-Chuan Hsieh [a], Yi-Hsuan Hsieh [b], Yu-Hsin Huang [c], Fan-Ching Shen [a], Han-Ni Tsai [a], Jui-He Tsai [a], Yu-Ting Lai [c], Yu-Ting Wang [c], Woei-Jer Chuang [b,d], Wenya Huang [a,b,c,*]

[a] *Medical Laboratory Science and Biotechnology, College of Medicine, National Cheng Kung University, Tainan 70101, Taiwan*
[b] *Basic Medical Sciences, College of Medicine, National Cheng Kung University, Tainan 70101, Taiwan*
[c] *Molecular Medicine, College of Medicine, National Cheng Kung University, Tainan 70101, Taiwan*
[d] *Biochemistry, College of Medicine, National Cheng Kung University, Tainan 70101, Taiwan*

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Abstract

HHR23A and hHR23B are the human homologs of *Saccharomyces cerevisiae* Rad23. hHR23B is associated with the nucleotide excision repair (NER) factor xeroderma pigmentosum C (XPC) protein and is required for global genome repair. The function of hHR23A is not yet clear. In this study, the potential function of the hHR23A protein was investigated using RNA interference techniques. The hHR23A knock-down (KD) construct diminished the RNA level of hHR23A protein by approximately 60%, and it did not interfere with expression of the hHR23B gene. Based on Southwestern immunoblot and host-cell reactivation assays, $hHR23A^{KD}$ cells were found to be deficient in DNA repair activity against the DNA damage caused by UVC irradiation. In these $hHR23A^{KD}$ cells, the XPC gene was not normally induced by UVC irradiation, indicating that the hHR23A protein is involved in NER through regulation of the DNA damage recognition protein XPC. Co-immunoprecipitation experiments revealed that hHR23A was associated with a small portion of hHR23B and the majority of p53 protein, indicating that hHR23A regulates the function of XPC by its association with the NER activator p53.

Keywords: hHR23; Rad23; Nucleotide excision repair; DNA repair; RNA interference

Nucleotide excision repair (NER) is the major pathway for repairing DNA lesions that cause structural distortion [1]. The disease caused by an NER deficiency is designated xeroderma pigmentosum (XP), which is characterized by extreme cutaneous photosensitivity to ultraviolet light, and, ultimately, metastatic skin cancer [2]. Among the XP genes identified, XPC is the major factor for damage recognition in the subpathway of global genome repair (GGR), which is involved in repairing DNA lesions on non-transcribed genes or non-transcribed strands of active genes [3]. To target DNA lesions, XPC is tightly associated in a protein complex with hHR23B, which increases the DNA-binding activity of XPC on DNA lesions [4]. In addition to hHR23B, hHR23A is also a sequence homolog of *Saccharomyces cerevisiae* Rad23 in humans [5]. Although hHR23A is highly homologous with hHR23B, its in vivo

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☆☆ *Abbreviations:* NER, nucleotide excision repair; hHR23, human homolog of Rad23; RNAi, RNA interference; siRNA, small-interfering RNA; KD, knock-down; KO, knock-out.
* Corresponding author. Fax: +886 6 236 3956.
E-mail address: whuang@mail.ncku.edu.tw (W. Huang).

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role in NER has not been identified. Previous studies in hHR23A/B knock-out (KO) mice have reported that hHR23A was likely functionally redundant with hHR23B, and that a mutation in the hHR23A gene alone did not cause observable defects in NER [6]. It has also been reported, however, that the hHR23A and hHR23B proteins directly interact with each other in vivo and in vitro, suggesting that these proteins are cooperative rather than redundant [7]. hHR23B is involved in DNA repair and proteolysis; therefore, it is likely that hHR23A is also involved [8]. The mHR23A gene in mice is abundantly expressed in some tissues, especially the skeletal muscles and testes, indicating that hHR23A might be an important protein in some tissues [9]. It is therefore important to understand the cellular functions of hHR23A. Mutant phenotypes of the hHR23A and hHR23B genes are quite difficult to study, because there are no diseases associated with these mutations. hHR23B KO mice exhibit moderate UV sensitivity and NER deficiency, whereas hHR23A KO mice do not exhibit any observable defects in DNA repair activity. However, when both of hHR23A and B were deleted, the mice exhibited extremely strong phenotypes, including DNA repair defects and sterility [6]. These findings indicate that the hHR23A does not completely duplicate the function of hHR23B and must have a function of its own. To study the function of hHR23A protein in humans, we used RNA interference techniques to construct a knock-down (KD) model for hHR23A.

Materials and methods

Cell lines, antibodies, and reagents. HtTA-1 cells (derived from human cervical carcinoma HeLa cell line) were maintained in regular Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1× non-essential amino acid, and 1× of antibiotic/antimycotic mixture (Gibco-BRL, Grand Island, NY). The cells were grown at 37 °C with 5% CO_2. The anti-hHR23A antibody in rabbits was generated against the hHR23A peptide, LADISDVEGEVG (Cashmere Scientific, Taipei, Taiwan), and then purified using an affinity column containing the recombinant hHR23A protein. The anti-hHR23B antibody in rabbits was purchased from BD Biosciences (San Jose, CA). Most of the reagents used for the reverse transcription and PCR amplifications were purchased from Promega (Madison, WI). Common chemicals used were purchased from Sigma Chemical (St. Louis, MO).

RNA interference. The DNA oligomers used for RNA interference constructs against the hHR23A and B genes were cloned into pSUPER (a generous gift from Dr. R. Agami, The Netherlands) [10]. These DNA sequences were selected from the regions that were low in homology between the hHR23A and B genes (Table 1). DNA fragments of the interfering sequences were annealed into double-stranded sequences and then cloned into pSUPER at the *Hin*dIII and *Bgl*II restriction enzyme sites. These constructs for RNA interference were full-length sequenced for verification.

Plasmid transfection. Lipofectamine 2000 reagent (Gibco-BRL, Grand Island, NY) was used for plasmid transfection experiments. Briefly, 2–4 μg of the plasmid to be transfected was diluted to 50 μl with Opti-MEM 1 reduced serum medium, which contains 2% FBS. Two microliters of the reagent was also diluted to 50 μl with Opti-MEM 1 and then allowed to sit for 5 min at room temperature. Solutions of the plasmid and reagent were then mixed and allowed to sit for 20 min. The mixture solution was added to the culture cells, which had been transferred to the Opti-MEM 1 medium. After plasmid transfection, the cells were incubated at 37 °C for 20 h in 5% CO_2. The culture medium was then switched to regular DMEM for further incubation.

MTT assay. A 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) cell proliferation assay was used to detect cell sensitivity to UVC irradiation [11]. Briefly, HtTA-1 cells were grown to 1×10^5/well in a 24-well culture plate and then transfected with the RNAi constructs or pSUPER vector control. The culture medium was removed, and cells were washed once with phosphate-buffered saline (pH 7.4) and treated with 15 or 30 J/m^2 of UVC irradiation. After 48 h of incubation at 37 °C, half the medium was removed. 0.5 mg/ml of MTT labeling reagent (Sigma Chemical) was added to each well with the UVC-treated or mock-treated cells. After 4 h of incubation at

Table 1
Sequences of the primers used for RNA interference constructs

Oligomer	Sequence	Region
23Ai-1(774–792)-forward	5′-GATCCCCGCTCCAGCAGCTGG GCCAGTTCAAGAGACTGGCCCA GCTGCTGGAGCTTTTTGGAAA-3′	XPC-binding
23Ai-1(774–792)-reverse	5′-AGCTTTTCCAAAAAGCTCCAG CAGCTGGGCCAGTCTCTTGAACT GGCCCAGCTGCTGGAGCGGG-3′	
23Ai-3(534–552)-forward	5′-GATCCCCGCGGGTCGTGGCCG CCCTGTTCAAGAGACAGGGCGG CCACGACCCGCTTTTTGGAAA-3′	UBA1
23Ai-3(534–552)-reverse	5′-AGCTTTTCCAAAAAGCGGGTC GTGGCCGCCCTGTCTCTTGAACA GGCGGCCACGACCCGCGGG-3′	
23Bi-3(615–633)-forward	5′-GATCCCCGCAAGTAATTGCAG CCCTGTTCAAGAGACAGGGCTG CAATTACTTGCTTTTTGGAAA-3′	UBA1
23Bi-3(615–633)-reverse	5′-AGCTTTTCCAAAAAGCAAGTA ATTGCAGCCCTGTCTCTTGAAC AGGGCTGCAATTACTTGCGGG-3′	

37 °C, 10% SDS/0.01 M HCl solubilization solution was added to wells, and cells were then incubated at 37 °C overnight. On the next day, the optical density $(OD)_{590\ nm}$ was measured and converted to a cell-survival percentage compared with control cells.

Southwestern immunoblot. The HtTA-1 cells transfected with the RNAi constructs against the hHR23A or hHR23B genes were irradiated using a 254-nm ultraviolet lamp. The cells were allowed to sit for 2 h for DNA recovery. After the incubations, the genomic DNA in cells was extracted using standard phenol/chloroform methods and diluted to 1 μg/μL in 0.4 N NaOH to denature the DNA strands. Five micrograms of genomic DNA from each set of cells was spotted on a Hybond-N^+ membrane (Amersham Biosciences, Piscataway, NJ). The remaining DNA lesions caused by UVC irradiation were then probed with mouse 64M-2 antibody, which specifically recognized the (6–4) photoproducts (a generous gift from Dr. T. Matsunaga, Kanazawa, Japan), based on the Western blot protocol described previously [12]. Finally, signals of the (6–4) photoproducts were detected using a kit (ECL Western Blotting Detection kit; Amersham Biosciences).

Host-cell reactivation assay. To detect DNA repair activity, the cisplatin-damaged plasmids consisting of luciferase reporter gene were transfected into HtTA-1 cells. To construct the plasmid substrate, 100 μg of $pCMV^{Luc}$ DNA was treated at 37 °C for 5 h with *cis*-dichlorodiammineplatinum (II) (cisplatin) (Sigma Chemical). After the treatment, the $pCMV^{Luc}$ DNA was purified using ethanol precipitation. To test the DNA repair activity in HtTA-1 cells expressing various types of RNA interference constructs, cisplatin-damaged $pCMV^{Luc}$ as well as the hHR23A or hHR23B RNAi constructs were co-transfected into HtTA-1 cells. After transfection for 72 h, cell lysates were collected and the luciferase activities were detected using a luciferase assay kit (Applied Biosystems, Foster City, CA).

Co-immunoprecipitation. To screen for proteins associated with hHR23A, the affinity-purified anti-hHR23A antibody in rabbits was used for immunoprecipitation assays. Briefly, 500 μg HtTA1 cell-free extracts in RIPA buffer (150 mM NaCl, 10 mM Tris–HCl, 0.1% SDS, 1.0% Triton X-100, 1% deoxycholate, 5 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride; pH 7.2) was mixed with anti-hHR23A antibody and protein-A agarose beads (Sigma Chemical) and then gently shaken at 4 °C for 1 h. After serial washings with RIPA buffer, the final immunoprecipitant was eluted from the protein-A agarose beads using SDS–PAGE loading dye (0.25 M Tris–HCl, 0.2% SDS, 10% glycerol, 2% dithiothreitol, 0.1% 2-mercaptoethanol, and 0.001% bromophenol blue; pH 6.8). The protein eluates were separated using SDS–PAGE electrophoresis and then analyzed using Western blots, following the protocol previously reported [12].

Immunofluorescence staining. The HtTA1 cells transfected with the pSUPER/hHR23Ai knock-down plasmid were seeded onto chamber slides and left overnight. The slides were then fixed in standard fixative solution (methanol/acetone 1:1) for 15 min. To visualize the hHR23A and XPC proteins, the HtTA1 cells were incubated with rabbit anti-hHR23A and mouse anti-XPC antibodies for 1 h at room temperature. Secondary anti-rabbit antibody conjugated with fluorescent dye (Alexa Fluor 594; Sigma) and anti-mouse antibody conjugated with Alexa Fluor 488 were incubated with the cells on slides. Cell nuclei were stained with Hoechst 3324 fluorescent dye. Subcellular localizations of hHR23A and XPC were observed using fluorescent microscopy.

Results

Inhibition of hHR23A and hHR23B expression using RNA interference

To specifically inhibit expression of hHR23A or hHR23B, RNA interference constructs were generated. The sequences used to construct the RNAi plasmids for hHR23A and B were derived from regions that contained unique sequences. The RNAi construct against hHR23A knocked down the level of endogenous hHR23A by approximately 60% and the construct

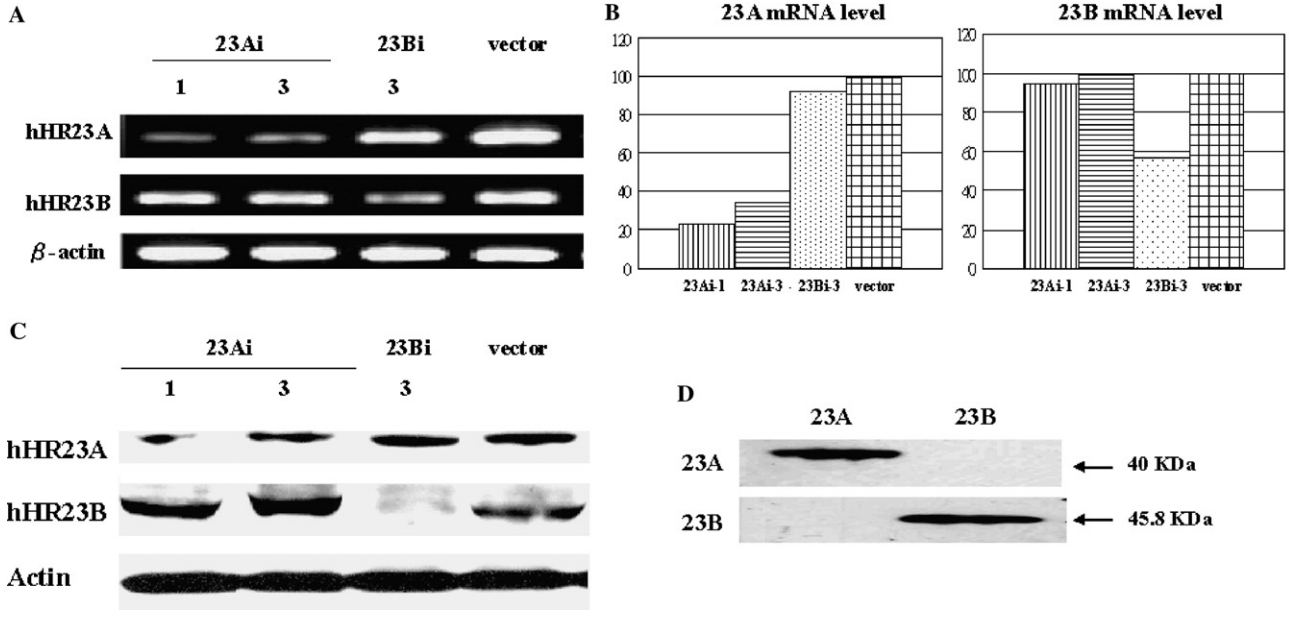

Fig. 1. Inhibition of expression levels of hHR23A and hHR23B using RNA interference. (A) RT-PCR of the hHR23A and B mRNA in HtTA1 cells transfected with hHR23A or hHR23B knock-down constructs. Vector: HtTA1 cells transfected with control plasmid pSUPER. (B) Quantification of RT-PCR products, based on DNA bands in agarose gels in (A). Amounts of DNA bands, stained with the fluorescent dye ethidium bromide, were analyzed using the Alpha Image System. (C) Western blot analysis of the hHR23A and hHR23B protein levels in $hHR23A^{KD}$ and $hHR23B^{KD}$ HtTA1 cells. (D) Specificities of the antibodies against hHR23A and hHR23B proteins. Recombinant hHR23A and hHR23B proteins in *Escherichia coli* were analyzed using anti-hHR23Aor B antibodies.

against hHR23B knocked down the level of hHR23B by 50% (RT-PCR and Western blot; Figs. 1A and B). There was no cross-inhibition between the RNAi constructs against either the hHR23A or hHR23B gene (Fig. 1C). This indicates that the specific RNA interference against hHR23A or B can be used as a model for functional study of hHR23A and B in vivo.

Inhibition of nucleotide excision repair using RNA interference against the hHR23A gene

The DNA repair activity in HtTA1 cells, in which RNA interference knocked down hHR23A or hHR23B gene expression, was examined. UV irradiation of the HtTA1 cells transfected with hHR23A or hHR23B RNAi KD constructs reduce their survival rate (Fig. 2). hHR23A^{KD} cells, although not as sensitive as hHR23B^{KD} cells to UV irradiation, were significantly more sensitive than wild-type HtTA1 cells to UV irradiation. Survival rates of hHR23A/B double KD cells were lower than for either of the single knock-down cells. These findings indicate that both hHR23B and endogenous hHR23A proteins are required for NER.

hHR23A^{KD} HtTA1 cells were then examined for the potential NER deficiency using a host-cell reactivation (HCR) assay on the cisplatin-damaged pCMV^{Luc} reporter plasmid. Cells transfected with hHR23A^{KD} and hHR23B^{KD} showed decreased DNA repair activity for recovery of cisplatin–DNA adducts (Fig. 3A). The hHR23A^{KD} construct reduced DNA repair activity by approximately 40%, and the hHR23A/B double knock-down constructs inhibited DNA repair activity more dramatically than what either of the single knock-down constructs did. To confirm these findings of the HCR assay, we used Southwestern immunoblot to analyze cells carrying the hHR23A^{KD} or hHR23B^{KD} constructs. After being transfected with hHR23A^{KD} or hHR23B^{KD} constructs, HtTA1 cells exhibited lower activities for repairing the (6,4)-photoproducts induced by the UVC irradiation, confirming that both hHR23A and hHR23B are involved in NER.

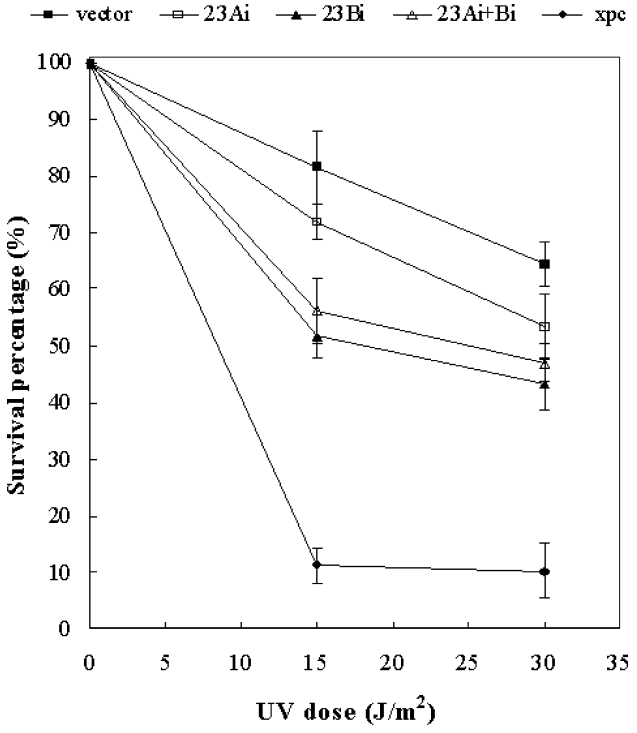

Fig. 2. Survival of hHR23A^{KD} and hHR23B^{KD} cells after UV irradiation. HtTA1 cells transfected with pSUPER plasmid (■, vector control); hHR23A^{KD} cells (□); hHR23B^{KD} cells (▲); HtTA1 cells transfected with both hHR23A^{KD} and hHR23B^{KD} constructs and (△); XPC mutant human fibroblasts (●, control cells for UVC irradiation).

HHR23A affects XPC subcellular localization after UV irradiation

Whether hHR23A is involved in regulating XPC protein was investigated using immunofluorescence analysis. In control HtTA1 cells, XPC gene expression was induced after UVC irradiation (Fig. 3C). In hHR23A^{KD} cells, however, XPC gene expression was greatly inhibited. These findings indicate that hHR23A is an important regulator of XPC in DNA repair.

Association of hHR23A with S5a and p53

The protein complex associated with hHR23A protein was detected using immunoprecipitation. A small portion of the endogenous hHR23B was associated with hHR23A, and the 26S proteasome factor S5A, previously reported [8] as associated with hHR23A and hHR23B proteins, appeared in the same complex (Fig. 4). Rpn12, an integral subunit of the 19S regulatory complex of the 26S proteasome, was not associated with hHR23A [13], however. Interestingly, p53 tumor-suppressor protein, an important activator for NER, was co-immunoprecipitated with hHR23A, suggesting that hHR23A links the signals from p53 to the other NER factors and regulates NER efficiency.

Discussion

We found that both hHR23A and hHR23B proteins regulate the induction of XPC protein by UVC irradiation and that both are required for NER. The functional involvement of hHR23A in NER is likely through the NER activator p53, because hHR23A is in the same complex as p53 protein. This complex also includes a minor portion of hHR23B. This finding indicates that the majority of hHR23B protein is in another protein complex. One study [7] reported that hHR23A and hHR23B directly interact with each other, probably through the binding of a ubiquitin-like (UbL) domain

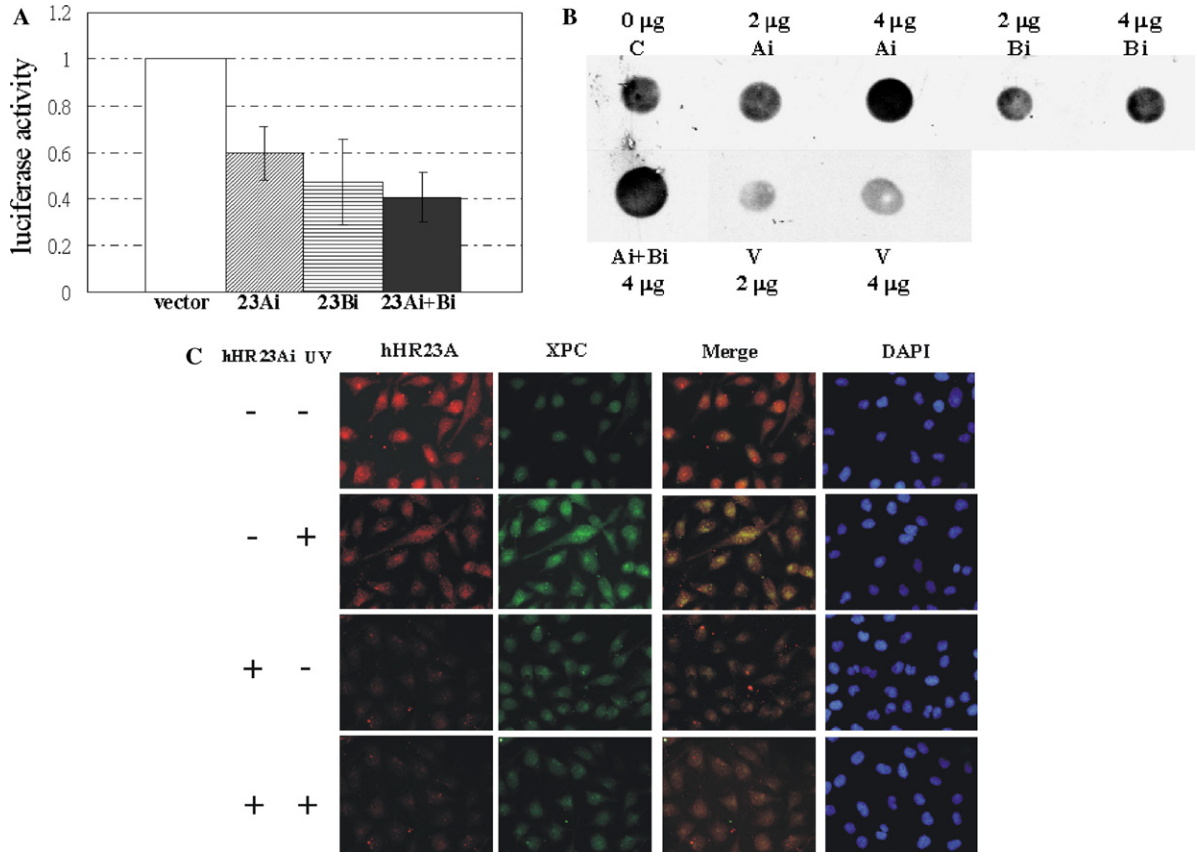

Fig. 3. hHR23A and hHR23B required for nucleotide excision repair. (A) Host cell reactivation assay on the cisplatin-damaged $pCMV^{Lux}$ plasmid. The *Y*-axis presents the luciferase activities in relative ratios to those in HtTA1 cells transfected with pSUPER (vector). 23Ai, HtTA1 cells transfected with the $hHR23A^{KD}$ construct; 23Bi, cells transfected with the $hHR23B^{KD}$ construct; 23Ai + 23Bi, cells transfected with both $hHR23A^{KD}$ and $hHR23B^{KD}$ constructs. (B) Southwestern immunoblot analysis detected removal of the 6–4 photoproducts induced by UVC irradiation. The DNA amounts of the knock-down plasmids used are indicated at the bottom of constructs. C, mock-treated control cells. V, cells transfected with indicated amounts of pSUPER. Ai + Bi/4 μg, the cells transfected with 2 μg $hHR23A^{KD}$ and 2 μg $hHR23B^{KD}$ constructs. (C) Subcellular localization of xeroderma pigmentosum C protein following UVC irradiation. The cells transfected or mock-transfected with the $hHR23A^{KD}$ construct were irradiated with 10 J/m^2 of UVC or mock-treated. hHR23A protein was visualized using immunofluorescent staining of rabbit anti-hHR23A antibody, followed with the secondary anti-rabbit antibody conjugated with the fluorescent dye Alexa Fluor 594. XPC protein was visualized using mouse anti-XPC antibody, followed with the secondary anti-mouse antibody conjugated with Alexa Fluor 488. Merge, fluorescent images of hHR23A and XPC proteins were merged to simultaneously examine their subcellular localizations. DAPI, the fluorescent staining of the cell nuclei.

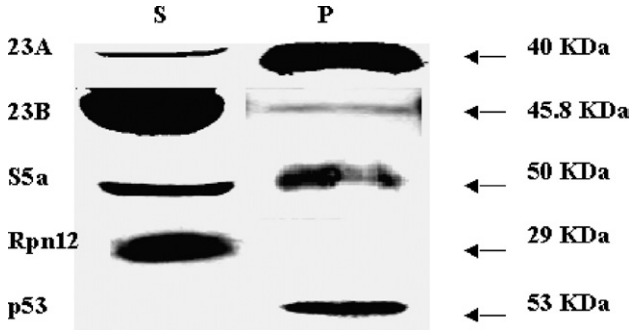

Fig. 4. Proteins associated with hHR23A using co-immunoprecipitation. S, supernatant; P, co-immunoprecipitant of the hHR23A protein. The proteins collected in the supernatant and immunoprecipitant were loaded in equal amounts in SDS–PAGE, in order to detect the relative level of each protein in the two portions.

with either one of the two ubiquitin-binding (UBA) domains in each protein [7]. Taken together with the findings in this study, this suggests that the hHR23A and B proteins are associated, the association is not essential for their functions.

We also found that the role of hHR23A in NER is regulating the induction of XPC protein following UVC irradiation, a role similar to that of hHR23B, which supports the claim of partial redundancy in another study [14]. In addition, the levels of total hHR23 proteins appear to be an important determining factor for NER activity in cells. The intracellular ratio of hHR23A to hHR23B protein is approximately 1:10 [15]. This difference presumably contributes to the following findings: (1) knock down of hHR23A protein causes mild deficiency of NER and (2) knock down of hHR23B protein causes a substantial inhibition of

NER activity. Therefore, the presence of both hHR23A and B proteins is important for efficient XPC induction in NER. It is not clear, however, why such a large amount of hHR23A and B proteins is needed for NER. Thus, based on the previous findings that the hHR23 proteins are players for multiple mechanisms including DNA repair and proteasome-mediated protein-degradation and apoptosis, it is conceivable that a large amount of hHR23A and B proteins is required to simultaneously perform these different tasks [16,17]. The multi-functionality of hHR23A and B proteins suggests that these proteins coordinate different cellular pathways that lead cells to different outcomes.

The role of the hHR23A/p53 complex remains to be elucidated. Overexpression of hHR23A and B proteins has led to the accumulation of ubiquitinated p53 and blocked p53 proteasome degradation [17]. hHR23A and B proteins downregulated the transactivating activity of p53 by interacting with the p300/cyclic AMP-responsive element binding (CREB)-binding protein [18]. These findings are consistent with ours that hHR23A is functionally linked to p53. However, the mechanism of how the hHR23A specifically affects each function that contributes to p53 remains unclear.

In addition to NER, base excision repair (BER) is associated with the XPC–hHR23B complex [19,20]. The xeroderma pigmentosum group C protein interacts physically and functionally with thymine DNA glycosylase, the enzyme that recognizes cyclobutane pyrimidine dimers (CPD) at the initial step of BER [19]. 3-Methyladenine-DNA glycosylase (MPG protein) also interacts with hHR23 proteins, suggesting that these proteins are involved in BER [20]. In this study, we found that $hHR23A^{KD}$, but not $hHR23B^{KD}$, cells, were hypersensitive to the treatment of methylmethane sulfonate (MMS), a major substrate for BER (data not shown) [1]. This suggests that hHR23A might be a player for BER, in addition to NER, coordinating the relative activities in BER and NER, the two major DNA repair pathways in cells.

In conclusion, this study clarifies the role of hHR23A protein in NER. Our data indicate that, like hHR23B, hHR23A regulates the expression levels of XPC NER factor following UVC irradiation. This is the first functional study of a human hHR23A protein. Based on this study, we believe that hHR23A protein should be categorized as a regulatory factor for NER.

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