

Potential of gene targeting in human cells by expression of *Saccharomyces cerevisiae* Rad52

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ABSTRACT

When exogenous DNA is stably introduced in mammalian cells, it is typically integrated in random positions, and only a minor fraction enters a pathway of homologous recombination (HR). The complex Rad51/Rad52 is a major player in the management of exogenous DNA in eukaryotic organisms and plays a critical role in the choice of repair system. In *Saccharomyces cerevisiae*, the pathway of choice is HR, mediated by Rad52 (ScRad52), which differs slightly from its human homologue. Here, we present an approach that utilizes ScRad52 to enhance HR in human cells containing a specific substrate for recombination. Clones of HeLa cells were produced expressing functional ScRad52. These cells showed enhanced resistance to DNA damaging treatments and revealed a different distribution of Rad51 foci (a marker of recombination complex formation). More significantly, ScRad52 expression resulted in an up to 37-fold increase in gene targeting by HR. In the same cells, random integration of exogenous DNA was significantly reduced, consistent with the view that HR and non-homologous end joining are alternative competing pathways. Expression of ScRad52 could offer a major improvement for experiments requiring gene targeting by HR, both in basic research and in gene therapy studies.

INTRODUCTION

Gene targeting by homologous recombination (HR) can be used to stably replace a chromosomal sequence with an exogenous one (1). This technique would be useful both in gene therapy, to correct a defined sequence restoring the normal function of a mutated gene, and in basic research, to introduce specific mutations for experimental purposes. The generation

of specific gene knockout mice strains by manipulation of mouse embryonic stem (ES) cells is the major example of success of this technology (2). Somatic cells are less proficient at gene targeting than ES cells, as targeting efficiency in the former is prevented by non-homologous end joining (NHEJ), which occurs at frequency 1000-fold higher than HR (1,3).

Although very little is known on how cells choose the recombination pathway to integrate an exogenous sequence, a growing body of evidence indicates that HR and NHEJ share some molecular components and can compete with each other (3). When cells are transfected, plasmid DNA is cleaved by cellular nucleases, producing free ends that mimic DNA double-strand breaks (DSBs) (4,5). Free ends are recognized and bound either by the Rad51/Rad52 complex (eventually leading to HR), or by Ku70/Ku86 [HDF1 and 2 in yeast (6)], which in mammalian cells is more abundant and results in NHEJ (7).

Several groups addressed the issue of targeting efficiency in mammalian cells by modulating the expression of recombination proteins. In cells defective for the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs), involved in NHEJ (8), gene targeting is increased, consistent with reports that in the same cells expression of the missing protein DNA-PKcs decreased spontaneous and induced HR (9). In mammalian cells deficient for Ku86 and Ku70, the rate of HR was similar to that of control cells, arguing for a role of DNA-PK complex downstream of the binding of Ku to DNA free ends (8).

In other studies, overexpression of human Rad51 and Rad52 has been used as a means to promote HR. Conflicting results have been obtained: in human fibrosarcoma HT1080 and in CHO cells, Rad51 overexpression increased the frequency of HR by 2.2-fold, whereas human Rad52 overexpression inhibited HR (10–12).

Recent experiments have demonstrated that the induction of a DSB in the target sequence, achieved by constructing a site-specific chimeric endonuclease, strongly increases the frequency of gene targeting (13,14). Although the system is

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very elegant, the necessity to construct a site-specific nuclease for every target gene constitutes a major limiting step.

We reasoned that the key to switch the balance towards HR might be found in the *Saccharomyces cerevisiae* model, where integration via HR mediated by the Rad52 epistasis group is the default fate for exogenous DNA. Yeast strains *rad52* null are defective in HR, accumulate mutations and chromosomal aberrations, and show increased sensitivity to DNA damaging agents (15,16). The *RAD52* gene product, ScRad52, seems to be the essential factor for HR, as it can promote DNA strand exchange, both dependent and independent of Rad51 (17). This capacity is characteristic of the yeast protein, whereas the mammalian homologous protein is dispensable and necessarily depends on Rad51 interaction for activity (18). Biochemically, the N-terminal DNA-binding domain of the mammalian and yeast proteins share 45% homology; the Rad51 interaction domain shares only 6% of amino acid, and there is an uncharacterised C-terminal tract present only in the yeast protein (19). Both proteins can promote strand invasion in an *in vitro* assay, but the efficiency of the yeast protein is almost 2-fold higher (20). These results indicate that human and yeast Rad52 play different roles, and that ScRad52 may contain additional functions promoting HR instead of NHEJ.

Based on these data and on our previous observation that yeast strains defective for Rad52 show a 10-fold reduction in intrachromosomal recombination (16), we reasoned that overexpression of the yeast Rad52 could possibly induce an increase in the HR rate in human cells. To this purpose, we derived several HeLaS3 clones expressing ScRad52 and carrying a new genetic system, based on hygromycin resistance (21), to detect gene targeting events.

The results confirm that the expression of ScRad52 in human cells significantly increases the frequency of HR.

MATERIALS AND METHODS

Plasmid DNAs

The recombination plasmids were constructed from pTPSN (from R. Michael Liskay, Oregon Health Science University, Portland, OR), which contains two copies of hygromycin resistance gene (*hyg*), one (*hyg1*) inactivated by an insertion of 10 bp generating a HindIII site at the unique PvuI site and the other (*hyg2*) inactivated in the same way at the SacII site (21,22). Plasmid pHygB⁻Neo, the HR substrate with the *hyg1* mutation, was obtained by pTPSN digestion with BamHI and re-ligation (Figure 1). Plasmid pHygB⁻, the targeting construct with the *hyg2* mutation, was made by cloning the 2.2 kb BamHI fragment, containing the SacII insertion, from plasmid pTPSN into the BamHI site of Litmus 28 (New England Biolabs).

The pMSGY52 plasmid containing the ScRad52 gene under control of mouse mammary tumour virus long terminal repeats was kindly provided by Colin Campbell, Minnesota Medical School, Minneapolis, MN (23).

The plasmid pBlue-puro (a kind gift from R. Kanaar, Erasmus University, Rotterdam, NL) contains the puromycin resistance gene driven by cytomegalovirus promoter. It has no homology with the genome of HeLaS3 cells; therefore, it can integrate randomly.

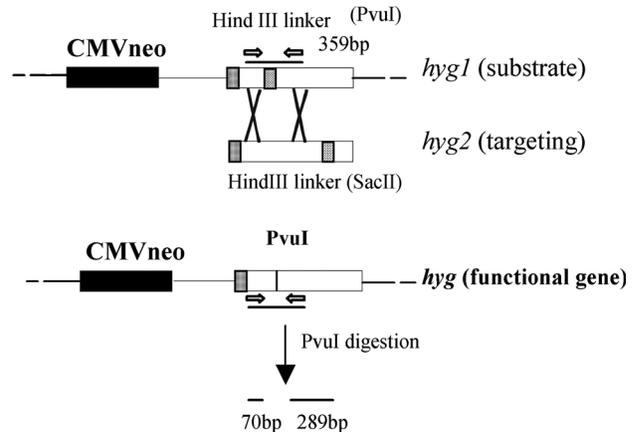


Figure 1. Genetic system for the detection of HR. In the upper part of the figure, the top line represents the plasmid used to deliver the *hyg1* defective gene to the genome. It contains a selectable marker (CMVneo) and has a 10 bp insertion at the PvuI site of the *hyg* gene. The targeting DNA fragment *hyg2* contains the same insertion at the SacII site. An event of HR (represented by the two couples of crossing lines) determines the restoration of an active *hyg* gene as represented in the bottom line. Horizontal arrows indicate the primers used to amplify a fragment of 359 bp which, after recombination, contains the PvuI diagnostic site.

Construction of cell strains

Plasmid pHygB⁻Neo and plasmids pMSGY52–pHygB⁻Neo were transfected in HeLaS3 cells. Transfection by electroporation was carried out as follows: 3.5×10^6 cells were suspended in 250 μ l DMEM, without serum and antibiotics, and mixed with 10 μ g DNA (pMSGY52, pHygB⁻Neo or pHygB⁻). Cells were transferred to 1 ml cuvette (Equibio), incubated for 10 min on ice, electroporated at 330 V/cm, 1000 μ F and 200 Ω with Electroporator II (Invitrogen) connected to a power supply (330 V, 25 mA, 25 W), incubated again on ice for 10 min and seeded on 10 cm dishes in complete medium. Transfection was considered successful when cell survival approached 50%. G418 (1 mg/ml) was added 24 h later and resistant colonies were isolated and expanded. Clones named HeLa.1B, HeLa.B2, HeLa.B5, HeLa.2A and HeLa.4A were used in the experiments of HR.

Plasmid pMSGY52 was co-transfected with plasmid pBlue-puro in clone HeLa.1B by electroporation. Cells were seeded on 10 cm dishes in complete medium. Puromycin (0.2 μ g/ml) was added 24 h later and resistant colonies were isolated and expanded. Clones named HeLa.1B-C and HeLa.1B-P were used in the experiments of HR.

Detection of ScRad52 expression by RT-PCR

G418^R clones were characterized for ScRad52 expression by RT-PCR. Total RNA from 2×10^6 cells was extracted with RNeasy Mini KIT (Qiagen), quantified by ultraviolet (UV) absorbance at 260 nm and treated with DNase I (Invitrogen). An aliquot of 1 μ g of total DNA-free RNA was reverse transcribed using SuperscriptIITM (Invitrogen) and subjected to PCR. Amplification of ScRad52 cDNA required a semi-nested PCR to allow successful detection. In the first round, the forward primer 5'-TTGCCAAGAACTGCTGAA-3' (S1) and the reverse primer 5'-GGAATATGCTTGGACGTA-3', specific for ScRad52 (23), were used to amplify a region of

1100 bp. An aliquot of 1 μ l of the first PCR product was subjected to a second round of PCR using primers S1 and y1779 (5'-CTTTATTACTGCTGTTTTACTGTTAG-3') to amplify a region of 500 bp.

MMS assay, X-irradiation and short interfering RNA

ScRad52 expressing cells (3×10^3 cells/well) were seeded in 96 multi-well plate. After 24 h, cells were incubated for 60 min with 1, 2 or 3 mM methyl methanesulfonate (MMS, Sigma). Cell proliferation at various MMS concentrations was determined by the proliferating cell assay (Promega) at the end of 1 h treatment and after 24 h. Values from the assay were used to calculate the proliferation curve, using GraphPad Prism v. 3.03.

For survival assay after X-irradiation, 200 cells were seeded in 30 mm dishes and irradiated 36 h later at the doses of 1.25, 2.5, 5 and 7.5 Gy using a Siemens Stabilipan irradiator (2 Gy/min, 18 mA and 200 kV). Cells were kept for further 10 days in culture and stained for colony counting.

ScRad52 was silenced using short interfering RNAs (siRNAs) (24). Briefly, a fragment of 700 bp located in the coding region of the ScRad52 gene was amplified using primers 5'-TCTGGTGGCCTTTGGTGTG-3' and 5'-CTTTATTACTGCTGTTTTACTGTTAG-3', both preceded at 5' ends by 23 nt (TAATACGACTCACTATAGGGAGA) encoding the T7 RNA polymerase promoter to allow *in vitro* transcription with the TurboScript T7 Transcription Kit (Gene Therapy System). The double-stranded RNA obtained was treated with recombinant Dicer enzyme to generate diced-siRNAs (d-siRNAs) according to the manufacturer's recommendations. Purification was performed using d-siRNA Purification Columns and quantified by UV light absorbance. d-siRNAs against enhanced green fluorescent protein (EGFP) unrelated gene were used as control. To silence ScRad52 gene, 4×10^5 expressing cells were seeded in 30 mm dishes and the day after were transfected with 2 μ g/dish d-siRNAs against ScRad52 or EGFP using Gene Silencer reagent (Gene Therapy System). After 24 h, cultures were trypsinized and seeded in 96 multi-well plate for MMS experiment, or on cover-slips for the Rad51 immuno-cytochemistry assay.

Determination of HR frequencies

HeLa.1B, HeLa.2A, HeLa.4A, HeLa.1B-C and HeLa.1B-P clones were electroporated as described above with the targeting plasmid pHygB⁻, collected and seeded in complete medium. After 24 h, hygromycin (300 μ g/ml) was added to the dishes. Selective medium was changed twice and after 15 days dishes were stained with crystal violet and colonies counted. The frequency of HR was calculated by dividing the total number of hygromycin-resistant colonies by the number of viable cells seeded. Cell viability was measured by scoring the number of colonies formed after seeding 200 cells in 60 mm dishes in non-selective medium, followed by 7–10 days incubation.

To demonstrate the occurrence of HR, genomic DNA of independent hygromycin-resistant clones was extracted and purified. A 359 bp fragment surrounding the 10 bp linker insertion at the PvuI site of the *hyg1* mutant gene was amplified by PCR with primers 5'-TGATGCAGCTCTCGGAGG-3' and 5'-AGTGTATTGACCGATTCCTTG-3'. PCR products

were purified, checked for the presence of PvuI diagnostic site by overnight digestion and analysed on a standard agarose gel. The same PCR product was used for the sequencing of selected clones.

Southern blot analysis was performed on DNA extracted from parental and recombinant clones, according to the standard procedures. Briefly, DNA was extracted by ethanol precipitation after Proteinase K treatment, resuspended overnight in 1 M Tris-HCl, pH 8.0, and digested sequentially with HindIII and BamHI. Digested DNA was separated on 0.8 % agarose gel, blotted onto positively charged nylon membrane and hybridized with Digoxigenin-labelled probe (Boehringer Mannheim, DIG labelling kit). The probe fragment covers the entire 2.2 kb hygromycin resistance gene.

Specific PCR to detect targeting events was performed using a pair of primers specific for the recombination product: primer 1 (5'-GATCGGCCGAGCGATCG-3') sits on the PvuI site (CGATCG) and primer 2 was 5'-TGATGCAGC-TCTCGGAGG-3' in the Hyg coding region. The PCR was performed using *Taq* polymerase (Invitrogen) that does not have a 3'→5' exonucleolytic activity.

Determination of non-homologous (random) integration frequencies

Frequency of non-homologous integration was determined by transfecting 2 μ g of pBlue-puro vector in HeLa clones (4×10^5 cells each) containing (HeLa.B2, HeLa.B5, HeLa.2A and HeLa.4A) or not (Hela.1B) ScRad52. Transfections were carried out with Polyfect transfectant (Qiagen) according to the manufacturer's recommendations. One day after transfection, cells were collected and plated (2×10^5 cells/dish) in 10 cm dishes containing 0.2 μ g/ml puromycin. Culture medium was changed after 7 days and replaced with puromycin-free fresh medium. The colonies were stained and counted 7 days later and the frequency of recombination was calculated by dividing the number of puro^R colonies by the number of viable cells seeded.

X-ray treatment and visualization of Rad51 nuclear foci

X-ray treatment for immuno-histochemistry was performed as follows: 4×10^5 cells were seeded in 30 mm dishes containing two 12 mm round cover-glasses. Dishes were irradiated (10 Gy) and after 6 h treated and control cells were fixed with paraformaldehyde 2% in phosphate-buffered saline (PBS) for 15 min at room temperature.

Rad51 foci were detected following a protocol by Roland Kanaar (25): cover-slips were washed with 0.1% Triton in PBS, incubated for 1 h with rabbit anti-human Rad51 serum (from R. Kanaar, Erasmus University, Rotterdam, NL) diluted 1:5000 in blocking buffer (0.15% glycine and 0.5% BSA in PBS), washed again and incubated with secondary antibody conjugated with Alexa-fluor 594 (Molecular Probes).

RESULTS

Experimental system and characterization of the ScRad52 expressing clones

The selection strategy developed to detect and quantify recombination events is based on two DNA elements each containing an inactive form of the gene conferring hygromycin

resistance (Figure 1). The fragment pHygB⁻Neo (target gene) contains, downstream of the Thymidine Kinase promoter, a hyg gene with a 10 bp insertion in the PvuI site, together with a neomycin phosphotransferase expression cassette (CMVneo); individual cellular clones with the fragment integrated in the genome are obtained through G418 selection. The plasmid HygB⁻ (the targeting fragment) contains a hyg gene with an intact PvuI site and a 10 bp insertion in a different location. The linearized targeting plasmid is delivered to the cells by electroporation. If a recombination event takes place, the resulting chromosomal gene will be functional (allowing for selection) and will contain the diagnostic PvuI site. This transfection set-up represents the genetic system used to test the hypothesis that ScRad52 affects the rate of HR.

HeLaS3 cells were transfected with pHygB⁻Neo, pMSGY52, or both, and several single cell clones were selected and characterized. Using Southern blot analysis, one copy of the hyg1 gene was found in clones HeLa.1B and HeLa.2A, and three or more copies in clone HeLa.4A (data not shown). Expression of the transgene was assessed on RNA extracted from individual clones by RT-PCR (or PCR as control, data not shown). All clones, except 1B, expressed ScRad52 (Figure 2A). Observation of cell cycle distribution, as measured by Propidium Iodide incorporation and FACS analysis, showed no interference of ScRad52 with progression of the cell cycle in all clones (data not shown).

The presence of ScRad52 in human cells can enhance the efficiency of DNA repair after treatment with DNA damaging agents (23,26,27), such as MMS. Therefore, we tested the rate of cell proliferation after exposure to 1, 2 or 3 mM MMS for 1 h. Figure 2B shows that all clones expressing ScRad52 were more resistant than the control clone. The possibility of a non-specific effect due to the insertion of ScRad52 was ruled out in the experiment shown in Figure 2C and D. The four clones containing the transgene were pooled and ScRad52 expression was silenced using siRNA/DICER technology (24). Figure 2C shows that ScRad52 RNA was reduced to undetectable level, and Figure 2D reports that loss of Rad52 expression restores the original level of sensitivity to DNA damage.

Further confirmation of increased resistance of ScRad52 expressing cells to DSB was obtained by measuring cell survival following X-irradiation. As shown in Figure 2E, clones expressing ScRad52 show increased survival at all X-ray tested doses.

Therefore, we have obtained a robust experimental set-up in which it is possible to assess the role of a functional ScRad52 in a human genetic background.

Gene targeting is increased by ScRad52 expression

As described in the Introduction, when exogenous DNA is transfected in human cells NHEJ occurs up to 1000 times more efficiently than HR (4). To investigate whether the expression of ScRad52 could enhance HR, we compared ScRad52 positive and negative clones. Cells were electroporated with the hyg2 fragment and clones were selected in medium supplemented with hygromycin as described in Materials and Methods.

The results in Figure 3A show that in the ScRad52 expressing clone HeLa.2A, the frequency of HR was 15-fold higher

than in the control clone HeLa.1B. Interestingly, clone HeLa.4A that contains more copies of the substrate showed even higher (37-fold) gene targeting frequency. This represents a remarkable efficiency in this system, especially when compared with the effect of the expression of other components of the DNA repair pathway (see Discussion).

In cells with an inactive copy of the gene hyg1, resistance to hygromycin can be acquired, under appropriate selection conditions, by a spontaneous mechanism of gene rearrangement in the absence of a targeting event. If a targeting fragment is present, the resistant phenotype is more efficiently acquired due to recombination via gene targeting. In our system, the two possibilities can be resolved, because only in true recombinants the hyg locus acquires PvuI sensitivity. Therefore, we extracted genomic DNA from resistant clones and performed a PCR followed by PvuI digestion as described in Materials and Methods. In control cells (Figure 3B, HeLa.1B), three clones out of the four are true recombinants, as demonstrated by loss of the 359 bp band after digestion.

For analysis of cells expressing ScRad52, sub-clones derived from clone HeLa.4A were selected because of the higher recombination rate. The result of PCR and PvuI digestion (Figure 3C) indicates that clones 2, 3, 4, 6 and 8 underwent gene targeting, as confirmed by the presence of the 289 bp fragment. However, DNA derived from these recombinant clones was only partially digested; this suggests that only one of the integrated substrates needs to be corrected to confer hygromycin resistance. The PCR results (Figure 3C) indicate that some of the clones acquire hygromycin resistance yet do not appear to have PvuI-sensitive DNA (clones 5, 7 and 9). To address this point, we also performed Southern blot analysis on clones 2, 3, 5, 7 and 9, as shown in Figure 4. Genomic DNA extracted from the parental and recombinant clones was digested with BamHI, which cuts outside of the target sequence hyg1, and with HindIII. The Hyg1 sequence integrated in the genome is sensitive to HindIII, but after successful targeting the HindIII site is replaced by PvuI site, and a gene targeting event is revealed by the loss of HindIII site.

DNA from the parental clone HeLa.4A shows at least six discrete bands, confirming a minimum of three integrated substrate fragments. All other clones show a lower number of bands, which result from the loss of the HindIII site after HR. Lack of PvuI sensitivity suggested in the PCR experiment (Figure 3C) could be explained by the relative abundance of uncorrected substrate and the amplification dynamics of PCR, which is strongly skewed towards the most represented substrate. To further confirm this point, we also performed a PCR using a different primer (see Materials and Methods), located over the PvuI site as illustrated in the scheme at the bottom of Figure 4. DNA from all recombinant clones was positive, indicating the presence of at least one HR product (Figure 4B). The locations of the target sequence in HeLa.1B, HeLa.2A and HeLa.4A clones are unlikely to be the same since they represent different events of random integration, which may undermine the clarity of results. We, therefore, transfected ScRad52 into HeLa.1B, which already contains the recombination substrate. Puromycin-resistant clones were submitted to RT-PCR analysis to check the expression of ScRad52 and to X-rays treatment to check their ability to repair DSBs. Since two clones, HeLa.1B-C and HeLa.1B-P, showed a transcriptionally active ScRad52 sequence

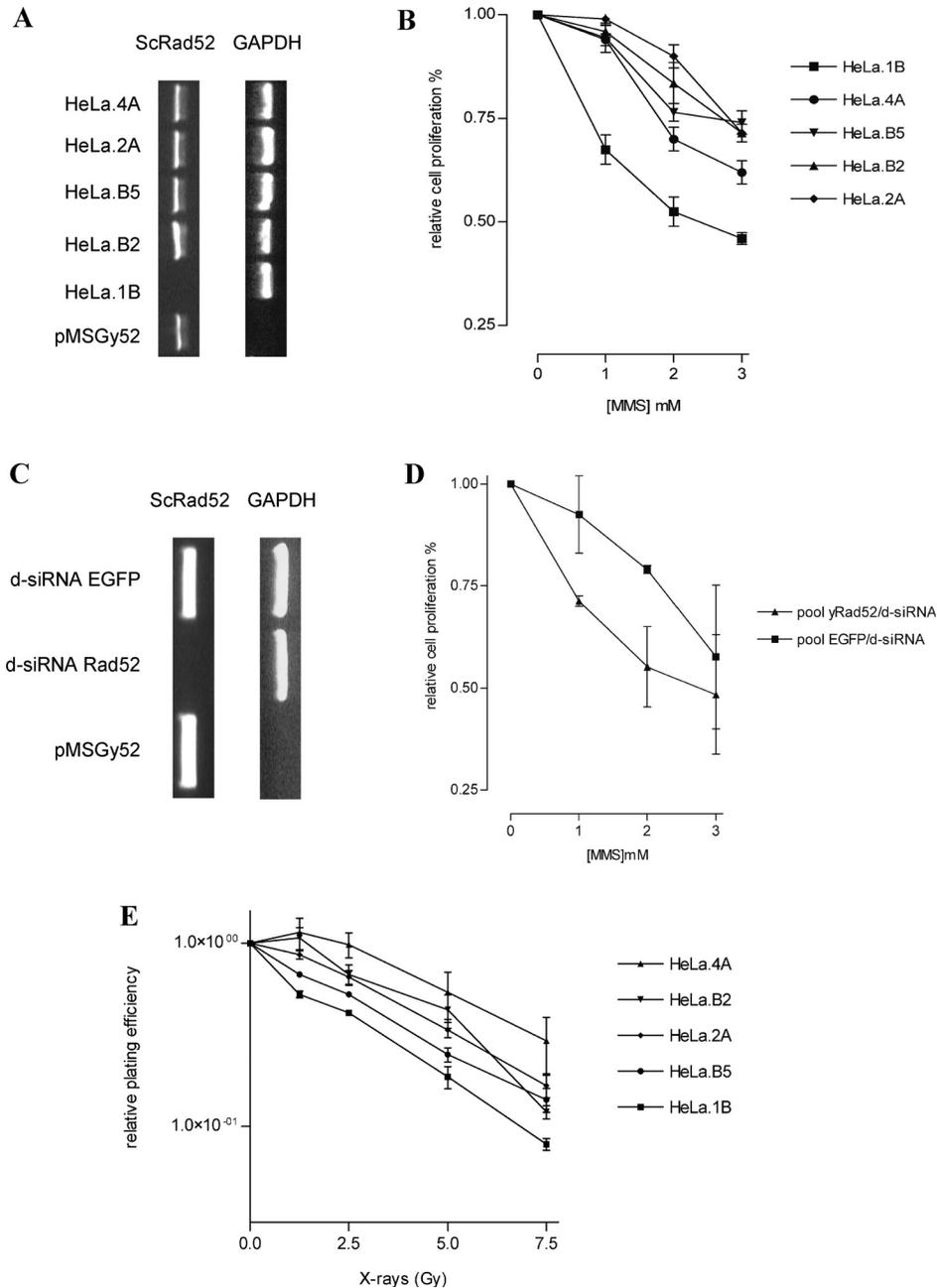


Figure 2. Characterization of HeLaS3 clones expressing ScRad52. (A) Detection of ScRad52 expression by semi-nested RT-PCR. All selected clones, except HeLa.1B, express the transgene. pMSGY52 DNA was used for PCR marker and GAPDH was used as RT-PCR control. (B) MMS sensitivity curves. Cells were treated for 1 h with 1, 2 or 3 mM MMS, and proliferation was measured at the end of treatment and after 24 h. Each point represents the ratio between the measures at times 1 h and at 24 h and reports the mean value \pm SD of six experiments. (C) Silencing of ScRad52 by d-siRNA. The pool of all ScRad52 expressing clones was transfected with 2 μ g of ScRad52/d-siRNA or EGFP/d-siRNA; RNA was extracted and used for RT-PCR as above. (D) MMS sensitivity in ScRad52 positive cells transfected with d-siRNAs. The plot shows the relative cell proliferation after treatment with MMS. Each point represents the mean value \pm SD of at least four independent experiments; in some cases the SD is smaller than the printed symbol. (E) Cellular survival after X-irradiation. Cells were irradiated at the indicated dosages and colonies were stained and counted after 10 days. Relative survival was calculated by dividing the number of colonies by the number of seeded cells. Each point represents the mean value \pm SD of three independent experiments performed in triplicate and is shown on a logarithmic scale.

(Figure 5A) that conferred an enhanced resistance to X-rays (Figure 5B), they were used to detect the true contribution of ScRad52 to HR. The results are reported in Table 1. HeLa.1B-C and HeLa.1B-P clones showed a frequency of HR that was 12- and 11-fold greater than control clone HeLa.1B, thus demonstrating that the expression of ScRad52 is sufficient to potentiate the gene targeting in human cells.

ScRad52 decreases NHEJ

In human cells, the introduction of exogenous DNA can mimic an instance of DNA damage (4). The generally accepted hypothesis argues that free DNA ends can be recruited by proteins that belong to either of the two alternative pathways leading to HR or NHEJ (3). To verify that the observed

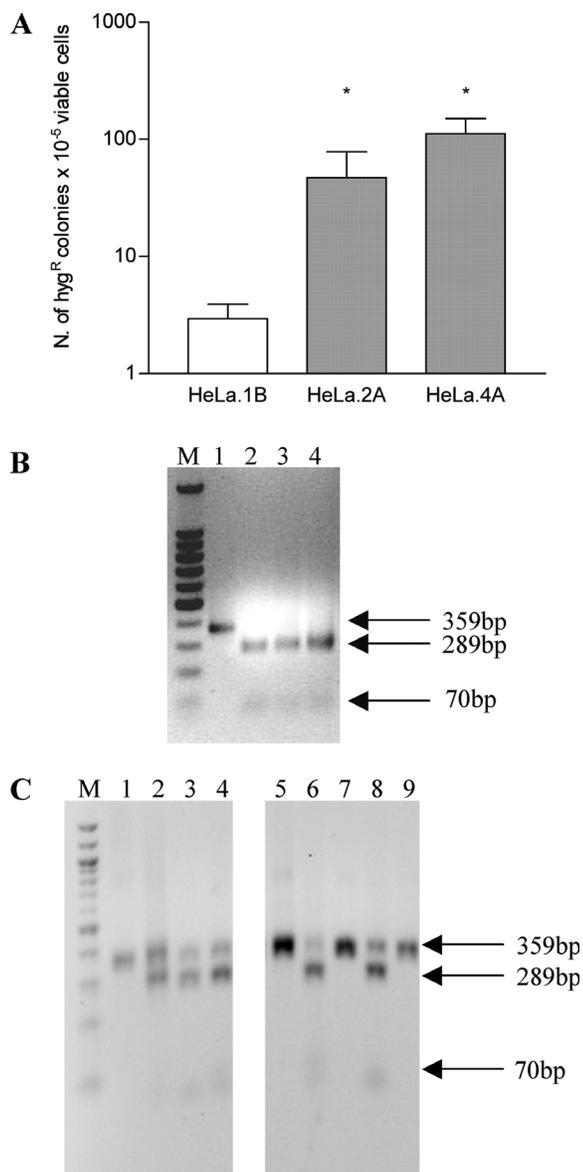


Figure 3. Effect of ScRad52 expression. (A) Frequency of HR. HR was determined following electroporation of the *hyg2* targeting plasmid and selection of hygromycin resistant colonies. Results are reported in logarithmic scale as mean value \pm SD of three independent experiments. Asterisks indicate that the difference from the control is significant ($P \leq 0.05$). (B) DNA analysis of clones recombined in the absence of ScRad52 (clone HeLa.1B). PCR was performed on genomic DNA from single hygromycin-resistant colonies and followed by PvuI digestion. The size of the fragments is shown on the right. M, 100 bp marker; lane 1, false recombinant (PvuI resistant); lanes 2–4, true recombinants (PvuI sensitive). (C) Analysis of clones derived from HeLa.4A. M, 100 bp marker; lane 1, non-digested PCR product; lanes 2–9, PvuI-digested PCR products. Note that undigested DNA is present even when recombination has occurred, as shown by partial PvuI sensitivity.

increase in HR was due to a preferential engagement of HR proteins rather than NHEJ, we measured the efficiency of random integration in cells with or without ScRad52 expression. We transfected HeLa clones with pBlue-Puro plasmid. The ScRad52 expressing clones HeLa.B2, HeLa.B5, HeLa.2A and HeLa.4A showed a reduction in the number of resistant clones obtained down to approximately one-third with respect to the control clone HeLa.1B (Figure 6). This reduction was

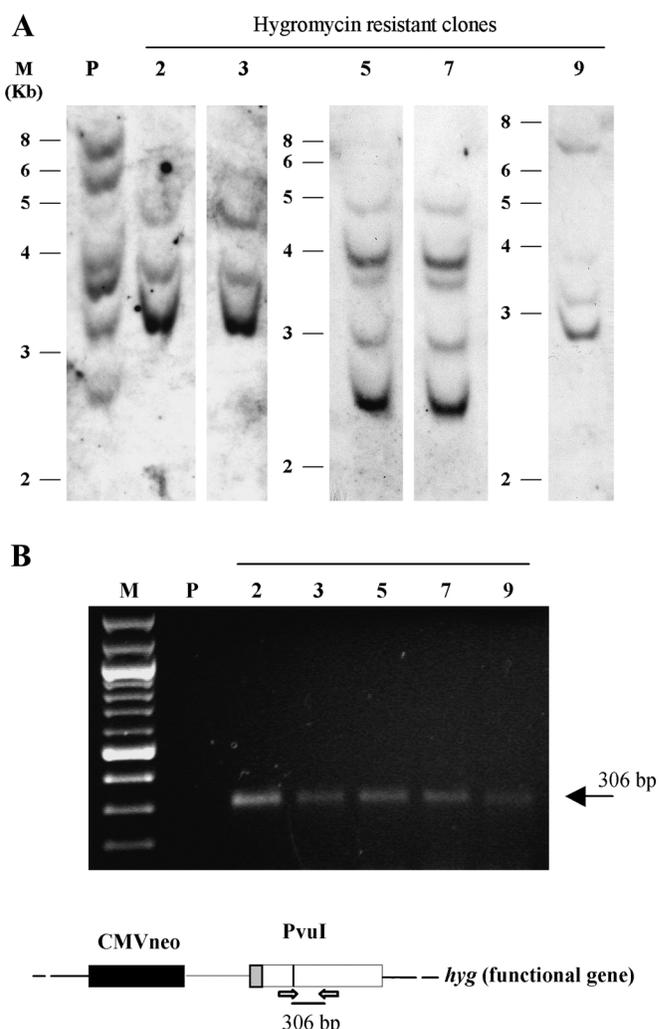


Figure 4. Molecular analysis of hygromycin-resistant clones. (A) Southern blot of recombinants. DNA from indicated clones was digested with HindIII and BamHI to reveal the number of corrections on the integrated substrates (see Figure 1). P, in the first lane, represents the parental clone HeLa.4A, which shows at least six bands, resulting from at least three copies of substrate. Clones 2 and 3 show half the number of bands, while clones 5, 7 and 9 (refractory to PvuI digestion), >4 bands each, indicating that at least one copy was targeted. (B) Recombination-specific PCR. DNA was subject to PCR using specific primers able to anneal on the PvuI site, only available after correction, as illustrated in the scheme. The expected PCR fragment of 306 bp was obtained from recombinant clones, but not from the parental.

significant ($P < 0.05$) indicating that ScRad52 inhibited random integration.

ScRad52 decreases Rad51 nuclear foci

It is well established that after DNA damage the endogenous mammalian Rad51 protein, a major player in HR, re-locates in distinct nuclear foci considered centres of molecular repair (25,28,29). DNA-free ends are first recognized by Rad51 together with its partner Rad52, leading to nuclear foci formation. These large multimolecular structures contain numerous other proteins rather than Rad51 and Rad52, including Rad50, Mre11, Nbs1 and PCNA. It has also been demonstrated that Rad52 and Rad51 proteins physically interact in the

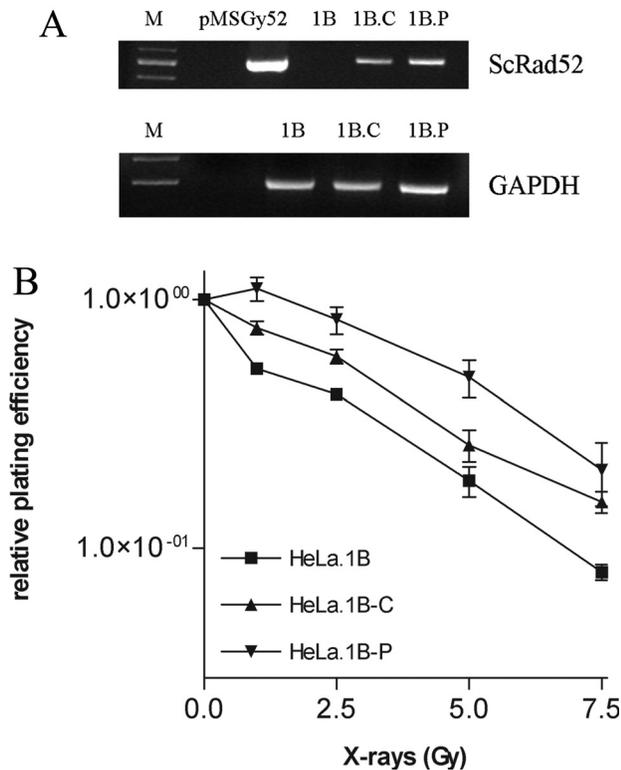


Figure 5. Characterization of clones derived from HeLa1B and expressing ScRad52. (A) Detection of ScRad52 expression by semi-nested RT-PCR. All selected clones, except parental HeLa.1B, express the transgene. pMSGY52 DNA was used for PCR marker and GAPDH was used as RT-PCR control. (B) Cell survival after X-irradiation. Cells were irradiated at the indicated dosages and colonies were stained and counted after 10 days. Relative survival was calculated by dividing the number of colonies by the number of seeded cells. Each point represents the mean value \pm SD of three independent experiments performed in triplicate and is shown on a logarithmic scale.

Table 1. Effect of ScRad52 on HR frequencies ($\times 10^{-5}$ viable cells)

Clones	Survival (%)	hyg ^R colonies	n-Fold stimulation
HeLa.1B	65.3 \pm 5.48	2.4 \pm 1.2	1
HeLa.1B-C	46.3 \pm 15.32	29.0 \pm 13.1*	12
HeLa.1B-P	49.0 \pm 11.4	26.7 \pm 8.6**	11

Results are reported as the mean of three or more experiments \pm SD.

* $P < 0.05$;

** $P < 0.01$.

two-hybrid system, co-immunoprecipitate and form stoichiometric complexes (30,31).

Recent reports, however, indicate that Rad52 (both human and yeast) can initiate a strand exchange reaction *in vitro* in the absence of Rad51 (20). If this is the case also in the nuclear environment *in vivo*, it should be possible to observe a different nuclear foci dynamics after DNA damaging treatments in the presence or absence of exogenous Rad52.

Cells expressing ScRad52 and control cells were exposed to X-rays (10 Gy) and fixed after 6 h. Rad51 foci were identified by indirect immuno-fluorescence as shown in Figure 7A and counted (Figure 7B). A reduction in the number of Rad51 foci due to ScRad52 expression was evident in both exposed and unexposed cells, indicating an effect of the protein prior to

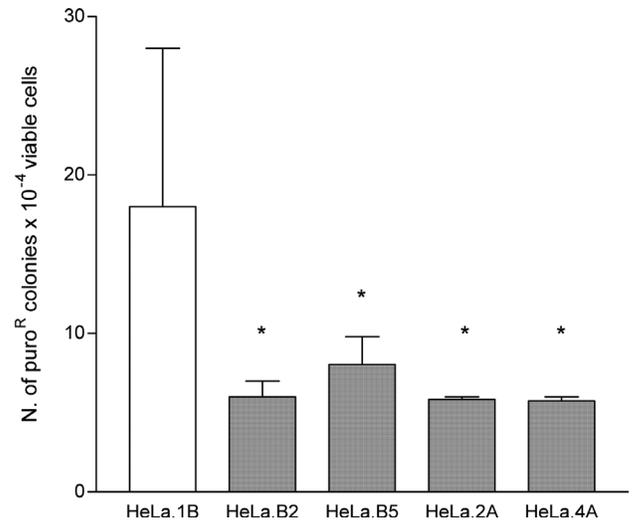


Figure 6. Effect of ScRad52 on non-homologous (random) integration. NHEJ was measured after transfection of pBlue-Puro vector and selection of colonies by puromycin resistance as described in Materials and Methods. Results are the mean value \pm SD of four independent experiments. Asterisks indicate significant differences from the control (HeLa.1B) ($P \leq 0.05$), calculated with Student's *t*-test.

DNA damage. This prompted us to investigate the number of nuclear foci in each clone of cells expressing ScRad52 in the absence of any treatment. Figure 7C reports the number of Rad51 foci per nucleus: the reduction is significant in each of the expressing clones. To exclude the possibility of a general decrease of Rad51 protein, we quantified the amount of Rad51 by western blot of cells expressing ScRad52. The results (data not shown) demonstrated that the amount of protein was comparable.

Silencing of ScRad52 by DICER/siRNA in clone HeLa.2A and in the pool of all clones demonstrated that the effect is specifically due to the presence of ScRad52, as shown in Figure 7D.

In previous studies (29), it was observed that overexpression of murine Rad52 in primary murine spleen cells induced an increase in the number of Rad51 foci. The opposite effect that we have obtained suggests that the yeast protein carries additional and/or different functions, as further discussed below.

DISCUSSION

Unlike mammalian cells, in *S.cerevisiae* DSB repair by HR is strongly preferred over NHEJ (4,32). A major component of the yeast recombination mechanism is Rad52 (15,19), which is responsible for homologous pairing and strand exchange, leading to the integration of an exogenous DNA fragment in the target chromosomal locus. *In vitro*, the yeast protein has been demonstrated to be more efficient in the strand exchange reaction than its human homologue, a property that could be associated with additional domains present in ScRad52 (20).

To test whether the protein alone can enhance HR in mammalian cells, we developed a system to quantify HR in human HeLa cells. A mutated hygromycin resistance gene (hyg1) was first inserted as single or multiple copies in the genomic DNA of HeLa cells; in order to measure HR, these cells were then transfected with a linear hyg2 fragment containing a different

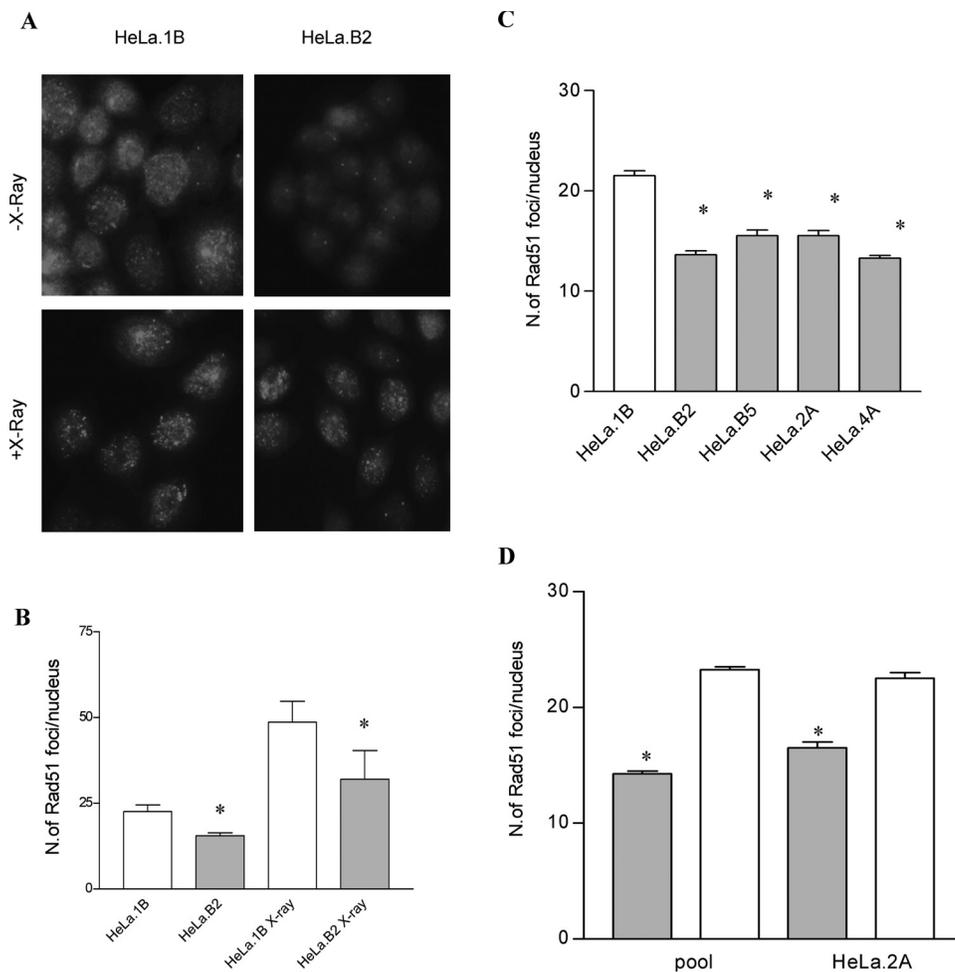


Figure 7. Effect of ScRad52 on Rad51 nuclear foci in negative and positive clones. (A) Effect of ScRad52 on X-ray-induced Rad51 nuclear foci. Rad51 nuclear foci were detected by immuno-fluorescence before (top) and after (bottom) X-ray exposure in cells expressing (right) or not (left) ScRad52. (B) Quantification of Rad51 foci per nucleus before and after X-ray exposure. Foci of 50 nuclei per clone were counted and are represented as mean value \pm SD of three independent experiments: cells expressing yeast Rad52 (grey bars) have a reduced number of foci, both before (left) and after (right) X-ray treatment. (C) Number of Rad51 foci in single ScRad52 expressing clones. Spontaneous Rad51 nuclear foci were counted after immuno-cytochemistry in negative and positive clones. Results are reported as the mean \pm SD of five experiments. Asterisks indicate significant differences from the control (HeLa.1B) ($P \leq 0.05$). (D) Silencing of the transgene restores the spontaneous number of Rad51 foci. Cells [pool of all clones (left) or single clone (right)] were transfected (white bars) with d-siRNA to silence exogenous Rad52, and foci were counted after immuno-staining. Treated cells show a number of foci comparable with wild type.

mutation. The frequency of HR was $2.95 \pm 0.96 \times 10^{-5}$ viable cells, in agreement with HR frequency of other cell lines ranging from 10^{-5} to 10^{-7} . Therefore, the system is a reliable tool to measure HR in human cells and is suitable to evaluate the effect of ScRad52 and other proteins of interest.

HR in cells is dependent on the interaction of several factors: the amount of target, which in natural condition would be one or two copies (for dominant and recessive characters, respectively), the amount of targeting construct delivered, the efficiency of the HR machinery and other factors, such as homology length (33,34) and cell cycle phase (35,36). In the HeLa system used in this study, we only addressed the effect of the presence of a single factor, namely ScRad52. To this end, we considered both a clone with one substrate only (HeLa.2A) and a clone with multiple copies (HeLa.4A).

We first tested the effect of ScRad52 on survival after both MMS and X-ray-induced damage, and show that its presence induces a more efficient recovery. This is consistent with the results by Johnson *et al.*, (23), who also observed a higher

efficiency of interplasmidic recombination upon overexpression of ScRad52 in human fibroblasts.

In all clones expressing ScRad52, the frequency of HR was between 11- and 37-fold the frequency of control cells as shown in Figures 3 and 4 and Table 1. The Southern blot analysis demonstrated that even in clones apparently negative in the PCR/PvuI digestion assay (Figure 3C), at least one copy of substrate is converted by HR (Figure 4A). A further confirmation of the correction was obtained by specific PCR in Figure 4B. Therefore, ScRad52 induce a genuine increase of HR. This represents a very remarkable effect, particularly when compared with the 2- to 3-fold stimulation obtained by overexpression of human Rad51 in human cells (12).

In order to assess the effect on NHEJ, we used a construct with no sequence homology with chromosomal DNA. When cells expressing ScRad52 were tested for random integration efficiency, we found that they had an NHEJ frequency reduced to one-third relative to the control line, supporting the hypothesis that ScRad52 interferes with NHEJ and actively

promotes HR. Our findings are compatible with a mechanism in which ScRad52 binds the free DNA ends, and in the absence of homology keeps them from recruiting components of the NHEJ pathway. In this view, our data represent a further support for the interactive competition model as discussed below.

The analysis of Rad51 nuclear foci formation added insights into ScRad52 function. Surprisingly, in cells expressing ScRad52, the number of Rad51 foci was reduced (but not the protein amount), yet DNA repair was not affected, as indicated in results shown in Figure 2. This suggests that ScRad52 performs HR more efficiently, despite the displacement of Rad51 and that ScRad52 could work in a Rad51-independent way establishing a yeast-like nuclear dynamics in human cells.

The leading view of recombination proposes that the two pathways, homologous and non-homologous, while sharing some components, diverge and compete with one another (3). The effort to increase the relative efficiency of HR has seen several groups engaged in studies of overexpression and repression of proteins involved in recombination and DSB repair.

For example, Kim *et al.* (10) overexpressed human Rad51 and Rad52 in human and hamster cells to find out that the two genes, singly or in combination, caused a reduction, rather than an increase, of the frequency of intrachromosomal HR after DSB. Significant inhibition of gene targeting in HT1080 human fibroblasts after overexpression of human Rad52 protein was also reported by Yanez and Porter (11), who found that in their system, random integration was slightly increased. In contrast, the expression of ScRad52 in human cells induced a 12-fold increase in inter-plasmid HR (23). In a study involving Rad52 from the yeast *Kluyveromyces lactis*, Milne and Weaver (40) show that its overexpression in a RAD52 wt strain of *S.cerevisiae* confers a dominant negative phenotype, probably due to the strict homology among the two proteins, which would interact in a less active hybrid form. From our data, it seems likely that interaction among the human and yeast proteins either does not occur (due to the very low homology), or is overwhelmed by the large amount of the exogenous protein produced.

From the literature and our data, we conclude that ScRad52 is more effective than the human protein in promoting HR, and at the same time is able to inhibit random integration.

Gene targeting events resemble to DNA repair after DSB induction. It is common knowledge [for examples see (32,37)] that in mammalian cells Rad52 binds single-strand and double-strand DNA ends, recruits Rad51 and carry out HR. The location of this event is identified with nuclear foci that are seen as Rad51 positive spots. In these foci, several components of the recombination machinery (RPA, BRCA1 and 2, Rad50, 52 and 54 and others) were demonstrated to converge in a dynamic series of interactions (25,28,29,38).

Liu and Maizels (29) observed that mouse cells expressing murine GFP-Rad52 showed an increased number of Rad51 nuclear foci. This prompted us to analyse the distribution of Rad51 foci in our cells overexpressing ScRad52. To our surprise, Rad51 foci per cell were reduced, while the number of focus-positive cells was unaffected.

This finding can be explained by supposing that ScRad52 can organize DNA repair/recombination structures excluding endogenous Rad51. In this case, we propose that ScRad52 acts

in a Rad51-independent fashion also in human cells, thereby promoting homology search and strand invasion leading to HR at the expense of random integration. The exclusion of Rad51 from the recombination sites would implicate that ScRad52 may also prevent binding of Ku, a complex involved in the early binding of DNA breaks and in the NHEJ pathway (39), explaining the reduction of random integration.

In conclusion, we demonstrate that the expression of ScRad52 protein in human cells results in higher resistance to DNA damaging agents MMS and X-rays, a marked increase of HR both as absolute number and frequency relative to NHEJ. The identification of ScRad52 as a tool that drives HR could be of great impact to develop new strategies for gene therapy of single gene disorders. Moreover, these results make ScRad52 a possible 'recombination enzyme' to be used in studies on the mechanism of recombination, and wherever a permanent disruption of a chromosomal locus is desired.

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