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Functional characterization of *RAD52* as a lung cancer susceptibility gene in the 12p13.33 locus

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Abstract

Recent genome-wide association studies have identified variations in the recombination repair gene, *RAD52*, that are associated with increased lung cancer risk, and particularly with the development of lung squamous cell carcinomas (LUSC). LUSC development is strongly associated with smoking. DNA repair is increased in the lung tissues of smokers, presumably because of ongoing DNA damage from exposure to tobacco smoke. A key player in the DNA damage response, *RAD52* plays a role in DNA strand exchange and annealing during homologous recombination (HR) in mammalian cells. In this study, we discovered two cis-expression quantitative trait loci (eQTL) SNPs in the *RAD52* gene that are associated with its expression and are also associated with LUSC risk. In addition, we report that amplification of the genomic region 12p13.33, which contains the *RAD52* gene, is significantly associated with the development of LUSC in the TCGA database and that somatic overexpression of *RAD52* was confirmed to be significant in LUSC tumors from our own patient cohort. Consistent with these genetic findings, we demonstrate that blockade of Rad52 slows cell growth and induces senescence in mouse bronchial epithelial cells. In contrast, overexpression of Rad52 leads to an increased rate of cell proliferation. We show that depletion of Rad52 in mouse lung tumor cells alters cell cycle distribution and increases DNA damage accumulation associated with increased tumor cell death. Our genetic and functional data implicate *RAD52* as a significant determinant of risk in the development of LUSC.

Keywords

eQTL; Non-small cell lung carcinoma; lung cancer; DNA damage; Cancer susceptibility gene; senescence; gene amplification

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Introduction

Lung cancer currently ranks as the foremost cause of cancer deaths among men and women in the United States with more than one-quarter of all cancer deaths due to this disease [1]. Squamous cell lung cancer (LUSC), which is particularly associated with smoking, comprises about 25% to 30% of all lung cancers and begins in squamous cells which are flat cells that line the inside of the bronchial airways. Furthermore, while 80–90% of lung cancer patients have a history of smoking, only about 10% of heavy smokers develop lung cancer [2]. This suggests that although tobacco smoke undoubtedly increases one's risk for lung cancer, genetic risk factors most likely predispose certain individuals to disease development as part of a complex interplay between carcinogens and genetic determinants. One would expect certain genes which influence exposure to, metabolism of, or response to carcinogens to play a role in lung cancer susceptibility, especially in LUSC.

Recent genome-wide association studies (GWAS) have identified a region on chromosome 12p13.33 as harboring a susceptibility locus for LUSC. This locus includes the recombination repair gene *RAD52* which harbors a specific single nucleotide polymorphism (SNP) - rs6489769 that is associated with LUSC [3]. *RAD52*, originally described in yeast as playing a key role in recombination repair, is involved in strand exchange and annealing of strands during homologous recombination and is predominantly recruited for DNA repair during S phase of the cell-cycle [4,5].

Similarly, recent additions to the 1000 genomes project, including four genome-wide association studies of lung cancer in populations of European ancestry, identified large-effect genome-wide associations for squamous cell lung cancer with the rare variant of *BRCA2*-K3326X [6]. While *BRCA2* is the more recognized factor in human HR, studies performed in *BRCA2*-deficient mammalian cells implicate *RAD52* as an independent and alternate participant in HR [7]. Upon *RAD52* depletion, both the frequency of double-strand break-induced HR and ionizing radiation-induced *RAD51* foci decreased significantly. Likewise, *RAD52*-*RAD51* foci were shown to form equally in the presence or absence of *BRCA2* [8]. As well, inactivation of *RAD52* in *BRCA2* competent mouse ES cells did result in a modest reduction in HR [9].

In humans, *RAD52* is a key player in the regulation of HR-related genomic instability that may lead to an increased LUSC risk. Furthermore, when combined with depletion of *BRCA2* or *PALB2*, depletion of human *RAD52* is synthetically lethal [10]. Thus, in human cells deficient in the *PALB2* or *BRCA2* genes, *RAD52* depletion may decrease cell survival by reducing rates of homologous recombination and by increasing damage-induced chromosomal abnormalities [3]. However, the exact role of *RAD52* in human lung cancer remains elusive and more effort is needed to fill this gap in the knowledge base.

In this study, we sought to investigate the role of *RAD52* in lung cancer susceptibility. As mentioned previously, squamous cell lung carcinoma is particularly associated with smoking. Thus, it fits that variation in this DNA repair gene would impact one's risk for lung cancer, suggestive of a potentially decreased ability to repair carcinogen-induced damage. Surprisingly, our copy number variation (CNV) analysis of LUSC tumors revealed a copy

number gain in the region of *RAD52*, resulting in a statistically significant level of amplification for *RAD52* in lung squamous cell carcinoma patients. We also identified two correlated cis-eQTL SNPs that exhibit cis-regulation of *RAD52* expression and significant association with LUSC. Elevated *RAD52* protein expression was also significant in LUSC tumors compared to that in matched normal lung tissues.

In line with the statistical evidence portraying a role for *RAD52* in developing lung cancer, we functionally demonstrate that upon *Rad52* depletion, the rate of cell proliferation was attenuated and senescence was induced in non-tumorigenic bronchial epithelial mouse cells. We also show that loss of *RAD52* slows cell proliferation while overexpression of *RAD52* enhances proliferation in mouse lung cancer cells. Additionally, in mouse tumor cell lines we demonstrate that depletion of *RAD52* alters cell cycle distribution by decreasing the fraction of cells in G0/G1 and significantly increasing the fraction of cells in G2/M. Finally, we demonstrate that loss of *Rad52* expression increases DNA damage accumulation in mouse normal bronchial and tumor cells.

Materials and Methods

Study subjects

The cis-eQTL analysis of *RAD52* was conducted on a cohort of 70 normal lung tissue samples provided by the Mayo Clinic [11]. The genetic association study conducted between *RAD52* SNPs and lung cancer phenotypes was performed by analyzing a patient population of European ancestry (n=28,998) used in a recent large-scale GWAS study for lung cancer [6]. Gene expression analyses for *RAD52* used the RNA-seq data for a cohort of tumor-normal paired RNA samples from 45 non-small cell lung cancer patients (36 lung adenocarcinoma (LUAD) and 9 lung squamous cell carcinoma (LUSC) recruited at the Medical College of Wisconsin.

eQTL and expression analysis of *RAD52*

The genotyping of the *RAD52* SNPs for cis-eQTL analysis was part of a previous GWAS project using Illumina HumanHap 370k and 610k BeadChips [11] (Illumina, San Diego, CA, USA). CEPH DNA samples (a family trio) were included in each 96-well plate to monitor genotyping, and the concordance between the replicates was 99.5%. The *RAD52* gene expression data in 70 normal lung tissues were part of the results of the genome microarray analysis done with the Illumina Human WG DASL array (Illumina, Inc, San Diego, CA, USA) as detailed previously [11]. The genetic association analysis between *RAD52* and lung cancer was part of the recent large-scale lung cancer GWAS research detailed previously [6]. RNA sequencing for the tumor-normal paired RNA samples from 45 NSCLC patients followed the protocol from our previous publication [12]. Briefly, for data analysis, we used TopHat to conduct alignment of reads to the human reference genome (hg19). We used Cufflinks to compute expression levels of transcripts. Fragments per kilobase of exon per million fragments mapped (FPKM) values were summarized to the gene level by taking the sum of Cufflinks FPKMs estimated for the alternative transcripts of each Ensemble gene [12]. We specifically extracted expression data for *RAD52* and

conducted a pairwise comparison of the expression of this gene for the 45 lung tumor-normal pairs.

Statistical analysis

The linkage disequilibrium analysis for the *RAD52* regions was conducted using Haploview software (<http://www.broadinstitute.org/scientific-community/science/programs/medical-and-population-genetics/haploview/haploview>) and the data from the Caucasian population of the 1000 Genomes Project. For the association testing between *RAD52* SNPs and lung cancer risk, we derived joint odds ratios (ORs) and 95% confidence intervals (CIs) under a fixed-effect model for each SNP and the associated per-allele P values. To explore variability in associations according to tumor histology, we derived ORs for all lung cancer, LUAD and LUSC in the same way as in our previous study [6]. For the eQTL analysis, a linear regression model adjusted for age and sex was used to assess the correlation between genotypes (independent variable, coded as 0, 1, or 2) and transcript expression levels of *RAD52* (eQTL, dependent variable) in 70 samples of normal lung tissue. The analysis was done with SAS version 9.0. Confounding factors that may be associated with lung cancer risk or gene expression were adjusted in the same way as in our previous publications (9,10). Therefore, pulling data from different cohorts did not introduce confounding information. In terms of RNA-seq based differential gene expression analysis of *RAD52*, a paired Student's T-test was done to compare *RAD52* expression between tumor samples and samples of adjacent normal tissue. We analyzed 36 LUAD (data not shown) and 9 LUSC tumor samples with matched normal tissue. The Wilcoxon signed rank test for the *RAD52* gene showed differential expression using the tumor-normal pairs. We also downloaded somatic copy number alteration data in the 12p13.33 region containing *RAD52* and somatic gene expression data of *RAD52* generated by TCGA (The Cancer Genome Atlas, <https://cghub.ucsc.edu/>) to check the somatic copy number change and its association with somatic *RAD52* expression changes in LUSC samples.

Cell culture

The immortalized, nontumorigenic mouse lung epithelial cell line, E10, as well as the mouse lung tumor cell lines obtained from spontaneous lung tumors in the A/J mouse (Spon2 and Spon6), as well as MC7 cells established from a methylene chloride-induced nonmetastatic lung tumor from B6C3F1 and the metastatic nicotine-derived nitrosamine ketone (NNK)-induced A/J mouse lung tumor (CL25M cell line) were cultured as previously described [13].

Lentivirus construction and transduction for short hairpin RNA (shRNA) expression

The pLKO.1 lentiviral system (GE Healthcare, PA, USA) was used to generate replication-deficient lentiviral particles for stable transduction of shRNA. The mature anti-sense strand of the shRNA sequences are as follows, Rad52-01: 5'-TAACTGCACCTTTACAAATGC -3', Rad52-03: 5'-TAGTCCTCTAAAGAGTTCAGC -3', Rad52-04: 5'-ATTCATCCGCTGTATACTGGC -3', shSCRAM: 5'-CCTAAGGTTAAGTCGCCCTCGCTCGAGCGAGGGCGACTTAACCTTAGG -3'. Lentivirus stocks were prepared in 293T cells, infected into target cells with 8 ug/ml

polybrene and selected with 2 ug/ml puromycin. Puromycin selected lines were used for experimental analysis from 4–25 days post-infection.

Overexpression of RAD52

The plasmids encoding empty vector pOPUR and pOPUR/Rad52 were a generous gift from Dr. Jac A. Nickoloff and were constructed according to his protocol [14]. Briefly, mammalian expression vector pOPUR was constructed to carry the RSV LTR promoter and triplicated lac operators from pOPI3CAT (Stratagene, La Jolla, CA) and the puromycin resistance gene from pPUR (Clontech, Palo Alto, CA). Human Rad52 cDNA was inserted downstream of the RSV LTR promoter in order to create a Rad52 overexpression plasmid that would retain its stability and not degrade post-transcription due to mRNA degradation signals in the 3'-untranslated region [14].

Immunoblot analysis of cellular lysates

Protein from E10, CL25M, Spon6 and Spon2 cells with shRNA targeting RAD52, constructs for overexpression of RAD52, scrambled vector control shRNA or empty pOPUR vector were extracted at 72–96 hours post transfection for transient transfections and in line with each experiment for stable transfections. Cells were lysed with 100 µL of 1X RIPA lysis buffer containing proteinase inhibitor cocktails (Fisher Scientific, Pittsburg, PA), sonicated for 10 seconds, and spun at 13,000 RPM for 30 minutes at 4°C. Purified protein concentration was then calculated by the Bradford method, and boiled for 5 min. Normalized lysate was resolved by 4–12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Invitrogen, Carlsbad, CA) and transferred to PVDF membranes. Commercial antibodies used included: Rad52 (Cell Signaling, Danvers, MA, USA, 2762), Rad52 (Santa Cruz, Dallas, TX, USA, SC20044), Anti-phospho-Histone H2A.X (Ser139) (EMD Millipore, Billerica, MA, USA), p21 (F-5) (Santa Cruz, Dallas, TX, USA, SC20044), and beta-Actin (Santa Cruz, Dallas, TX, USA, SC20044). Immunoblots were developed using Clarity Western ECL Substrate (BioRad, Hercules, CA, USA) and quantitated on a ChemiDoc XRS+ Imaging System (BioRad, Hercules, CA, USA).

Cellular Senescence by SA-beta-gal staining

Cellular senescence was assayed as described previously [15]. Briefly, equal numbers of E10 Immortalized, nontumorigenic mouse lung epithelial cells stably infected with either scrambled pLKO.1 vector or shRNA constructs targeting the Rad52 gene (shRad52-03,04) were plated in 6-well sterile tissue culture plates 48 hours prior to analysis and at a confluence of approximately 30%. Senescence was measured according to manufacturer's protocol (Cell Biolabs, San Diego, CA). Briefly, the cells were washed with PBS. After washing, 1x Fixing Solution (25% Glutaraldehyde) was added to each well and the cells were incubated at room temperature for 5 minutes. Fixation solution was removed, the cells were washed in PBS and cell staining working solution was prepared fresh and added to each well. The cells were then incubated overnight at 37°C, protected from light. The development of blue color was detected visually under a light microscope. Data correspond to the percentage of SA-beta-Gal-positive cells counted for each cell population. Numbers on the graphs indicate a percentage of SA-beta-Gal-positive cells and were derived from counting 200 cells in triplicate plates.

Apparent Doubling Time

E10 cells were infected with either Scr. pLKO.1 or shRad52-03. Cells underwent puromycin selection for 48 hours until all cells in the mock infected plate were dead. Puromycin selected populations were then seeded at equal densities, while remaining in media with puromycin, and incubated under normal growth conditions for 48–72 hours. Cell number and viability were determined by the Countess Automated Cell Counter (Invitrogen, Carlsbad CA) and apparent doubling time was calculated. $2^x = n_{\text{final}}/n_{\text{start}}$ for the number of doublings (x) and (h/x) for the apparent doubling time, where h is the varying periods of time.

Cell Proliferation Curves and Plating Efficiency

Cells were harvested with trypsin, counted on the Countess automated cell counter (Invitrogen, Carlsbad, CA) and plated at a density of 3,000 cells per well in multiple replicates on a 96-well tissue culture plate. Cell numbers were measured starting 4 days after infection and 2 days after completing selection in puromycin, although cells remained on puromycin throughout the length of the experiment to ensure measurement of the correct population. Cell confluence was measured every two hours on an Incucyte live cell imager (Essen Biosciences, Ann Arbor, MI), through photomicrographs. Cell culture confluence was measured using Incucyte software (Essen Biosciences, Ann Arbor, MI).

Analysis of cell cycle distribution by propidium iodide staining

Mouse tumor cell lines were cultured in RPMI with 10% serum over a period of 14 days. Flow cytometry was employed to analyze cell cycle distribution on triplicate samples of cells using ethanol fixing and propidium iodide staining with RNase to assess relative DNA content. Sub-confluent cultures of cells were harvested by trypsinization. Post-harvest, cells were centrifuged in 15 ml conical tubes at 1000 rpm for 5 min, re-suspended in cold PBS, then centrifuged again in the 15 ml conical at 500 ×g for 20 minutes. Next, the supernatant was discarded and 1 ml cold PBS was added to wash the cells. The cells were then centrifuged again at 500 ×g for 20 minutes, the supernatant was discarded and the cells were thoroughly suspended in 500 ul cold PBS, avoiding formation of cell clumps. Next, using the Cell Countess, cells were measured to a concentration of 2×10^6 cells/ml in PBS. For fixation, 5 ml of ice cold 95%/Absolute ethanol was added to the cells in PBS while gently vortexing. Cells were then held on ice for 2 hours to complete fixation. For staining, cells were spun down at 1500 rpm for 5 minutes to pellet. The ethanol was aspirated and the cells were washed twice with PBS, leaving a small amount to re-suspend the cells in. One ml of PI staining solution (900 ul PBS, 50ul PI stock soln, 50 ul RNase stock soln) was then added to the cell pellet. Finally, the cells were incubated in the dark for 3 hours at 4 degrees Celsius to ensure the RNase had digested all the RNA. Flow cytometry was performed on the Accuri C6 cytometer (BD Biosciences, San Jose CA). Data were compiled and analyzed using CFlow Plus and FlowJo software.

Comet Assay

A Comet Assay kit (Trevigen, Helgermen CT) was used to quantify and analyze DNA damage in individual E10 and Spon2 cells. The image that is obtained resembles a “comet”

with a distinct head and tail. The head is composed of intact DNA, while the tail consists of damaged DNA. Cells are scored based on their Olive Tail Moment (OTM). The OTM is defined as the product of the tail length and the fraction of total DNA in the tail. One group of shSCRAM cells in each cell line was treated with Hypochlorous acid (HOCL) at 4°C in suspension to serve as a positive control for DNA damage accumulation.

Results

eQTL containing RAD52 is associated with lung cancer development

We found two SNPs within RAD52 that are associated with RAD52 gene expression in both lung tumor tissue as well as with non-small cell lung cancer phenotypes, especially LUSC. The first SNP, rs6413436, is significantly associated with LUSC in our GWAS cohort ($p = 1.0 \times 10^{-3}$ after multiple testing adjustment, odds ratio (OR) = 1.06 for overall lung cancer (95% CIs: 1.024–1.10), $n=28,998$; $p = 2.7 \times 10^{-3}$, OR=1.09 for LUSC (95% CIs: 1.03–1.15), $n=19,437$). Although this SNP is not genotyped in our eQTL cohort of 70 lung tissues, it is in strong linkage disequilibrium (LD) ($D'=1$ and $r^2=0.82$) with a nearby SNP, rs10744729, which we have genotyped in our eQTL cohort and found to be significantly associated with RAD52 gene expression ($p = 0.01$, $n=70$). Figure 1A shows the linkage disequilibrium plot of the tagged SNPs for the RAD52 region based on data from the Caucasian population of the 1000 Genomes Project. SNPs rs6413436 and rs10744729 are located in RAD52 intron 11 and intron 9, and the distance between them is relatively small, at about 1.9 kb. These are the two tagged SNP markers in Block 1 of RAD52 (marker #4 and #6 in Figure 1A red box). The SNP rs10744729 also showed a significant association with LUSC in our GWAS study ($p = 0.02$ after multiple testing adjustment). And the linkage disequilibrium between rs10744729 and rs6413436 in our dataset is close to complete LD ($r^2 = 0.82$). There is complete LD between these 2 SNPs as revealed by HapMap data for whites ($r^2 = 1$, Figure 1A). It is thus reasonable to conclude that rs10744729 is a proxy SNP for rs6413436. The significant association of both rs10744729 and rs6413436 with LUSC could be explained by the strong LD between these 2 SNPs. This plus that rs10744729 was significantly associated with RAD52 expression in lung tissues suggest that these 2 SNPs were eQTL SNPs that associate with LUSC risk by influencing RAD52 gene expression.

Figure 1B demonstrates that the risk allele 'A' of rs10744729 (in LD with the lung cancer risk allele 'C' of rs6413436) is associated with increased expression of *RAD52* in a dose-dependent manner (homozygous reference alleles 'CC' < heterozygous 'AC' < homozygous mutants 'AA'). ANOVA analysis showed that this SNP explains 9.2% of the expression variance of *RAD52*. Thus, the eQTL for rs10744729 and its associated SNP, rs6413436, accounts for a small proportion of the variance in *RAD52* expression in lung tissues, an observation consistent with the relatively modest genetic risk observed with these two variants for LUSC (OR=1.09). By contrast, no significant association with lung adenocarcinoma (LUAD) was found for these two SNPs ($p = 0.24$, $n=19,589$), suggesting that these two eQTL SNPs were not related to the LUAD etiology.

Somatic copy number analysis in the *RAD52* gene region using TCGA data

Using GISTIC software, somatic copy number analysis in 358 TCGA lung squamous cell carcinoma patients showed that the genomic region of 12p13.33 containing *RAD52* reached a statistically significant level for focal amplification ($q\text{-value} = 2.56 \times 10^{-3}$, much less than the genome wide cutoff $q = 0.25$, Figure 2). Thirty-eight of 358 lung SCC tumors (11%) showed copy number gains in the *RAD52* region (segments were reported as amplified if the corresponding estimated copy number ratio was greater than 1.25, i.e., $\log_2$ ratio > 0.32). These 38 identified TCGA LUSC tumors and their copy number amplification at the *RAD52* gene region are listed in Supplemental Table 1. Gene expression levels of *RAD52* are also positively correlated with somatic copy number gains in the *RAD52* region in the TCGA LUSC samples (Supplementary Figure 1), suggesting that the somatic copy number gains in the LUSC tumors led to the corresponding increase in *RAD52* expression.

Other significant *RAD52* SNPs influencing LUSC

We identified a number of *RAD52* SNPs which showed more significant P-values (P-values $< 10^{-6}$ (Supplemental Table 2) for the associations with squamous cell lung cancer phenotype. However, these SNPs were not associated with *RAD52* gene expression in our samples, suggesting that other mechanisms may underlie their associations with LUSC. The *RAD52* SNPs we presented here are the ones associated with both *RAD52* gene expression and LUSC risk. As well, these are cis-eQTL SNPs that may influence LUSC phenotype by affecting the expression level of *RAD52*. We replicated the significant association between *RAD52* SNP rs6489769 and the risk of squamous cell carcinoma $P = 9.4 \times 10^{-7}$, (Supplemental Table 2) which was originally reported by Shi et al [3]; however, this SNP does not show significant association with our *RAD52* gene expression data, suggesting it may not be a cis-eQTL SNP in *RAD52*. In fact, Shi et al. [3] did not find that the SNP rs6489769 was associated with *RAD52* gene expression. Current evidence suggests that rs6489769 may influence one's risk of developing LUSC via mechanisms other than *RAD52* expression.

Increased *RAD52* gene expression in LUSC samples from NSCLC cohort

Based on our independent non-small cell lung cancer (NSCLC) patient dataset, we found that *RAD52* was significantly overexpressed in the tumors of LUSC patients ($p=0.05$, $n=9$, Figure 3). Specifically, there was an average of a 50% increase in *RAD52* expression in LUSC tumors compared to that in normal tissue samples (Figure 3). However, there was no significant difference in *RAD52* expression between the tumor and normal lung tissues of LUAD patients ($p=0.29$, $n = 36$). This data suggests that *RAD52* overexpression could contribute to LUSC etiology but not to LUAD carcinogenesis, which is consistent with our eQTL study showing that the identified eQTL SNPs in *RAD52* are significantly associated with LUSC but not LUAD.

Loss of Rad52 induces senescence in mouse immortalized lung epithelial cells

Although Rad52 acts in a parallel fashion to the known tumor suppressor BRCA2, depletion of Rad52 has actually been shown to slow cell proliferation in MCF7 breast cancer cells [8]. This suggests that although the inherent function of Rad52 is to repair damaged DNA, in a

pro-tumorigenic setting, it may actually function to enhance cell proliferation or to protect tumor cells from apoptosis. Given Rad52's recent identification as a susceptibility candidate associated with squamous cell lung carcinoma, we hypothesized that depletion of Rad52 might be a factor in the survival and proliferation of lung and lung tumor cells. To determine the effect of Rad52 depletion in mouse bronchial cells, we treated immortalized, non-tumorigenic mouse bronchial epithelial cells with either shRNA targeting *RAD52* or a scrambled vector. RAD52 was depleted at the protein level from the E10 cells by 2.63 fold and 1.89 fold using shRNA constructs 03 and 04, respectively. The cells were cultured for 7–10 days post-infection before they stopped dividing, became morphologically round and flattened, and stained positive for senescence associated beta-galactosidase activity, indicating that loss of RAD52 resulted in senescence (Figure 4).

Phosphorylation of gamma H2AX is an early sign of DNA damage induced by replication stalling [16]. The role of γ -H2AX foci in the DNA damage signaling pathway induced by double-strand breaks is to act as docking areas for the recruitment of repair factors and to bring the broken DNA ends closer so that DNA repair is accomplished [17]. Beard et al. found that upon induction of DNA damage, γ -H2AX-proficient cells, as well as H2AX-complemented H2AX^(-/-), cells reacted by increasing p21 levels and arresting the cell cycle. As our results also suggest, (Figure 4D), H2AX is required for p21-induced cell cycle arrest after replication stalling. In addition, γ -H2AX has been shown to be essential for concentrating repair proteins and maintaining the integrity of the DNA damage response foci [18]. Finally, the significance of H2AX in cell cycle arrest was previously demonstrated by Fernandez-Capetillo et al. [19]. This serves to confirm the observed senescence in our E10 bronchial epithelial cells and to suggest a further role for the DNA damage response in the aforementioned growth arrest discovered upon Rad52 depletion.

Expression of RAD52 affects growth in mouse lung epithelial and tumor cells

Utilizing distinct viral transfections, equal numbers of viable Spon2, Spon6, CL25M and E10 wild-type and RAD52-depleted cells were seeded in the Incucyte live cell imager, and the growth rate was observed over a period of 3 days. Upon depletion of RAD52 in mouse lung epithelial cells, we observed the rate of cell proliferation begin to decrease around 96 hours post infection and 48 hours post completion of puromycin selection, prior to the appearance of a senescent phenotype. Cellular proliferation in lung tumor cell lines (Spon2, Spon6 and CL25M) also decreased with depletion of RAD52 (Figure 4A). Admittedly, we recognize that percent confluence is not a pure measure of cell number; however, doubling time assays in E10 cells (Figure 4) along with MTS measurements of cell proliferation confirm that cell morphology did not affect proliferation rates. In effect, any error caused by differences in cell morphology would likely function to enlarge cells depleted of RAD52, enhancing their rate of confluence.

In comparing the apparent doubling time of E10 cells, we determined that depletion of RAD52 led to a significant increase in apparent doubling time (*P<0.05), increasing 1.83 fold from those infected with a scrambled control shRNA (Figure 4C). Moreover, although E10 cells overexpressing RAD52 did not show a growth difference, all tumor cell lines

overexpressing RAD52 showed a significant increase in growth rate around 5 days post-infection.

Depletion of RAD52 alters cell-cycle distribution in lung tumor cells and induces accumulation of cells in G2/M

To further investigate whether changes in cell-cycle regulation correlate with the observed changes in cell growth, we compared cell-cycle profiles of different mouse lung tumor cell lines through flow cytometry with propidium iodide (PI) staining. The E10 mouse bronchial epithelial cell line became senescent post-RAD52 knockdown, which prevented the acquisition of enough cells for flow cytometry. Results from these studies demonstrate accumulation of tumor cell populations in the G2/M fraction and a decrease in cell populations in the G0/G1 fraction consistent with deficient DNA repair-induced senescence (Figure 6). These data strongly support a role for RAD52 in G2/M progression and/or passage through the G2/M checkpoint in mouse lung tumor cells. This G2 delay is likely to be the major determinant of the slower growth rate seen for the RAD52-depleted mouse cells (Figure 5). A less severe profile was seen in the Spon2 cell line (Figure 6A). This indicates that other factors besides Rad52 expression may be rate-limiting in the passage of Spon2 cells beyond the G2 phase.

Loss of RAD52 induces spontaneous DNA damage in immortalized bronchial epithelial and lung tumor cells

Highly efficient and accurate DNA repair throughout the replication cycle of a cell is critical in maintaining genome stability and thwarting disease-causing polymorphisms. When DNA damage accumulates, a cell may either die because of excessive damage, or the cell may mechanistically sidestep the damage through modulation of other checkpoints or tumor suppressive proteins, leading to replication of the damaged cell and disease.

In order to determine if the observed changes in cell growth and cell cycle distribution may be due to a deficiency in the DNA damage response, E10 and Spon2 cells were depleted using multiple shRNA constructs and subjected to the Comet assay to quantify DNA damage in individual cells (Supplementary figure 2).

Analysis of olive tail moment (OTM) showed that both wild type E10 and Spon2 cells displayed little endogenous DNA damage. However, Rad52 depleted cells cultured for 6–11 days contained relatively high levels of DNA damage accumulation (Supplementary figure 2). These results demonstrate that under cell-culture conditions where the repair system is impaired, RAD52 deficiency is associated with the accumulation of DNA damage.

Discussion

In between cycles of replication, eukaryotic cells will undergo damage to their genome by both endogenous and exogenous means. In order to survive, cells have developed different means of damage control and repair systems. As the main mechanism in the eukaryotic DNA damage response, homologous recombination serves as a safeguard in cell cycle progression, ensuring proper signaling of chromosome damage and accurate chromosome segregation during mitosis [20]. If unsuccessful in a critical repair process such as the DNA

damage response, a cell may either undergo apoptosis or accumulate mutations that will ultimately evolve into genomic instability and cancer [7]. The complementary evidence determined by our genetic and functional studies suggests a potential role for the DNA repair gene, *RAD52*, in lung tumorigenesis. Our research into the role of *RAD52* in susceptibility to lung carcinogenesis emerged from prior findings implicating variation in *RAD52* as a factor in LUSC development [3]. Here, we have identified two candidate regions in *RAD52* where variations in the gene are associated with increased copy number as well as increased *RAD52* expression in lung tumor tissue compared to normal.

A study performed by the Barlow group demonstrated that by reducing homologous recombination in ATM-deficient mice through genetic deletion of the *Rad52* gene, not only did the development of T-cell lymphomas decrease, but mice depleted of *Rad52* also lived longer [21]. This report suggests that excessive homologous recombination, termed hyperrecombination, by a member of the normal DNA damage response may contribute to tumorigenesis. This hyperrecombinogenic, phenotype is known to be toxic to the cell, leading to chromosomal instability and disease through an accumulation of lethal recombination variants [21,22]. In A-T, disease phenotypes are believed to arise from a loss of ATM kinase activity as well as disrupted signaling to proteins such as p53, chk2 and DNA-repair proteins, such as *Rad52*, resulting in cell cycle checkpoint defects and impaired DNA repair [21]. Barlow's group tested the hypothesis that excessive recombination might be responsible for the overlapping phenotypes in A-T and Blooms in vivo by crossing *Atm*^{-/-} mice with mice deficient in the HR protein *Rad52* [21]. Her group clearly demonstrated that by reducing homologous recombination through genetic deletion of the *Rad52* gene, they were able to significantly decrease and delay the incidence of thymic tumors in *Atm*^{-/-} mice: more than half of the *Atm*^{-/-} mice developed tumors by 4 months of age compared to less than 20% of the *Atm*^{-/-} *Rad52*^{-/-} mice [21].

Therefore, when considering current GWAS studies, our eQTL analysis, and recent experimental studies, it is reasonable to suggest that although chromosomal instability is not rescued in the absence of *Rad52*, genetic deletion of *Rad52* may result in a reduction of abnormally high levels of intrachromosomal recombination and that this reduction by *Rad52* reduces tumorigenesis by protecting genome integrity. Given the recent genomic data linking *Rad52* to lung cancer along with our data demonstrating *Rad52* amplification in LUSC cases, along with the known role of *Rad52* in recombination, it is rational to suggest that overexpression of *Rad52* would aid in the development of a lung cancer phenotype.

As with that study, we found that not only did lung tumor tissue samples express higher levels of *RAD52* compared to normal lung tissue, but depleting *RAD52* in functional *in vitro* studies led to phenotypes associated with decreased tumorigenesis. As our functional studies show, overexpression of *Rad52* leads to an increased rate of cell growth, a prominent tumorigenic phenotype, further supporting a role for *Rad52* in the development of lung cancer.

As mentioned above, in this study we sought to determine the effect of loss and overexpression of *RAD52* on such phenotypes associated with lung tumorigenesis including induction of senescence, rates of cell proliferation, altered cell cycle distribution and

accumulation of DNA damage. Current literature implicates a connection between a high frequency of structural variation in the genome with phenotypic diversity and predisposition to disease [23]. Hence, it would be reasonable to believe that DNA repair genes such as *RAD52* may modulate oncogenic phenotypes related to DNA damage and genomic variation. We observed that E10 immortalized mouse lung epithelial cells went into a state of senescence 7–10 days post infection with shRNA constructs targeting Rad52. E10 cells express high levels of the known oncogene MDM2 and are ARF/p53-inactivated [13]. Thus, it makes sense that depletion of Rad52 in E10 cells lead to upregulation of p21 and phospho-gamma H2AX expression, similar to observations made by the MacLaren group upon knockdown of c-Jun [24]. This group found a potential role for c-Jun in the DNA damage response, linking its depletion to premature senescence, an accumulation of cells in G2/M phase of the cell cycle, and a persistence of DNA damage [24].

Furthermore, in mouse lung tumor cells, knockdown of Rad52 curbed proliferation and led to an accumulation of cells in the G2/M phase of the cell cycle (Figures 5,6). Alteration of cell-cycle regulation by RAD52 has not been demonstrated previously. The observed G2/M-accumulation upon RAD52 depletion may be due to variation in expression of other proteins or increased sensitivity to culture conditions.

Little is known of the effects of variation in *RAD52* in regards to susceptibility to lung cancer and tumor development. However, genetic variations that alter homologous recombination, coupled with a carcinogenic environment, may plausibly contribute to tumorigenesis. Further understanding of the role that *RAD52* plays in the development of NSCLC will ultimately yield clinically significant results through determination of prediction markers and prognostic factors in regard to LUSC therapeutics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 2. Somatic copy number analysis using the GISTIC program on 358 TCGA lung squamous cell carcinoma patients showed that the genomic region of 12p13.33 containing RAD52 reached a statistically significant level for focal amplification ($q\text{-value} = 2.56 \times 10^{-3}$, much less than the genome-wide cutoff $q = 0.25$).

Figure 3.

Overexpression of RAD52 in lung squamous cell carcinoma tumors observed in our independent NSCLC patient dataset. RAD52 was significantly overexpressed in the tumors of LUSC patients ($p=0.05$, $n=9$). There was an average of a 50% increase in RAD52 gene expression in LUSC tumors compared to normal tissues.

Figure 4.

Immortalized E10 mouse lung epithelial cells underwent spontaneous senescence upon Rad52 depletion. (a, top, middle) Senescence is demonstrated through senescence-associated β -galactosidase activity by staining cells with the chromogenic substrate 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal). (a, bottom) Representative phase-contrast images documenting the observed cells are shown for each population. (b, top) Indicated bronchial epithelial cells were infected with control shRNA or Rad52-shRNAs (03 or 04). Cells were fixed and stained for senescence-associated beta-galactosidase (SA-beta-Gal) activity 10 days after the infection. Numbers on the graphs indicate percentage of SA-beta-Gal-positive cells compared to total cell count and were derived from counting 200 cells in triplicate plates. (b, bottom) Densitometry using ImageJ software demonstrated 2.63 and 1.89 fold decreases in expression levels of Rad52 in E10 cells infected with shRad52-03 and shRad52-04 compared to scrambled control, respectively. (c) E10 cells infected with either Scr. pLKO.1 or shRad52-03 were seeded at equal densities and incubated under normal growth conditions for 48–72 hours. Apparent doubling time was calculated. (d) E10 Scr. pLKO.1 and shRad52-03,04 populations were cultured under normal growth conditions and cell lysates were prepared and subjected to immunoblot analysis. (ns, no significance; * $p < 0.05$; ** $p < 0.005$)

Figure 5.

Cellular proliferation of lung tumor and bronchial epithelial cells with altered RAD52 expression. (a) Cellular proliferation in lung tumor cells (Spon2, Spon6, CL25M) and bronchial epithelial cells (E10) transfected with shRNA against Rad52 compared to scrambled control. (b) Cellular proliferation in lung tumor cells (Spon2, Spon6, CL25M) and bronchial epithelial cells (E10) transfected with the pOPUR Rad52 overexpression plasmid compared to vector. (c–d) Transfected populations from all cell lines were cultured under normal growth conditions for 7–10 days, Cell lysates were prepared and subjected to immunoblot analysis. (ns, no significance; * $p < 0.05$; ** $p < 0.005$)

Figure 6.

Depletion of Rad52 alters the cell-cycle distribution of lung tumor cells inducing a decrease in cells in G0/G1 and an accumulation of cells in G2/M. (a) Cell cycle progression was assessed by propidium iodide staining as a measure of DNA content. Flow cytometry was performed on CL25M, Spon2 and MC7 Rad52-depleted and scrambled vector control cells. Fixed cells were acquired using the Accuri C6 Cytometer. (b) Cells were then analyzed using FlowJo software and the Watson-Pragmatic cell cycle method and percentage values for G0/G1 (2 NDNA content), S (intermediate DNA content) and G2/M (4 N DNA content) cells. (c) Confirmation of knockdown of Rad52 in mouse tumor cell lines using the

shRad52-03 construct and scrambled control shRNA by western blot. (ns, no significance; *
 $p < 0.05$; ** $p < 0.005$)

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