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Recombinant cells in the lung increase with age via *de novo* recombination events and clonal expansion

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Abstract

Homologous recombination (HR) is a critical DNA repair pathway, which is usually error-free, but can sometimes lead to cancer-promoting mutations. Despite the importance of HR as a driver of mutations, the spontaneous frequency of such mutations has proven difficult to study. In order to gain insight to location, cell type, and subsequent proliferation of mutated cells, we utilized the Rosa26 Direct Repeat (RaDR) mice for *in situ* detection and quantification of recombinant cells in the lung. We developed a method for automated enumeration of recombinant cells in lung tissue using the Metafer 4 slide-scanning platform. The mean spontaneous HR frequencies of the lung tissue in young and aged mice were 2×10^{-6} and 30×10^{-6} respectively, which is consistent with our previous reports that mutated cells accumulate with age. In addition, by using the capability of Metafer 4 to mark the position of fluorescent cells, we found that recombinant cells from the aged mice formed clusters in the lung tissue, likely due to clonal expansion of a single mutant cell. The recombinant cells primarily consisted of alveolar epithelial type II or club (previously known as Clara) cells, both of which have the potential to give rise to cancer. This approach to tissue image analysis reveals the location and cell types that have undergone HR. Being able to quantify mutant cells *in situ* within lung tissue opens doors to studies of exposure-induced mutations and clonal expansion, giving rise to new opportunities for understanding how genetic and environmental factors cause tumorigenic mutations.

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Statement of author contributions

TK and JEK contributed equally to this work. Conceived and designed the experiments: TK BPE. Performed the experiments: TK JEK NL. Analyzed the data: TK JEK. Contributed reagents/materials/analysis tools: TK JEK NL BPE. Wrote the paper: TK JEK BPE. TK was a visiting scientist of Department of Biological Engineering, MIT.

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Keywords

ROSA26 Direct Repeat GFP (RaDR) Mice; Homologous Recombination; Metafer 4; Lung; Genotoxicity

Introduction

DNA double strand breaks (DSBs) are highly cytotoxic and can give rise to large-scale sequence rearrangements (Krejci et al. ; Lord and Ashworth 2012). Homologous recombination (HR) is a critical pathway for repair of DSBs (Holthausen et al. ; Kass and Jasin 2010; San Filippo et al. 2008). While HR is essential for survival in mammals, excessive HR increases the risk for genomic misalignments that can cause insertions, deletions, or loss of heterozygosity (LOH) (Bishop and Schiestl 2000; Bishop and Schiestl ; Costantino et al. ; Gu et al. ; Jonnalagadda et al.). These classes of large-scale sequence rearrangements have been observed in multiple cancers, and have been attributed to aberrant HR (Gupta et al. ; Loeb et al. ; Pal et al. ; Roy et al. ; Shao et al. ; Strout et al.). Given that HR events are linked to cancer, being able to detect such mutations in mammals opens doors to studies of potentially carcinogenic genetic and environmental factors.

Until recently, *in vivo* models for mutation analyses were either limited to a subset of tissues (*e.g.,* p^{un} (Lebel ; Schiestl et al. ; Schiestl et al.) and Pig-a (Bryce et al.) or require digestion of the tissue for analysis (*e.g.* Gpt-delta (Nohmi et al.), BigBlue® (Piegorsch et al.), Muta™mouse (Cosentino and Heddle), and Random Mutation Capture (RMC) (Wright et al.). Digesting the tissue necessarily results in loss of information about the context of the mutation, such as cell type, location or clonal expansion. Other mouse models have been developed that utilize a fluorescent reporter to detect mutations (Kass et al. ; Noda et al.), though each has different utility. The Jasin laboratory has developed a mouse model that allows in depth interrogation of DSB repair mechanisms in primary cell cultures, but this model requires artificial induction of a DSB at an I-SceI restriction site and has not been used for *in situ* analyses (Kass et al.). On the other hand, the Nakamura HPRT-dup-GFP model allows detection of *in situ* mutations (Noda et al.), but neither the extent of expression nor potential for silencing of their transgene has been determined with a positive control. Transgenic animal models have also been developed to spontaneously acquire functional mutations, such as the latent activated K-ras ($K\text{-}ras^{LA}$) model developed in the Jacks lab, wherein mutant K-ras is not expressed unless there is a homologous recombination event at an integrated transgene (Johnson et al.). However, when the mutation confers a growth advantage, the spontaneous frequency of that mutation cannot be quantified in the dysplastic outgrowth. A method to detect functionally inert, spontaneous mutations *in situ* greatly expands the amount and type of information accessible from a single animal by allowing analysis of cell type, location and clonal expansion.

The fluorescence yellow direct repeat (FYDR) mouse model, developed in the Engelward laboratory, was the first to show that HR events can be detected via fluorescence *in situ* in the pancreas (Hendricks et al. ; Kiraly et al. ; Kiraly et al. ; Wiktor-Brown et al. ; Wiktor-Brown et al. ; Wiktor-Brown et al. ; Wiktor-Brown et al. ; Wiktor-Brown et al.). Based on

prior studies of recombination-derived mutation events at a natural direct repeat that controls pigmentation (Lebel ; Schiestl et al.), FYDR mice were designed to exploit the same mechanism with a visible reporter as well. FYDR mice contain a direct repeat of an *Eyfp* expression cassette, one with a deletion at the 5′ end, and the other with a 3′ deletion. Normally, expression of these genes leads to production of an incomplete, non-fluorescent protein. However, following recombination between the internal homologous sequences of *Eyfp*, a complete EYFP coding sequence and thus protein can be produced, making it possible to detect mutations from aberrant HR by the appearance of a fluorescent signal. It is noteworthy that recombination via several different mechanisms can produce the full-length sequence, including gene conversion, unequal sister chromatid exchange and replication fork repair (Figure 1A). FYDR has been used for many studies, including the first demonstration that HR events occur in adult animals and accumulate with age (Wiktor-Brown et al.).

While the FYDR mice have been effective for several studies of genetic and environmental factors that impact genomic stability, this model is limited to analysis of the pancreas and skin, since the recombination substrate was integrated randomly at a locus that can be epigenetically silenced. Since HR cannot be detected unless the recombined *Eyfp* gene is expressed, the FYDR mice could not be used for studies of HR in many tissues, including the lung. To overcome this limitation, we recently created another mouse model, the *Rosa26*Direct Repeat (RaDR) mice, which have a direct repeat comprised of truncated *Egfp* sequences that has been targeted to the *Rosa26* locus (Sukup-Jackson et al.), similar to the FYDR mouse design. The *Rosa26* locus was selected based on previous data showing that genes inserted into this locus are virtually ubiquitously expressed (Jonnalagadda et al. ; Soriano). The RaDR mice offer an exciting opportunity to assess the spontaneous frequency of HR-derived mutations in previously inaccessible tissues, such as the lung.

Using the RaDR mice, we have developed methodology to detect and quantify recombinant mutated cells within intact pancreatic, liver, and colon tissue (Sukup-Jackson et al.). We subsequently found that aging and exposure to genotoxic compounds caused an increase in recombination events in the RaDR mice (Kiraly et al. ; Sukup-Jackson et al.). We aimed to extend our studies of mutagenicity to the lung, since most lung genotoxicity assays focus on evidence of DNA damage rather than mutagenesis (Dalrymple et al. ; Lindberg et al. ; Morita et al.), making a method to detect mutant cells potentially valuable.

Due to the airspace in lung tissue, we had previously been unable to image RaDR lung mutations with our traditional whole mount imaging method (Sukup-Jackson et al.). Here, we show an alternative automated method for enumeration of recombinant cells using scanning fluorescent microscopy, which has allowed detection of HR in lung tissue *in situ*. Furthermore, we recapitulate the finding that mutation frequency and clonal expansion of those mutant cells increase with age in the lung, as previously observed in the pancreas and colon (Sukup-Jackson et al. ; Wiktor-Brown et al.). Finally, we demonstrate that the observed mutations occurred in cell types that have the potential to give rise to cancer, and that those cells clonally expanded to increase the total burden of mutations in the tissue.

Materials and Methods

Animals

The RaDR mice on the C57Bl/6J background were bred in house. The design of the integrated HR reporter is shown in Figure 1A. Mice constitutively expressing EGFP (129S-*Gt(ROSA)26Sor*$^{tm1(CAG-EGFP)Luo}$/J, developed in the Luo lab at Stanford University and donated as a kind gift from Dr. Susan E. Erdman, MIT, MA) were used as a positive control for histology analysis and flow cytometry. Animals with no EGFP expression were used as a negative control for flow cytometry. All animals were housed and handled in Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) accredited facilities with diets, experimental methods, and housing as specifically approved by the Institutional Animal Care and Use Committee (IACUC). The MIT CAC (IACUC) specifically approved the studies as well as the housing and handling of these animals.

Antibodies

Anti-mouse CC10 antibody (T-18) and anti-mouse SP-C antibody (M-20) were purchased as a primary antibody from Santa Cruz, TX. Alexa Fluor 546 labeled donkey anti-goat IgG and ProLong® Gold Antifade reagent with DAPI were purchased from Life Technologies, CA.

Flow cytometry

Lungs were harvested from mice after euthanasia by CO_2 and placed in 5 mL of 2 mg/mL Collagenase V (Sigma-Aldrich, MO) in Hank's Balanced Salt Solution (HBSS) (Life Technologies). The tissues were dissociated in GentleMACS™ (Miltenyi Biotec, MA) C tubes and immediately placed in a 37°C incubator on a gentle shaker for to allow further dissociation by collagenase. After 40 minutes, the dissociated tissue solution was triterated, filtered through a 70 μm cell strainer into Dulbecco's Modified Eagle Medium (DMEM, Life Technologies) containing 10% fetal bovine serum (FBS, Atlanta Biologicals, GA), and centrifuged. The supernatant was decanted, then pellets of tissue were resuspended in 350 μL of OptiMEM™ (Life Technologies) and filtered through a 35 μm cell strainer into a 5 mL flow cytometry tube. These suspensions were analyzed by a FACScan flow cytometer with an excitation laser at 488nm, with filters to measure green fluorescence (530nm) and autofluorescence (585nm).

Imaging analysis of lung histology

The left lobe of the lung was harvested from mice after euthanasia by CO_2 asphyxiation and fixed with 4% paraformaldehyde (PFA) in PBS overnight at 4°C. The PFA/PBS solution was replaced with 20% sucrose/PBS for 1.5 days, and then the lobe was sliced into 3 pieces and embedded in O.C.T compound. Embedded samples were frozen with liquid nitrogen and stored at −80°C until preparation of 10 μm of histology sections.

Sections for immunofluorescent staining were incubated with primary antibodies diluted at a ratio of 1:50 in PBS/3% Bovine serum albumin/0.2% Triton X100 for 1 hour at room temperature. The sections were washed with PBS and then stained with secondary antibody for 1 hour at room temperature. Anti-fade reagent with DAPI (Life technologies) was dropped on samples for all fluorescent images prior to microscopy. Hematoxylin and eosin

staining was also used to identify the EGFP-positive regions of tissue. All histology samples were processed for taking images with the fluorescent microscope (Nikon, NY) and Volocity 5.5 software (PerkinElmer, MA). Images were captured with 10x, 20x, and 40x objectives in bright field, DAPI, FITC, and TRITC channels. Fluorescent and H&E images were overlaid manually with Photoshop CS3 (Adobe, CA).

Analysis of homologous recombination frequency in lung histology

Lungs were processed as indicated above. Histology sections were treated with anti-fade reagent with DAPI overnight and then whole lung histology was scanned with Metafer 4 v3.6.7 RCDetect (Metasystems, MA). Scanned area was set manually to cover whole lung histology. During the scan, both the nuclei and EGFP-positive cells were enumerated under a 20x objective in the DAPI and FITC channels, respectively. After the scan, each EGFP-positive cell was marked manually. The frequency of EGFP-positive cells in total cell count (DAPI-positive) was calculated by using the data on Detailed Search Report in RCDetect. The analysis protocol of RCDetect was validated with histology sections of both positive control (defined as $>370 \times 10^{-6}$ in EGFP constitutively expressing mouse) and negative control (defined as 0 in wild type 129 background).

Clusters of EGFP-positive cells were also enumerated in the lung histology within the scanned area, which consisted of assembled image grids. Two or more EGFP-positive cells in one grid were defined as one cluster. The mean area of each grid space was 0.13 mm^2.

Statistical analysis

Mann-Whitney *U*-test was conducted between young and aged group using GraphPad Prism 5. The distribution of EGFP-positive cells in RaDR mice is non-normal across tissues and among individual animals; therefore, a nonparametric rank comparison test is appropriate.

Results

Detection of rare fluorescent recombinant cells in RaDR lung tissue by flow cytometry and histology

To learn about HR-derived mutations in the lung, we first used flow cytometry to assay disaggregated lung samples. As shown in Figure 1B, cells from WT mouse lung show autofluorescence, producing approximately equal signals at 530 nm (detecting green fluorescence) and 585 nm (detecting red, or autofluorescence). In contrast, cells from positive control EGFP expressing mice (EGFP-Tg) show higher fluorescence at 530 nm, causing a shift to the right. We created a bracket that excludes autofluorescent cells and captures brightly fluorescent cells for a conservative quantification of mutated cells in the sample. Analysis of lung tissue from RaDR mice shows that rare cells (average 1.2×10^{-5}, n = 2) are positive for green fluorescence (Figure 1B, right panel). This is consistent with previous studies of RaDR mouse lung samples, where the mutant frequency in lung tissue was observed to range from 1×10^{-6} to 1×10^{-4} (Sukup-Jackson et al.).

While analysis by flow cytometry provides an unbiased approach for estimating the proportion of recombinant cells, information about cell type and context is lost. To learn

about recombination in the context of lung physiology, we extended this study to analyze green fluorescent cells within lung tissue sections. Figure 2A shows a representative image of lung tissue from WT mice with very little fluorescence. In contrast, most of the cells in the section from the positive control mouse are brightly fluorescent. Consistent with flow cytometry results, lung tissue from a RaDR mouse contains a small subpopulation of fluorescent green cells. RaDR mouse lung sections were also stained for H&E, and images showing green fluorescence were overlaid. These sections displayed healthy lung morphology, with alveolar air space bounded by thin layers of cells. We observed, as shown in Figure 2B, that healthy lung tissue contained rare fluorescent cells which appeared to be either Alveolar Epithelial Type I (AEI) or Alveolar Epithelial Type II (AEII) based on their locations and morphology. (Additional cell type analyses are described below.)

Interestingly, green fluorescent cells are often relatively close to each other, but not necessarily in direct contact. Previously, we have seen in other tissues that a single recombinant cell can clonally expand, leading to a cluster comprised of recombinant daughter cells (Sukup-Jackson et al. ; Wiktor-Brown et al.). However, we do not see such tight clustering of recombinant cells in the lung, which may be a consequence of lung tissue morphology. For example, Figure 2C illustrates how a band of fluorescent cells across an alveolus may appear as two distinct foci when analyzed by cross section. This observation prompted us to interrogate the relationships of clustered cells using more detailed analyses, discussed below.

Development of a platform for automated detection of fluorescent recombinant cells within RaDR lung tissue

It is now well established that mutant cells accumulate with age in several tissues, including pancreas, liver, and colon (Sukup-Jackson et al. ; Wiktor-Brown et al. ; Wiktor-Brown et al.). To explore the possibility that mutant cells accumulate in the lung, we quantified the proportion of fluorescent recombinant cells in frozen lung sections using the Metafer 4 automated slide-scanning system. The overview of our approach is diagrammed in Figure 3. Briefly, the left lobes of RaDR mouse lungs were excised and frozen in OCT for histology sectioning. The sections were stained for DAPI and scanned for all cells (DAPI-positive) as well as green fluorescent cells (DAPI-positive, EGFP-positive) using Metafer 4. Based on our flow cytometry data, the proportion of recombinant cells in the lung was expected to be low, on the order of 1×10^{-5} (see above and (Sukup-Jackson et al.)). To identify rare green fluorescent cells, we developed parameters for the RCDetect application in Metafer 4 that enable their detection. After setting the scan area, both recombinant cells and total nucleated cells in the area were automatically enumerated by Metafer 4 and the frequency of mutated cells was calculated to be on the order of 1×10^{-5}, consistent with the estimated mutant frequency from flow cytometry. This non-biased approach made it possible to quantify the proportion of mutated cells, calculated as the number of recombinant cells per million nucleated cells in the scanned area.

To learn about the impact of aging on the frequency of recombinant cells, we created cohorts with young (7–9 weeks) or aged (8–15 months) mice, with five animals per cohort. The experiment was conducted twice and the data was pooled for a total of ten mice per cohort.

Remarkably, we observed a significant increase in the proportion of recombinant cells in aged mice compared to young mice (Figure 4A, $p < .0001$, Mann-Whitney *U*-test). In both young and aged mice, the number of EGFP-positive cells is ~10^{-5}, within the previously reported range of 1×10^{-6} to 1×10^{-4} mutant cells in the lung as measured by flow cytometry (Sukup-Jackson et al.). Thus, we show for the first time that mutant cells accumulate with age in the lung, which may be an important factor when considering the underlying cause for increased risk of cancer with age.

Spatial distribution analysis reveals that fluorescent recombinant cells clonally expand during aging

In addition to knowing the overall proportion of recombinant cells, we were also interested in the possibility of clonal relationships among such cells, as had been previously observed in the pancreas, liver and colon (Sukup-Jackson et al. ; Wiktor-Brown et al.). To study clonal expansion, we again exploited the Metafer 4 platform, which can both quantify recombinant cells and also show the location of the recombinant cells within the lung tissue. Figure 4B shows Metafer 4 scans of young and aged mice (the three highest HR frequencies in each group are shown). Results confirm the initial quantification in Figure 4A, that lungs from aged mice have many more fluorescent cells than young mice.

Importantly, we observed that mutated cells are not evenly distributed, but rather occur in clusters. As described above, a recombinant cell can potentially divide to form many daughter cells in relatively close proximity. The chance that two cells located within the same field (one grid square) would both undergo independent RaDR mutation events is extremely unlikely, given that these events are known to be rare. Thus, to estimate clonal expansion from single mutant cells, we define a cluster as fluorescent cells either within the same square or in adjacent squares, indicated in Figure 4B by colored circles. Quantification shows that samples from aged mice have an approximately nine-fold increase in the number of clusters within the same scanning area of tissue (Figure 4C). Taken together, studies of the distribution of fluorescent cells indicate that the overall proportion of recombinant cells increases with age as a result of both *de novo* recombination and subsequent clonal expansion.

While our approach does not definitively show that nearby cells are clonally related, we aimed to estimate clonal expansion given the information available. Although it is possible that these cells could have undergone independent mutation events, it is improbable that such rare recombination events would occur in such close proximity and within clusters consisting almost entirely of the same cell type. (For example, if the RaDR transgene undergoes recombination in 1×10^{-5} cells, two adjacent cells independently developing the RaDR mutation has a probability of 1×10^{-10}) Supporting this inference, others have observed with lineage labeling that expansion can result in clusters of non-adjacent cells (Giangreco et al. ; Rawlins et al.), similar to clusters observed here. This may be an artifact of tissue sectioning, as proposed above, or a result of cell migration or tissue maintenance. Considering statistical likelihood and previous studies of lineage tracing, it is highly likely that these clusters of recombinant cells arose from clonal expansion of a single mutation rather than by independent mutation events.

Immunohistochemistry shows that both club cells and AEII cells can mutate and clonally expand

Virtually all cancers have mutations at tumor suppressor loci, and more than half of those loci lose function as a result of mitotic homologous recombination (Bishop and Schiestl ; Gupta et al.). Although we are assessing recombination from an integrated reporter, key aspects of the underlying molecular processes that drive HR at our reporter and at tumor suppressor loci are shared. It is therefore of great interest to know whether or not it is possible to detect recombination in cells that can give rise to cancers.

To learn which cell types can undergo mutagenic recombination to express the fluroescent reporter, immunohistochemical (IHC) staining was performed using antibodies against the well-established cell markers CC10 (Garcia-Sanmartin et al.) and SP-C (Ruiz et al.) to detect club and AEII cells, respectively (Figure 5). We selected to stain lung tissue from an aged mouse (15 months old) that had one of the highest HR frequencies to capture the largest possible number of mutated cell types. As can be seen in Figure 5A, a cluster of EGFP-positive cells counterstained for CC10, a club cell marker. In Figure 5B, we also observed many green fluorescent cells in relatively close proximity that stained positively for SP-C, the AEII cell marker (EGFP-positive cells that stained for SP-C are denoted by white circles, upper right box). Although not all of the green fluorescent club or AEII cells are touching, as described above, these cells are likely to be clonally related given their close proximity (note that the image of EGFP-positive AEII cells corresponds to the largest cluster in Figure 4Bvi), and it may be that this particular cross section did not capture all of the cells within this cluster. Taken together, we see that two major cell types, both of which have been shown to be associated with carcinogenesis (Cho et al. ; Kim et al. ; Mainardi et al. ; Moghaddam et al. ; Sutherland and Berns ; Xu et al.), can be monitored both for *de novo* mutations and clonal expansion – two major drivers of cancer.

Discussion

In this study, we describe the first *in vivo* model that enables detection of rare spontaneous recombinant cells and clusters resulting from their clonal expansion in the lung *in situ*. While recombination events are generally accurate, misalignments can cause sequence rearrangements, and HR-driven sequence rearrangements have frequently been observed in cancers (Bishop and Schiestl ; Costantino et al. ; Gu et al. ; Haigis et al. ; Pal et al.). Extensive studies show that conditions that promote HR are often tumorigenic (Heyer et al. ; Krejci et al. ; Schiestl et al. ; Schiestl et al.).

Here, we compared young mice and old mice and quantified the proportion of recombinant cells. Consistent with results from the pancreas (Wiktor-Brown et al. ; Wiktor-Brown et al.), we observed more than an order of magnitude increase in the accumulation of recombinant cells in the older mice. If we consider that any RaDR mutation could have easily been a mutation in a cancer gene instead, these results indicate that the chance of a second mutation arising in a pre-existing mutant cell continually increases during aging. Furthermore, lung cancers display genomic instability but require only a small number of mutations to begin tumor development (McFadden et al.). Profiling of Alu sequences (a repetitive element found throughout the genome) in lung cancers has demonstrated that aberrant HR between

homologous regions of the genome is associated with increased malignancy (Furmaga et al.). Thus, regardless of the type of mutation that initiates lung cancer, evaluation of HR-derived mutations in the lung provides important information about the potential for progression of such cancers.

Knowledge about which cell types can mutate and/or undergo clonal expansion is fundamental to understanding the underlying causes of cancer. Here, we have shown that at least two different cell types can undergo spontaneous mutation: club and AEII cells. Furthermore, we show that both club cells and AEII cells can likely undergo clonal expansion. Although we were unable to confirm clonality by lineage tracing, previous lineage studies have shown that clonal expansion in the lung can lead to clustered non-adjacent cells as observed here (Giangreco et al. ; Rawlins et al.). Furthermore, the fact that no significant clonal expansion was observed in young mice and clusters mostly consisted of the same cell type in aged mice, it is most likely that these cells acquired the RaDR mutation and proliferated during adulthood. In addition, because the clusters of mutated cells consist of the same cell type, our findings support the model that the adult lung secretory and epithelial cell populations appear to be maintained locally by distinct sets of progenitors (Giangreco et al. ; Rawlins et al.). Given the evidence that club, AEI and AEII cells as well as bronchioalveolar stem cells (BASCs) may be the cell(s) of origin for lung tumors (Cho et al. ; Kim et al. ; Mainardi et al. ; Moghaddam et al. ; Sutherland and Berns ; Xu et al.), our observations of mutation and clonal expansion in club and AEII cells hold significance with regard to lung carcinogenesis.

In previous studies, we have shown that as mice age, mutations accumulate and the extent of clonal expansion increases (Sukup-Jackson et al. ; Wiktor-Brown et al. ; Wiktor-Brown et al.). For the pancreas, aging leads to a higher frequency of *de novo* recombination events, which can be identified by isolated clusters of fluorescent cells. Additionally, in collaboration with the So Laboratory, we found extensive clonal expansion in the pancreata of aged mice, and discovered that the overall proportion of fluorescent cells is mostly a reflection of clonal expansion from isolated recombination events (Wiktor-Brown et al.). While the accumulation of mutations has also been demonstrated in the liver, bladder, heart, small intestine, and other tissues (Dolle et al. ; Hill et al. ; Ono et al. ; Stuart et al.), analogous studies had never been performed in lung tissue until now. Interestingly, different tissues have been found to accumulate different types of mutations with age; for instance, Dollé et al. demonstrated that the small intestine primarily develops point mutations, while the heart appeared to develop more large-scale sequence rearrangements (Dolle et al.). Other studies utilizing different transgenic mouse models for mutation detection reported that different tissues tend to develop similar types of point mutations with age (Hill et al. ; Ono et al.), but these studies did not incorporate analyses of sequence rearrangements. Furthermore, none of these studies were capable of *in situ* mutation analyses, and it is therefore impossible to determine how much of the observed mutation frequency resulted from increased *de novo* mutations or clonal expansion. It will be interesting to integrate RaDR sequence rearrangement data with studies of other types of mutations for a more complete picture of the age-dependent accumulation of different types of spontaneous mutations in different tissues.

Genomic instability has been identified as one of the “enabling characteristics” of cancer, without which the mutations required for carcinogenesis are statistically unlikely to ever occur (Hanahan and Weinberg ; Loeb). The spontaneous frequency of mutations is very low, on the order of about 10^{-6} for a mutation in a single allele, and approximately 10^{-5} for mutations that arise in the remaining wild type allele (e.g., LOH). If the spontaneous mutation rate is taken as 10^{-5}, then the probability of three independent sequential mutations in a single cell is 10^{-15}. Given that the total number of cell divisions in an adult is on the order of 10^{13}, cancers would never arise if the mutation rate were stable. Therefore, Loeb has proposed that cancer requires a hypermutable state (Loeb ; Loeb et al.). This well-substantiated model indicates that the mutation rate must be increased in order to accumulate the necessary mutations to form a metastatic cancer (Loeb ; Loeb). Importantly, clonal expansion also increases the odds of sequential mutations. If one mutant cell divides to become 100 cells with the same initiating mutation, the chance of a second mutation in that lineage increases by 100X (the same impact as increasing the mutation rate by 100X).

One of the most prevalent approaches to analyze fluorescent cells is flow cytometry. Our previous report on the RaDR system confirmed that all 11 tissues analyzed from RaDR mice showed a number of rare fluorescent recombinant cells when analyzed by flow cytometry (Sukup-Jackson et al.). However, most tissues require disaggregation to prepare cell suspensions, which causes loss of information about the context of the mutation. We therefore also described a whole mount imaging analysis method for three intact RaDR organs (liver, pancreas, and colon), which permits spatial resolution of mutations (Sukup-Jackson et al.). While the same approach would ideally be taken for the lung, the tissue’s unique morphology includes a great deal of air space, making it difficult to capture fluorescent foci. Here, we have shown that the Metafer 4 imaging platform offers an effective alternative approach for whole tissue imaging analysis with intact organs.

The Jacks laboratory has previously demonstrated the potential for lung cells to acquire K-ras mutations via spontaneous HR (Johnson et al.), providing an excellent model of orthotopic lung tumors arising from a common carcinogenic mutation. It would be very interesting to know how often recombination happens at the transgene, since this would give rise to new understanding regarding the likelihood that K-ras mutant cell goes on to form a tumor. However, due to the fact that recombination frequency is dependent on the design of the reporter (e.g., extent of homology, distance between repeats etc.) as well as gene expression, our models are not directly comparable. Furthermore, the frequency of mutations in this model is difficult to assess because the activated K-ras mutation is only identifiable by subsequent changes in tissue morphology that arise well after the mutation event and cannot be normalized to cell number or tissue area. Alternatively, in our model, the development of a mutation is neither advantageous nor harmful to the cell, so the accumulation of RaDR mutations is unbiased by phenotype of the mutation itself. Our study complements the Jacks model, which has revealed progressive genomic instability in K-ras-driven lung cancer (To et al.). Thus, the accumulation of mutations with age is compounded when one of those mutations is oncogenic. Having a tool that enables detection of recombinant cells within lung tissue thus enhances our ability to identify conditions that are likely to lead to lung carcinogenicity.

Development of an approach for quantifying HR in the lung tissue provides new avenues for evaluating the risk of genotoxic compounds, in terms of both *de novo* mutagenesis and proliferation of mutated cells. RaDR provides a method for studying spontaneous genetic sequence rearrangements in single cells of any tissue *in situ*, and can be multiplexed with other transgenic mutation detection systems, such as Gpt-Δ/Spi$^-$, permitting mutation analyses of sequence rearrangements (RaDR), point mutations (Gpt-Δ) and deletions Spi$^-$) from the same sample. The potential granularity of mutagenicity analyses at the whole-tissue level has never been possible without single-cell deep sequencing of entire tissues. This model has utility not only in the realm of genotoxicity testing, but also as a tool for studying the impact of environmental exposures and airway inflammation, which may individually or together put cells at risk for sequence rearrangements. In particular, explorations of the sequence rearrangement potential in established models of lung damage will be valuable, such as determining whether bulky adducts from smoking are recombinogenic. Since the RaDR mouse can detect sequence rearrangements in virtually every tissue, we also anticipate novel insights as to whether different exposures have mutagenic potential in “off-target” tissues. It will be interesting to use these mice in future studies to examine how various exposures lead to new mutations and impact the ability of pre-existing mutant cells to undergo clonal expansion.

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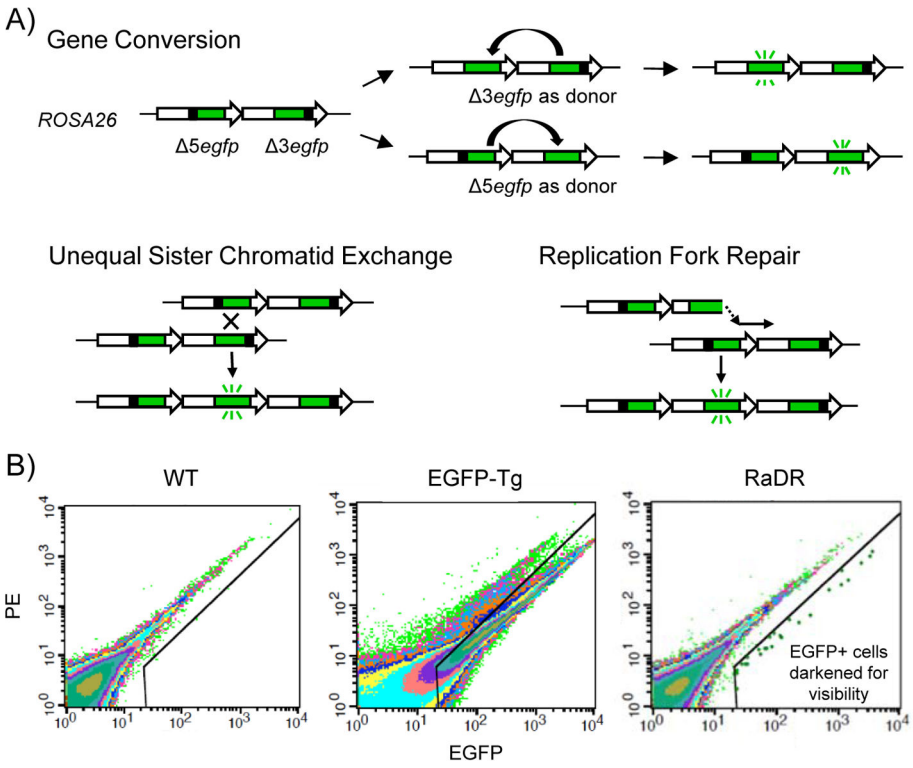

Figure 1.

Fluorescence detection of recombinant cells in RaDR lung sections. A) Mechanisms by which the RaDR transgene can undergo HR to produce a full length EGFP gene. B) Flow cytograms of lung cells from wild type (WT), EGFP constitutively expressed (EGFP-Tg), and RaDR mice. The horizontal axis measures fluorescence at 530 nm for EGFP, and the vertical axis measures autofluorescence at 585 nm. Cells with significantly higher levels of fluorescence at 530 nm than 585 nm are shifted right and fall into the bracket for “green fluorescence”. Cells in the bracket of the RaDR cytogram have been darkened for visibility.

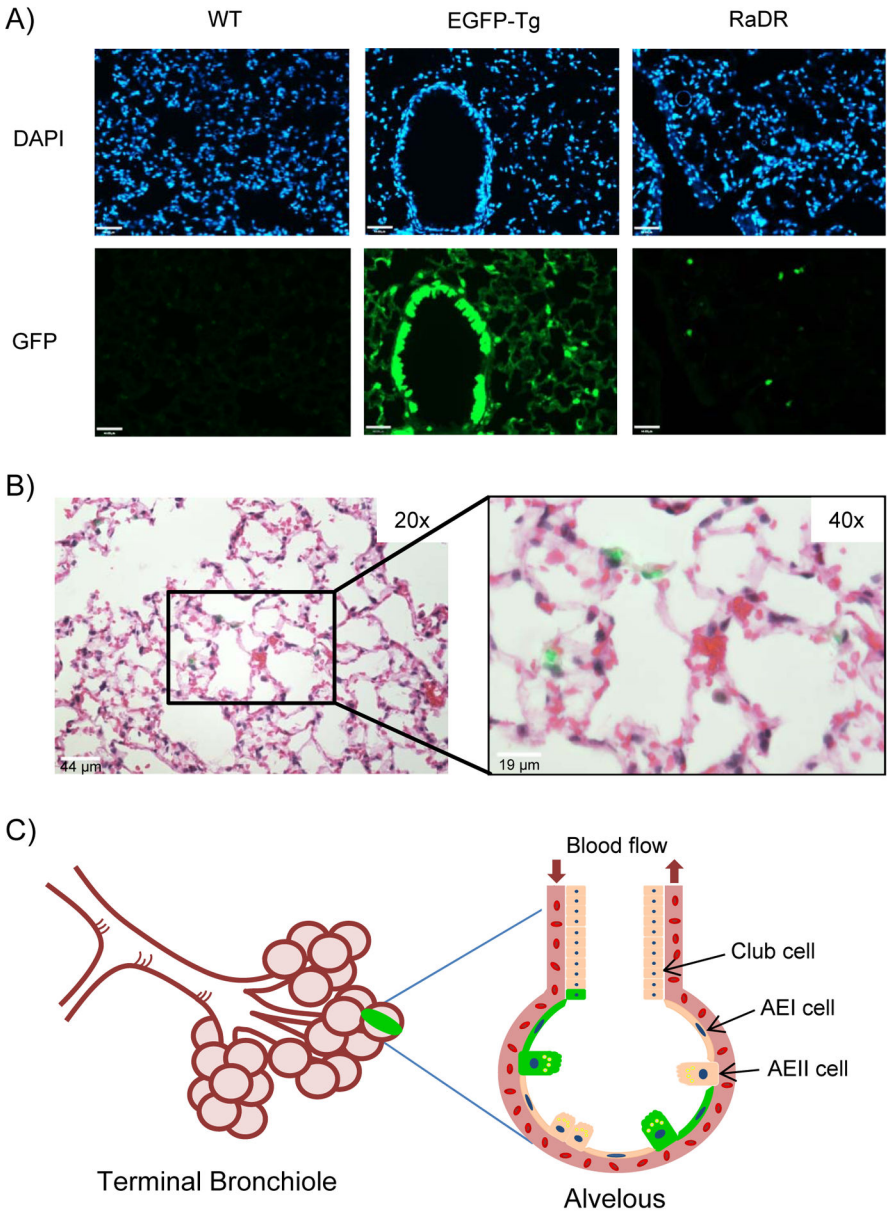

Figure 2.

A) Fluorescence imaging analysis of lung tissues from WT, EGFP-Tg, and RaDR mice. Cells that have undergone homologous recombination at the RaDR substrate in the *ROSA26* locus can form EGFP-positive foci (lower right, bar = 44 μm). B) Hematoxylin and eosin (H&E) staining of RaDR lung. A section from a RaDR mouse lung was stained with H&E after imaging for fluorescence. Overlaid images are consistent with some cells having undergone HR to produce EGFP. The left image was taken with the 20x objective (bar = 44 μm), and the right image was taken separately with the 40x objective (bar = 19 μm) to magnify the region enclosed by the black box. C) Example diagram showing how a strip of EGFP-positive cells may appear to be multiple foci after sectioning.

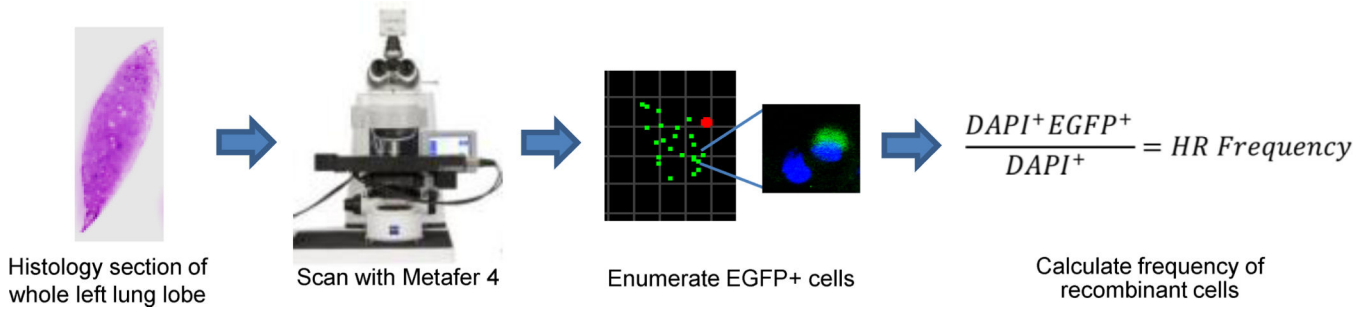

Figure 3.

Schema for automated analysis of recombinant cell frequency in lung tissue with Metafer 4. The left lobe of the lung was removed from RaDR mice, fixed with 4% paraformaldehyde, and embedded with OCT compound to make cryopreserved tissue sections. Sections were stained for DAPI with an anti-fade reagent and then scanned with Metafer 4. The scan result was analyzed to calculate the recombinant cell frequency in the lung.

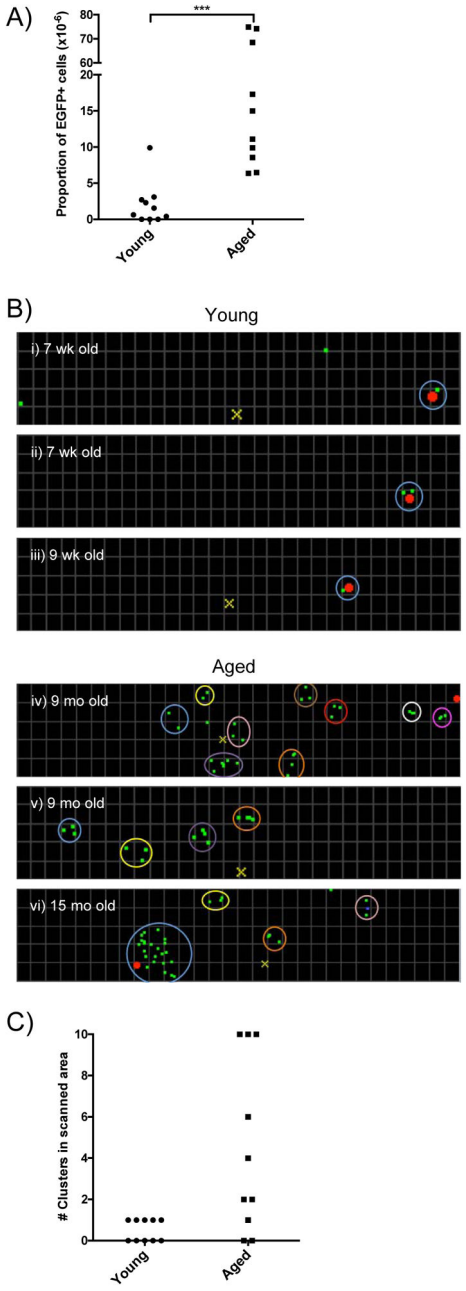

Figure 4.

Analyses of clusters of mutated cells in scanned lung sections. A) Comparison of spontaneous HR frequencies between young mice and aged mice (n = 10/group). The proportion of recombinant cells was statistically different between the two groups (Mann-Whitney *U*-test, $p < 0.0001$). B) Sample data from three young mice (i–iii) and three aged mice (iv–vi) with each cluster of fluorescent cells designated by a colored circle. The mean area per grid square was 0.13 mm^2. C) Number of clusters observed in samples from young (white bars) and aged (black bars) mice. Each bar represents the number of clusters in the scanned area of one left lung lobe.

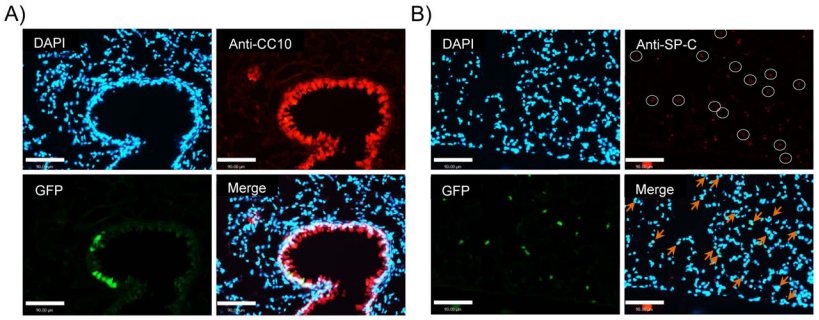

Figure 5.

Immunohistochemical analysis of RaDR lung. Lung histology sections from an aged mouse (15 months old) were stained with DAPI and using antibodies to either CC10 or SP-C. All images were taken with the 10x objective (bar = 90 μm). A) Detection of CC10 positive cells, consistent with club cells. B) Detection of SP-C positive cells, indicating AEII cells. White circles (top right) and orange arrows (bottom right) indicate the SP-C and EGFP double positive cells.