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Tissue Specific Differences in the Accumulation of Sequence Rearrangements with Age

Dominika M. Wiktor-Brown, Werner Olipitz, Carrie A. Hendricks, Rebecca E. Rugo, and Bevin P. Engelward*

Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, United States

Abstract

Mitotic homologous recombination (HR) is a critical pathway for the accurate repair of DNA double strand breaks (DSBs) and broken replication forks. While generally error-free, HR can occur between misaligned sequences, resulting in deleterious sequence rearrangements that can contribute to cancer and aging. To learn more about the extent to which HR occurs in different tissues during the aging process, we used Fluorescent Yellow Direct Repeat (FYDR) mice in which an HR event in a transgene yields a fluorescent phenotype. Here, we show tissue-specific differences in the accumulation of recombinant cells with age. Unlike pancreas, which shows a dramatic 23-fold increase in recombinant cell frequency with age, skin shows no increase *in vivo*. *In vitro* studies indicate that juvenile and aged primary fibroblasts are similarly able to undergo HR in response to endogenous and exogenous DNA damage. Therefore, the lack of recombinant cell accumulation in the skin is most likely not due to an inability to undergo *de novo* HR events. We propose that tissue-specific differences in the accumulation of recombinant cells with age result from differences in the ability of recombinant cells to persist and clonally expand within tissues.

Keywords

clonal expansion; mutation; aging; homologous recombination; pancreas; skin

1. Introduction

The accumulation of somatic mutations is considered to be a major cause of cancer and aging [1,2]. Mutations are believed to accumulate with age due to a combination of increased levels of endogenous DNA damaging agents, such as reactive oxygen species [3], and decreased efficiency and fidelity of DNA repair [4–8]. Double strand breaks (DSBs) are considered to be among the most toxic and mutagenic lesions that mammalian cells experience. In the context of aging, the steady-state levels of DNA DSBs have been shown to increase with age [9,10]. Furthermore, the improper repair of DSBs can lead to large scale genomic sequence rearrangements, such as translocations, insertions, and deletions [11], and an increased frequency of such rearrangements is often observed in aged cells [12–16]. Consistent with these findings, it has also been shown that deficiencies in the ability to

Corresponding Author: 77 Massachusetts Avenue, 56-631, Cambridge, MA 02139, Tel: 617-258-0260, Fax: 617-258-5424, Email: bevin@mit.edu.

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repair DSBs cause accelerated aging [17]. Together, these data suggest that DSBs and their repair are critical factors in the aging process.

Mammalian cells use two main pathways for the repair of DSBs: non-homologous end joining (NHEJ) and homologous recombination (HR). NHEJ directly rejoins DSBs in a sequence independent manner, often resulting in small sequence alterations [18,19]. In contrast, HR uses homologous sequences present on the sister chromatid or homologous chromosome as templates for repair, enabling the repair of DSBs with high fidelity [11]. NHEJ is the preferred pathway for the repair of DSBs during G₀/G₁ phases of the cell cycle, whereas HR is important during late S/G₂ [20]. Although HR is generally error-free, exchanges between misaligned sequences that lead to insertions, deletions, translocations, and loss of heterozygosity (LOH) can occur. Since over 40% of the genome is comprised of repeated elements [21], during G₀/G₁, NHEJ is preferred to minimize deleterious rearrangements [22]. In contrast, when a sister chromatid is present during S phase, HR is preferred since it plays an essential role in the repair of DSBs that arise as a result of replication fork breakdown (*i.e.*, replication fork encounter with a blocking DNA lesion or a single strand gap [23–27]). Indeed, HR is the only DNA repair pathway that can accurately reinsert the broken DNA end to restart the replication fork [11]. If DSBs at broken forks are instead acted upon by NHEJ, ends from independent loci may be joined, which will inevitably lead to large scale sequence rearrangements. Thus, in order to prevent mutation formation, it is critical that cells initiate the appropriate DSB repair pathway.

Germline mutations in genes that modulate DSB repair cause premature aging syndromes. For example, Werner syndrome is caused by a mutation in the RecQ-like DNA helicase WRN [28,29]. WRN is believed to play an important role in the resolution of HR intermediates [30,31], and loss of function of WRN is associated with an increased frequency of deleterious recombination events [32–34]. In addition, heritable mutations in ATM, a protein that plays a critical role in initiating DSB repair [35], result in ataxia telangiectasia, a disease that is associated with symptoms of premature aging [17]. Interestingly, ATM^{-/-} cells show an increased frequency of HR and are particularly susceptible to error-prone HR [36–39]. Finally, mice with germline mutations in Ku80 (Ku86), an integral protein in NHEJ, exhibit an accelerated aging phenotype [40]. Together, these results show that defects in DSB repair can promote aging.

Given its potentially pivotal role in suppressing aging and age-related diseases, there is great interest in understanding how DSB repair by HR changes in somatic cells with age. In previous studies, LOH events were analyzed to measure the accumulation of cells harboring recombined DNA during aging [41,42]. Since LOH can be caused by multiple mechanisms, careful analysis of DNA must be done in order to reveal the fraction of events resulting from HR. Using such analyses, it has been shown that HR is responsible for a large fraction of LOH events that accumulate with increasing age in both lymphocytes and kidney cells [41,42], suggesting that HR contributes significantly to DNA rearrangements that occur during aging. Due to technical limitations, little is known about the importance of HR in other cells types. In particular, unless a cell can be cultured *ex vivo*, the accumulation of recombinant cells cannot be studied using these approaches.

In addition to these studies of HR and LOH, the accumulation of point mutations in various tissues has also been measured by using mouse models for mutation detection [43–46]. Intriguingly, the effect of age on mutation frequency appears to be strongly tissue-dependent, and differences in mutation accumulation do not correlate with cell proliferation within the tissue. For example, in tissues with low proliferation rates, an increase in mutant cell frequency with age is seen in the heart [45,46] but not in the brain [43–45,47]. In addition, tissues that exhibit high proliferation rates, mutant cell frequency increases in the

small intestine [46,48] but not in the testis [43,45,49]. Thus, although the accumulation of mutations differs among tissues, the reasons for these differences are not yet understood.

Here, we set out to investigate the effects of aging on the frequency of HR events in two different tissue types *in vivo*. To study recombination *in vivo*, we developed the Fluorescent Yellow Direct Repeat (FYDR) mice, in which a HR event at an integrated transgene yields a fluorescent cell [50]. A comparison of pancreatic and skin tissues shows that while recombinant cells accumulate in the pancreas with age, the frequency of recombinant cells in skin does not change. Previously, by using *in situ* imaging analysis, we had shown that the accumulation of recombinant cells in aged pancreata results not only from *de novo* recombination events but also from clonal expansion of existing recombinant cells [51]. To determine if the lack of accumulation in skin results from a decrease in the demand for HR with age, we analyzed primary fibroblasts from FYDR mice *in vitro*. Neither the spontaneous rate of HR nor the ability of cells to use HR in response to an exogenous recombinogen changes with age, suggesting that fibroblasts within aged skin are able to undergo *de novo* recombination events *in vivo*. Thus, the lack of accumulation of recombinant cells in aged skin may be due to the absence of extensive clonal expansion in skin with age.

2. Materials and Methods

2.1 Animals

C57BL/6 FYDR mice were described previously [50]. Positive control FYDR-Recombined (FYDR-Rec) mice arose spontaneously from an HR event in a FYDR parental gamete, and all cells carry the full-length *EYFP* coding sequence [52]. FYDR cohorts had equal ratios of males to females. Controls were sex and age matched, except aged negative control C57BL/6 were 47–85 weeks old and the aged positive control FYDR-Rec mice were 52–68 weeks old.

2.2 Isolation of ear fibroblasts and ventral skin cells

Ventral skin or ears were isolated, minced, and incubated at 37°C in 4 mg/ml collagenase/dispase (Roche Applied Sciences). After 1 hour, two volumes of fibroblast medium was added [DMEM, 15% FBS, 100 units/ml penicillin, 100 µg/ml streptomycin, 5 µg/ml amphotericin B (Sigma)]. After 24 hours at 37°C and 5% CO₂, cells were tritured, filtered (70-µm mesh; Falcon), and analyzed by flow cytometry (ventral skin cells) or seeded into dishes (ear fibroblasts). For all *in vitro* studies, primary ear fibroblasts were isolated from 5 juvenile (4 weeks old) and 5 aged (62–89 weeks old) female FYDR mice in parallel. After pooling cells from mice within each cohort, ~1 million cells were analyzed by flow cytometry to determine the initial frequency of recombinant cells within each age group. The remaining cells were plated for *in vitro* studies.

2.3 Flow cytometry

Pancreatic cells were disaggregated as described previously [51]. Disaggregated pancreatic or skin cells were pelleted and resuspended in 350 µl OptiMEM (Invitrogen), filtered (35 µm), and analyzed with a Becton Dickinson FACScan flow cytometer (excitation 488 nm, argon laser). Live cells were gated by using forward and side scatter. On average, ~1 million cells were analyzed per sample for flow cytometry.

2.4 Calculation of recombination rate in primary fibroblasts

Using pooled primary ear fibroblasts isolated from 5 juvenile and 5 aged FYDR mice, 3 rate experiments were plated in parallel for each age cohort. For each rate experiment, ~10⁴ cells were seeded into 24 independent cultures. Cultures were expanded and analyzed by flow

cytometry once the density reached $\sim 10^6$ cells per well. The method of p_0 was used to determine the rate of recombination per cell division as described [53,54]. Rate experiments were repeated with fibroblasts from different cohorts of mice 3 times. For frequency analysis, the final frequencies for each culture of the rate experiment were averaged.

2.5 Comet assay

Alkaline comet assay experiments were performed using pooled primary ear fibroblasts isolated from 5 juvenile and 5 aged FYDR mice. Two independent experiments with 3 replicate slides per sample were carried out at dim light using a commercially available comet assay kit according to the manufacturer's protocol (Trevigen, Gaithersburg, MD). Briefly, cells were trypsinized, rinsed with ice cold Ca^{2+} and Mg^{2+} free PBS and counted. 2×10^4 cells were then suspended in 80 μl of 1% molten low melting point agarose and placed onto comet assay slides (Trevigen). After incubation at 4°C for 30 min slides were immersed in pre-chilled lysis solution (Trevigen) for 60 min at 4°C , rinsed with ice cold PBS and incubated in an alkaline solution ($\text{pH} > 13$) at room temperature for 40 min, allowing DNA unwinding. Next, slides were subjected to electrophoresis at 30V for 30 min followed by rinsing 5 min with 70% ethanol. Slides were kept in a moist chamber overnight at 4°C , stained with ethidium bromide and analyzed on a Nikon Fluorescence microscope using Komet 5.5 Software (Andor Technologies, South Windsor, CT). 99 cells per slide were quantified for percent comet tail DNA and Olive tail moment.

2.6 SCE analysis

Primary ear fibroblasts from 5 juvenile and 5 aged FYDR mice were pooled and seeded at 2×10^5 cells per well. After 24 hours, 10 μM BrdU was added to the culture media. Cells were harvested after undergoing two population doublings and analyzed for SCE frequency as previously described [55]. SCEs were counted in a blinded fashion from 2 independent experiments with each experiment containing cells combined from 5 mice per cohort.

2.7 Quantification of DNA damage-induced recombination

Primary ear fibroblasts from 5 juvenile and 5 aged FYDR mice were pooled and seeded at 5×10^5 cells per 100 mm dish. After 24 hours, triplicate or quadruplicate samples were exposed to 0.5 $\mu\text{g/ml}$ mitomycin-C (CAS No 50-07-7) in Dulbecco's Modified Eagle's Medium (Sigma) for 1 hour. After 72 hours, samples were analyzed by flow cytometry. Population growth was determined from the number of viable cells per dish. Experiments were repeated with fibroblasts from different cohorts of mice 3 times.

3. Results

3.1 Comparison of spontaneous recombinant cell frequency in pancreatic and cutaneous tissues

To study HR *in vivo*, we previously developed FYDR mice that carry a direct repeat recombination substrate containing two differently mutated copies of the coding sequence for enhanced yellow fluorescent protein (EYFP). An HR event can restore full length EYFP coding sequence, thus yielding a fluorescent cell (Fig. 1A). One method for determining the *in vivo* frequency of recombinant cells is to analyze disaggregated tissue by flow cytometry [51,56]. Briefly, for both pancreas and skin we compared fluorescence intensities in disaggregated tissue from negative control and positive control (FYDR-Recombined [52]) mice and established a region (R2) that excludes negative control cells (Fig. 1B, note: identical criteria were used to establish an R2 region for pancreas [51]). Over 21 million skin cells and over 34 million pancreatic were analyzed by flow cytometry and 0 and 1 cell appeared in the R2 region established for skin and pancreas, respectively, indicating an

extremely low frequency of false positives. Thus, while the actual number of fluorescent recombinant cells may be underestimated for both skin and pancreas, virtually all false positive cells are eliminated.

The distribution of spontaneous recombinant cell frequencies in pancreatic tissue from 100 mice aged 4–10 weeks was previously established and published elsewhere [51] (Fig. 1C, black bars). To compare homologous recombination between pancreas and skin, skin from 100 mice aged 4–10 weeks was similarly analyzed (Fig. 1C, gray bars). Although recombinant cell frequency is variable among mice for both tissues, the median recombinant cell frequency is statistically significantly higher for pancreas than for skin (Fig. 1D). Additionally, analysis of independent mice shows that ~20% of mice contained more than 20 recombinant cells per million in the pancreas, as compared to 4% for skin, suggesting that the distribution of recombinant cell frequencies among mice differs between tissues. High spontaneous frequencies of recombinant cells observed in some samples may result from an early HR event followed by clonal expansion of the resulting fluorescent cell. These data, therefore, raise the possibility that fluorescent recombinant cells within the pancreas may be more likely to clonally expand than those contained within the skin.

It is formally possible that differences in recombinant cell frequencies for pancreatic versus skin tissues may be due to differences in the expression levels of the *FYDR* transgene. Clearly, if *EYFP* is expressed at lower levels, it will be more difficult to detect recombinant cells. To determine if the increased frequency of recombinant cells in pancreatic tissue is due to increased *EYFP* expression, we analyzed pancreatic and skin tissues from positive control *FYDR*-Rec mice that carry the full-length *EYFP* coding sequence in every cell [52]. *EYFP* expression was, in fact, statistically significantly higher in pancreatic (~52%) versus skin (~30%) tissues (Fig. 1E). Given that the positive control *FYDR*-Rec mice express *EYFP* under an identical promoter and at the same locus as the *FYDR* recombination substrate, it is likely that there is higher expression in pancreatic tissue of the *FYDR* mice as well. This higher level of expression in the pancreas compared to skin may partially account for the higher median recombinant cell frequency in pancreatic compared to skin tissues and for the apparent differences in the distribution of recombinant cell frequencies between the tissue types. The observation that a larger proportion of the mice show very high frequencies of recombinant cells in the pancreas compared to skin raises the possibility that recombinant cells in the pancreas have the ability to persist and clonally expand, while those in the skin do not. We had previously established that recombinant cells accumulate in the pancreas with age [51]. We, therefore, extended these studies to explore the effects of aging on the accumulation of recombinant cells in the skin.

3.2 Tissue-specific effect of aging on recombinant cell frequency

To explore the effects of aging, recombinant cells were quantified in two different age groups: 'juvenile' (4 weeks old) and 'aged' (62–89 weeks old). For pancreatic tissue, the median frequency of recombinant pancreatic cells dramatically increased ~23 fold from juvenile to aged mice (Fig. 2A; shows previously published data [51] combined with new results). Given that we observed no statistically significant difference in *EYFP* expression with age in pancreatic cells (Fig. 2B), these data indicate that recombinant cells accumulate in the pancreas with age. For skin tissue, analysis of the recombinant cell frequency shows that the median is virtually identical between the juvenile and aged cohorts (Fig. 2C). Comparison of *EYFP* expression in skin with age shows no statistically significant difference between juvenile and aged mice (Fig. 2D). Thus, unlike in pancreatic tissue, recombinant fluorescent cells do not accumulate in cutaneous tissue with age.

3.3 Effect of age on HR in cultured primary fibroblasts

Previous studies [51] show that accumulation of recombinant cells in the pancreas is caused by both *de novo* recombination events and clonal expansion. Therefore, the fact that recombinant cells do not accumulate in skin can be explained by either a lack of *de novo* recombination events, a lack of persistence of cells that harbor recombined DNA, or a lack of clonal expansion with age (or some combination of these factors).

A lack of *de novo* recombination events may result from a reduced ability of aged cells to undergo HR at the FYDR substrate. To test this hypothesis, primary ear fibroblasts from juvenile and aged mice were cultured *in vitro*. When cells from juvenile and aged mice were allowed to expand *in vitro*, we observed that aged cells had a statistically significantly longer doubling time (Fig. 3A). While aged cells grew more slowly, cultures for both cohorts showed a similar increase in the frequency of recombinant cells as a consequence of *de novo* recombination events that occurred as the cells divided (Fig. 3B). Therefore, we conclude that HR is an active repair process in dividing cells *in vitro* for both age groups and that cells from both age cohorts are similarly susceptible to *de novo* recombination events. Furthermore, we estimated the rate of recombination in juvenile versus aged primary fibroblasts using the p_0 method [53,54]. We found that there is no statistically significant difference in the rate of recombination between juvenile and aged cells (Fig. 3B). To determine if differences in the *EYFP* expression levels exist, fibroblasts from juvenile and aged positive control mice were cultured in parallel with rate experiments. Results show that there is no significant difference in *EYFP* expression with age *in vitro* (data not shown), indicating that differences in *EYFP* expression do not affect the apparent rates of HR. Thus, these data suggest that juvenile and aged cells are comparable in their HR capacity.

The rate of HR is partially dependent on the site of integration of the recombination substrate [57]. To determine if the relationship between juvenile and aged cells is genome-wide, the frequency of another type of HR event, namely, sister chromatid exchanges (SCEs), was measured using an independent method. For SCE analysis, sister chromatids are differentially stained by culturing cells in the presence of the base analog 5-bromo-2'-deoxyuridine (BrdU) for two cell divisions. Recombination events that occur during these two replication cycles can be visualized in metaphase spreads (Fig. 3C). Blinded analysis of SCEs from juvenile and aged cells shows no statistical difference in the frequency of SCEs (Fig. 3D). Thus, since the rate of HR, as measured at the FYDR locus and by SCE analysis, is not statistically different between juvenile and aged fibroblasts, the lack of accumulation of recombinant cells in skin with age does not result from a suppression of HR.

DNA damage is known to induce HR, and therefore the rate of HR depends upon the amount of damage present within cells. To determine if the lack of recombinant cell accumulation in the skin might be due to diminished need to use HR, we assessed the amount of DNA damage within primary fibroblasts. For these studies, we evaluated the levels of single-strand breaks and alkali sensitive sites in juvenile and aged cells using the Comet assay. The average olive tail moment and percent tail DNA are two different ways to analyze the amount of DNA damage from Comet data [58]. The averages for both olive tail moment (Fig. 3E) and percent tail DNA (Fig. 3F) are not statistically different between juvenile and aged fibroblasts, suggesting that the number of spontaneous single-strand breaks and alkali sensitive sites does not differ between these age cohorts. Therefore, we conclude that it is unlikely that differences in spontaneous levels of DNA damage explain the lack of accumulation of recombinant cells in skin.

3.4 Effect of age on DNA damage-induced recombination

Although fibroblasts cultured from juvenile and aged mice are similarly able to recombine in the presence of spontaneous damage, the ability to respond to exogenous DNA damage may change with age. To determine if aged cells are differentially sensitive to exogenous DNA damage, juvenile and aged fibroblasts were treated *in vitro* with the cross-linking agent and potent recombinogen mitomycin-C (MMC). Cell proliferation and recombinant cell frequency were analyzed 72 hours post mock- or MMC-treatment. Compared to mock-treated cells, juvenile and aged cells treated with MMC exhibit similar decreases in cell densities (Fig. 4A), indicating that growth inhibition following MMC treatment is similar in both age cohorts. For both juvenile and aged fibroblasts, analysis of recombinant cell frequency by flow cytometry shows a statistically significant increase in the frequency of recombinant cells for MMC-treated as compared to mock-treated cells (Fig. 4B). However, there is no statistically significant difference in the magnitude of induction between juvenile and aged cells, suggesting that juvenile and aged fibroblasts are similarly able to respond to exogenous DNA damage.

4. Discussion

Many human progeroid syndromes, such as Werner Syndrome, Ataxia Telangiectasia, Cockayne Syndrome, and Trichothiodystrophy, are caused by defects in proteins that sense or repair DNA damage [17], indicating that the inability to accurately repair DNA damage contributes to aging. It is hypothesized that during the aging process, mutations accumulate within cells, causing diminished cell viability or capacity to carry out normal functions [2]. Over time, the number of mutant cells within a tissue can increase, resulting in an overall reduction of tissue function. An increase in mutant cell frequency with age can result from a combination of factors, including an increase in DNA damage levels, a decrease in DNA repair capacity and/or an increase in the persistence or clonal expansion of mutant cells within a tissue. In these studies, we analyzed the relative contribution of these factors in the context of mutant cell accumulation with age in pancreas and skin.

HR events are an important class of mutations that are believed to contribute to the aging process [41,42]. Using the FYDR mice, data presented here combined with previous studies [51] shows that the frequency of recombinant cells within pancreatic tissue increases ~23-fold with age, while in skin tissue there is no accumulation. Other mouse models, including Big Blue [43,44], Muta Mouse [45], and LacZ [46], have been used to examine changes in mutation frequency with age in various tissues. Consistent with the data presented here, the effect of age on mutation frequency appears to be strongly tissue-dependent. Although none of these studies have reported mutation frequency in the pancreas, increases in mutant cell frequency with age have been shown in a number of other gastrointestinal tissues including the liver and small intestine [43–48]. Interestingly, no tissues have shown such a dramatic increase in mutant cell frequency with age as observed in the pancreas. In terms of the skin, one study showed a slight increase (~1.5-fold) in mutant cell frequency with age [45]. While we cannot rule out that an increase in the frequency of recombinant cells in the skin may be seen in even older mice (the maximal lifespan of C57BL/6J mice is 110 ± 21 weeks [68]), this and other analogous studies [43–48] have shown little or no increase in mutant cell frequency is observed in skin with age, which is in sharp contrast to the large increase observed in pancreas and other gastrointestinal tissues.

We and others have found that the magnitude of the increase in mutant frequency with age did not correlate with cellular proliferation within the tissue. For example, very little accumulation of mutations was seen in testes [45,49], which contain highly proliferating cells [59]. In contrast, in liver and heart, which are slow or non-proliferating tissues [59], there was a significant accumulation of mutations with age [44–47]. These data suggest that

in addition to cell proliferation, other factors may also have a large impact on the accumulation of mutant cells with age. We hypothesize that the ability of mutant cells to persist and clonally expand within a tissue, rather than the rate of cell turnover, may have the greatest impact on the burden of mutant cell frequency with age.

For these studies, a comparison of pancreas and skin shows that while cells within the adult pancreas exhibit extremely low levels of proliferation (~1% of cells are in S phase [60]) and persist for long periods of time (turnover time of ~250–500 days [59]), cells within the skin have higher proliferation rates and relatively short persistence times (turnover time of ~7–60 days [59]). Despite the seemingly low level of proliferation in the pancreas, a dramatic increase in recombinant cell frequency is observed with age. In contrast to pancreas, skin does not appear to accumulate recombinant cells with age. Previously, we have analyzed FYDR pancreata by *in situ* imaging to show that the dramatic accumulation of recombinant pancreatic cells with age is caused by a combination of *de novo* recombinant events and clonal expansion [51]. In addition, since pancreatic cells have relatively long turnover times, once a recombinant fluorescent pancreatic cell appears, it can persist within the tissue for years. Therefore, the lack of accumulation of recombinant cells in skin may result from an absence of clonal expansion, an inability to undergo *de novo* HR events or a short persistence of recombinant cells. Here we find that primary fibroblasts from juvenile and aged mice showed no difference in their ability to undergo HR in response to spontaneous and exogenous DNA damage. While culturing cells *in vitro* may not directly reflect *in vivo* conditions, these results nevertheless suggest that an inability to undergo *de novo* HR events with age does not explain the lack of accumulation of recombinant skin cells. Therefore, these observations suggest that recombinant cells do not accumulate in the skin with age because they are either short lived or do not clonally expand.

Age is a risk factor for many diseases, including cancer [61]. Similar to aging, the accumulation of multiple mutations within cells is believed to cause cancer [1]. Furthermore, the clonal expansion of mutant cells has been shown to be a key step in tumor formation. In fact, analysis of tumors shows that key mutations in tumor suppressors and oncogenes are often shared by most, if not all, malignant cells within the tumor [62–64]. Because most mouse models for measuring mutations generally require tissue disaggregation, information regarding clonal expansion cannot be gathered. For example, if the same mutation is observed in multiple cells, it cannot be determined if the mutation results from multiple independent events (*e.g.*, at a mutation hot spot) or clonal expansion. Thus, many of the studies analyzing the accumulation of mutant cells with age either do not differentiate between the contribution of independent mutation events and clonal expansion [46,47,49,65] or completely remove the contribution of clonal expansion by specifically analyzing only independent mutation events [43,44]. Our previous studies [51], combined with the results presented here, indicate that clonal expansion contributes significantly to the overall increase in recombinant pancreatic cells with age. For example, we observe that in the pancreas >90% of the recombinant cells that accumulate in aged mice are due to clonal expansion (Wiktor-Brown *et al.*, in preparation). Thus, analyzing clonal expansion is important for ascertaining the underlying mechanisms that contribute to the increase in mutant cell frequency with age.

The effect of aging on a number of DNA repair pathways has been examined in multiple studies. The efficiency and fidelity of some DNA repair pathways such as base excision repair and NHEJ have been shown to decrease with age [4–7,66,67]. Here, we measured recombination using two independent methods: we assessed the rate of recombination at the FYDR locus (which is designed to measure not only exchanges between sister chromatids, but also gene conversions in the absence of crossovers), and we also measured the frequency of SCEs. Together, these data show that HR in primary fibroblasts does not change with age,

at least for mice up to 62 weeks of age. To note, the maximal lifespan of C57BL/6J mice is 110 ± 21 weeks [68]; thus, a change in HR may be seen in mice that are much older. Other studies have determined the effect of aging on HR by measuring SCEs in young and aged fibroblasts and lymphocytes. Interestingly, analyses of SCEs with age show conflicting results, with some studies showing no change in the spontaneous frequency of SCEs with age [69–71] and others indicating an increase in SCE frequency with age [72–74]. Thus, unlike some DNA repair pathways that exhibit decreased repair capacity with age, the ability of cells to undergo HR does not decrease with age, and, in fact, some studies suggest that the rate of HR may even be increased in aged cells [75].

HR is a critical DNA repair pathway known to contribute to aging. Here we have demonstrated that the accumulation of recombinant cells with age is tissue-specific, with pancreatic tissue showing a dramatic increase in recombinant cells and skin showing no increase. The differences in recombinant cell accumulation with age are most likely due to differences in the clonal expansion of recombinant cells within the tissues, indicating that analysis of clonal expansion is critical for understanding overall mutation burden within a tissue. Because of the ability to detect recombinant cells *in vivo* using fluorescence, the FYDR mice provide a unique tool to study the contribution of clonal expansion to recombinant cell frequency. Thus, in addition to age, the effects of other cancer risk factors, such as genetics and environment, on the clonal expansion of recombinant cells can be examined simply by crossing FYDR mice with transgenic mice predisposed to various cancers or by treating mice with different environmental agents. Determining the relative importance of clonal expansion to mutant cell frequency within different tissues is fundamental to our understanding of mechanisms that modulate susceptibility of various tissues to age associated degeneration and cancer.

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Fig. 1.

FYDR system and analysis of pancreatic and skin cells by flow cytometry. (A) Arrangement of the FYDR transgenic recombination substrate: large arrows indicate expression cassettes; yellow boxes show coding sequences; black boxes indicate positions of deleted sequences (deletion sizes not to scale). (B) Flow cytometry of disaggregated skin cells. Axes indicate relative fluorescence intensity at 515–545 nm (FL1) versus 562–588 nm (FL2). R2 region delineates *EYFP*-positive cells. Representative data are shown for a negative control C57Bl/6 mouse, a positive control FYDR-Rec mouse, and a FYDR mouse. For clarity, data for individual cells (dots) have been darkened in the FYDR R2 region. (C) Spontaneous frequency of recombinant pancreatic (black bars, data previously published [51]) and skin (grey bars) cells per million total cells analyzed as determined by flow cytometry for 100 FYDR mice aged 4–10 weeks. *n*, number of independent samples. (D) Median recombinant cell frequency for pancreatic and skin tissues from 100 FYDR mice aged 4–10 weeks.

*Median frequency of recombinant cells is statistically significantly higher for pancreatic versus skin tissues ($p < 0.0001$, 2-tailed Mann-Whitney). (E) Average percentage of fluorescent pancreatic ($n = 63$) and skin ($n = 33$) cells as determined by flow cytometry from 4–10 week old positive control mice. *Average percentage of fluorescent cells is statistically significantly higher for pancreatic versus skin tissues ($p < 0.0001$, 2-tailed student's *t*-test). Error bars indicate 1 standard deviation.

Fig. 2.

Effects of aging on the frequency of recombinant cells and expression of the FYDR substrate in the pancreas and skin. (A) Frequency of recombinant pancreatic cells per million as determined by flow cytometry for juvenile (n=49) and aged (n=41) mice. Previously, pancreata from a subset of juvenile (n=24) and aged (n=16) mice were analyzed [51]. Here, an additional 25 mice were added to each cohort. *Aged cohort is statistically significantly higher than juvenile cohort (p<0.0001, Mann-Whitney). Medians are indicated by black bars. Points on the x-axis indicate individual mice with 0 recombinant cells. (B) Average percentage of fluorescent pancreatic cells in positive control mice as determined by flow cytometry from juvenile (n=13) and aged (n=20) positive control mice. Error bars indicate 1 standard deviation. (C) Frequency of recombinant skin cells per million as determined by flow cytometry for juvenile (n=47) and aged (n=44) mice. Medians are indicated by black bars. Points on the x-axis indicate individual mice with 0 recombinant cells. (D) Average percentage of fluorescent skin cells as determined by flow cytometry from juvenile (n=17) and aged (n=11) positive control mice. Error bars indicate 1 standard deviation.

Fig. 3.

Effects of aging on spontaneous levels of HR and DNA damage in primary fibroblasts *in vitro*. (A) Average doubling times for juvenile and aged primary fibroblast cultures. *Aged cohort is statistically significantly higher than juvenile cohort ($p < 0.005$, 2-tailed student's t-test). Error bars indicate 1 standard deviation. (B) Frequency of recombinant cells per million at the time of plating (black bars) and after ~7 population doublings in culture (grey bars) of primary fibroblasts from juvenile and aged mice as determined by flow cytometry. Error bars indicate 1 standard deviation. (C) Average rate of HR in juvenile and aged cells as determined by the method of p_0 [53,54]. Error bars indicate 1 standard deviation. (D) SCEs per chromosome in juvenile ($n=138$) and aged ($n=138$) cells. Medians are indicated by black bars. (E) Average olive tail moment and (F) Average percent tail DNA in juvenile and aged cells. Error bars indicate 1 standard deviation.

Fig. 4.

Effects of aging on MMC-induced HR in primary fibroblasts *in vitro*. (A) Average cell density per million for mock- (black bars) and MMC-treated (grey bars) primary fibroblasts from juvenile and aged mice. *Cell densities for MMC-treated cohorts are statistically significantly lower than mock-treated cohorts ($p < 0.0001$, 2-tailed student's t-test). Error bars indicate 1 standard deviation. (B) Average frequency of recombinant cells per million for mock- (black bars) and MMC-treated (grey bars) primary fibroblasts from juvenile and aged mice. *Recombinant cell frequencies for MMC-treated cohorts are statistically significantly higher than mock-treated cohorts ($p < 0.001$, 2-tailed student's t-test). Error bars indicate 1 standard deviation.