The Impact of Age, Exposure and Genetics on Homologous Recombination at an Engineered Repeat Sequence in Mice

by

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Dedicated to my loving family

This work would not have been

possible without your constant

support

encouragement

and love

Preface

The work described in this thesis was done in collaboration with members of the Engelward and So laboratories. The Fluorescent Yellow Direct Repeat mice were created by Carrie A. Hendricks, Karen H. Almeida, Molly S. Stitt and others from the Engelward laboratory. I am extremely grateful to all those who contributed to this thesis project.

Chapter II was published in *PNAS* in August of 2006. The development of the assay for analyzing recombinant cells by flow cytometry and the collection of data for the analysis of recombinant cell type data was done in collaboration with Carrie A. Hendricks. Werner Olipitz, Carrie A. Hendricks and the author collected the data for the distribution of spontaneous recombinant cell frequencies in pancreatic tissue. Arlin B. Rogers performed the histological analysis to determine recombinant pancreatic cell type. The collection of data for the effect of mitomycin-C and age on homologous recombination was done in collaboration with Werner Olipitz. All other results and assays were contributed by the author.

Chapter III was published in *Cell Cycle* in December of 2006. Initial characterization of positive control FYDR-Recombined mice was done in collaboration with Carrie A. Hendricks. Werner Olipitz was instrumental in helping to collect data for the effect of mitomycin-C treatment on homologous recombination. Histological analysis of positive control FYDR-Recombined tissue sections was done by Arlin B. Rogers. All other results and assays were contributed by the author.

In Chapter IV, epifluorescence and two-photon microscopy techniques were developed in the laboratory of Peter T. So by Hyuk-Sang Kwon. Data for the number of cells per recombinant focus were collected by Yoon Sung Nam and Hyuk-Sang Kwon. Image analysis for quantification of cells per recombinant foci was done by Hyuk-Sang Kwon. The author contributed all other work.

In Chapter V, Carrie A. Hendricks developed the assays for detection of recombinant skin cells by flow cytometry. Werner Olipitz, Carrie A. Hendricks and the author collected the data for the distribution of spontaneous recombinant cell frequencies in skin and pancreas and for the frequency of recombinant skin cells with age. The assay for determining the effect of mitomycin-C on homologous recombination *in vitro* was developed in collaboration with Carrie A. Hendricks. The protocol used to measure the rate of homologous recombination was modified from an assay developed by Carrie A. Hendricks. Analysis of DNA damage by the Comet assay was done by Werner Olipitz. The sister chromatid exchange analysis was done in collaboration with Rebecca E. Rugo. The author contributed all other work.

Chapter VI will be submitted as a manuscript pending data collection for the effect of p53 status on DNA damage induced recombination. Data for the frequency of recombinant cells, the number of recombinant foci and the rate of homologous recombination was collected in collaboration with Saja A. FakhralDeen. The author was primarily responsible for all other work.

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Abstract

Mitotic homologous recombination is a critical pathway for the repair of DNA double-strand breaks and broken replication forks. Although homologous recombination is generally error-free, recombination between misaligned sequences can lead to deleterious sequence rearrangements, and conditions that stimulate homologous recombination are associated with an increased risk of cancer. To study homologous recombination *in vivo*, we used Fluorescent Yellow Direct Repeat (FYDR) mice in which a homologous recombination event at a transgene yields a fluorescent cell. To study homologous recombination using FYDR mice, we developed one- and two-photon *in situ* imaging techniques that reveal both the frequency and the sizes of isolated recombinant cell clusters within intact pancreatic tissue. We then applied these tools to analyze the effects of cancer risk factors such as exposure, genetic predisposition and age on homologous recombination *in vivo*.

To determine the effect of exposure to exogenous carcinogens on homologous recombination, FYDR mice were treated with two different chemotherapeutic agents, cisplatin and mitomycin-C. Results show that exposure to these DNA damaging agents causes an induction of recombinant pancreatic cells *in vivo*, indicating that homologous recombination is an active repair pathway in adult pancreatic cells and that exposure to certain carcinogens stimulates recombinational repair.

As a first step towards exploring the effect of genetic predisposition to genomic instability on homologous recombination *in vivo*, FYDR mice were crossed with mice carrying a defect in p53, a critical tumor suppressor that is mutated in almost 50% of all human tumors. Although loss of p53 is known to promote genomic instability, results show that p53 status does not significantly affect the spontaneous recombinant cell frequency in the pancreas *in vivo* or the rate of homologous recombination in cultured fibroblasts *in vitro*.

Age is a risk factor for many types of cancers. Here we examined the effect of age on homologous recombination in two tissues of FYDR mice, pancreas and skin. In the pancreas, a dramatic accumulation of recombinant cells is seen with age, resulting from

both *de novo* recombination events and clonal expansion of recombinant cells. In contrast, the skin shows no increase in recombinant cell frequency with age. *In vitro* studies using primary fibroblasts indicate that the ability to undergo homologous recombination in response to endogenous and exogenous DNA damage does not significantly change with age, suggesting that these skin cells are able to undergo *de novo* homologous recombination events in aged mice. Thus, we propose that tissue-specific differences in the accumulation of recombinant cells with age result from differences in the ability of these cells to persist and clonally expand within the tissue.

To further characterize the FYDR mice as a tool for studying homologous recombination, we exploited positive control FYDR-Recombined mice in which all cells carry the full-length coding sequence for enhanced yellow fluorescent protein. Studies show that expression of the FYDR transgene varies among mice, among tissues, and even among cells within a tissue. However, the variation in FYDR expression does not significantly change with age or exposure to exogenous carcinogens. Furthermore, positive control mice reveal that several tissues, in addition to the pancreas and skin, may be amenable for studying homologous recombination in the FYDR mice. Thus, our studies demonstrate that FYDR mice combined with *in situ* imaging technology provide powerful tools to study the effects of cancer risk factors on homologous recombination *in vivo*. Ultimately, by applying these techniques to study additional cancer risk factors, we may better understand the relationship between DNA damage, homologous recombination and cancer.

Thesis Supervisor: Bevin P. Engelward
Title: Associate Professor of Biological Engineering

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BIR	break-induced replication
bp	base pair
DSB	double-strand break
DSBR	double-strand break repair
EYFP	enhanced yellow fluorescent protein
FYDR	fluorescent yellow direct repeat
FYDR-Rec	fluorescent yellow direct repeat-recombined
H&E	hematoxylin and eosin
HR	homologous recombination
IP	intraperitoneal
kb	kilobase
LOH	loss of heterozygosity
MMC	mitomycin-C
NHEJ	nonhomologous end-joining
SCE	sister chromatid exchange
SDSA	synthesis-dependent strand anealing

Chapter I

Introduction

Chapter 1

1.1 Homologous Recombination and Genomic Stability

Mitotic homologous recombination is critical for the repair of genome destabilizing DNA damage, such as double-strand breaks and interstrand cross-links. These DNA lesions can be caused by environmental exposures, such as radiation or chemotherapy, and by endogenous cellular metabolites, such as reactive oxygen species (1-4). In addition, double-strand breaks can occur as the result of DNA processing. For example, replication fork encounter with a DNA lesion or a single strand gap can cause replication fork breakdown, creating a double strand end (5-9) (Figure 1-1). Regardless of how they arise, double-strand breaks are considered to be among the most cytoxic and mutagenic DNA lesions, since a single unrepaired double-strand break can result in the permanent loss of over 100 million base pairs of genetic information (10), and misjoining of double-strand breaks can lead to gross chromosomal rearrangements (11). By using homologous DNA sequences present on the sister chromatid or homologous chromosome as templates, homologous recombination can repair double-strand breaks with high fidelity (Figure 1-2A). In addition, homologous recombination provides the only pathway for the accurate repair of double-strand breaks that arise as a result of broken replication forks (12). Thus, homologous recombination is important for preventing mutagenic sequence rearrangements that can result from double-strand breaks.

In addition to homologous recombination, cells use another DNA repair pathway, nonhomologous end-joining, to recognize and repair double-strand breaks. In contrast to

homologous recombination which uses homologous sequences as templates for repair, nonhomologous end-joining directly rejoins double-strand breaks, regardless of sequence (13, 14) (Figure 1-2B). Nonhomologous end-joining is the preferred pathway for the repair of double-strand breaks during G_0/G_1 phases of the cell cycle, whereas homologous recombination is important during late S/G_2 (15). Although homologous recombination is generally error free, exchanges between misaligned sequences can lead to insertions, deletions, translocations, and loss of heterozygosity. Since over 40% of the genome is comprised of repeated elements (16), high levels of homologous recombination during G_0/G_1 can lead to increased recombination between misaligned sequences. Thus, nonhomologous end-joining is preferred during G_0/G_1 to minimize deleterious rearrangements (17). In contrast, when a sister chromatid is present during S phase, homologous recombination is preferred since it plays an essential role in the repair of double-strand breaks at broken replication forks (12). If replication fork-associated double-strand breaks are instead acted upon by nonhomologous end-joining, ends from independent loci can be joined, which will inevitably lead to large scale sequence rearrangements. Therefore, in order to prevent mutation formation, it is critical that cells initiate the appropriate double-strand break repair pathway.

Given its key role in the repair of double-strand breaks and broken replication forks, it is not surprising that loss of function of homologous recombination can promote genomic instability. Germline deletions of key homologous recombination proteins are embryonic lethal in mice (e.g., RAD51 (18-21), BRCA1 (22), BRCA2 (22, 23)). In addition, inherited mutations in genes that result in impaired homologous recombination function are associated with higher incidences of cancer. For example, germline

mutations in *BRCA1*, *BRCA2*, *FANCC*, and *NBS1* increase the risk of many types of cancer (24-28). Loss of function of these genes suppresses homologous recombination (29-32) and causes an increased frequency of tumor-promoting sequence rearrangements (33, 34).

While the suppression of homologous recombination can cause genomic instability, too much homologous recombination can also increase the risk of deleterious sequence rearrangements. The repair of a double-strand break by homologous recombination requires a repair template, *i.e.*, a sequence of duplex DNA that is homologous to the sequence surrounding the double-strand break. A sister chromatid, homologous chromosome or one of many repeated elements throughout the genome (16) can all potentially serve as repair templates for a double-strand break. Recombinational repair involves the nonreciprocal transfer of genetic sequence information from a donor template to the broken strand, a process known as gene conversion. In addition, the resolution of homologous recombination intermediates can lead to exchanges of flanking sequences. Thus, an inherent risk of deleterious sequence changes is associated with every homologous recombination event and depends upon which substrate is chosen as the repair template. For example, a sister chromatid carries genetic information identical to that of the broken strand; thus, the chance of deleterious sequence rearrangements is minimal. In contrast, transferring genetic information or exchanging flanking sequences from a homologous chromosome or repeated element can lead to mutations such as loss of heterozygosity and translocations, sequence changes that can initiate or promote cancer (35). Thus, increased levels of homologous recombination increase the likelihood of potentially deleterious gene conversion events.

Given that increased levels of homologous recombination can promote genomic instability, it is not surprising that germline mutations in genes that increase homologous recombination are associated with higher cancer incidence and often with an accelerated aging phenotype. For example, inherited mutations in two RecQ-like DNA helicases, WRN and BLM, cause Werner and Bloom syndromes, respectively (36-38). Both syndromes are characterized by increased cancer risk (24), and Werner syndrome patients also exhibit an accelerated aging phenotype (39). WRN and BLM are believed to play important roles in the resolution of homologous recombination intermediates, particularly those that arise at stalled replication forks (40-44), and loss of function of either WRN or BLM is associated with an increased frequency of deleterious recombination events (45-49). In addition to BLM and WRN, heritable mutations in ATM (ataxia-telangiectasia, mutated), a protein that plays a critical role in initiating double-strand break repair (50), result in ataxia telangiectasia, a disease that is associated with increased cancer incidence and symptoms of premature aging (51, 52). Interestingly, $ATM^{-/-}$ cells show an increased frequency of homologous recombination and are particularly susceptible to error-prone homologous recombination (53-56). Taken together, these data suggest that deregulation of homologous recombination can promote sequence rearrangements that cause genomic instability.

1.2 Recognition and Repair of Double Strand Breaks by Homologous Recombination

The repair of a double-strand break occurs via a series of complex signaling cascades that are initiated by the recognition of the double-strand break. Although the exact mechanism of double-strand break recognition is not known, one of the earliest repair factors present at the site of a double-strand break is the Mre11-Rad50-Nbs1 (MRN) complex, which recruits and activates ATM (57-61). Subsequently, activated ATM serves as a key facilitator of the double-strand break repair signaling cascade (for a review see (50)) as it phosphorylates a number of downstream targets, such as p53 (62-64), MDM2 (65, 66), CHK2 (67, 68), BRCA1 (69-71), and NBS1 (72-75). Importantly, loss of function of many of these proteins involved in the recognition of double-strand breaks results in impaired double-strand break repair and increased genomic instability (28, 50).

Once a double-strand break has been recognized, repair of the double-strand break by homologous recombination is initiated by the MRN complex, which contributes to the 5' to 3' resection of the double-strand ends to produce the single-stranded 3' overhang required for homologous recombination (76) (Figure 1-3A, 1). NBS1 of the MRN complex contains binding domains that recruit the complex to the double-strand break (77), RAD50 has long coiled coils that hold adjacent strands of DNA together (78-80), and MRE11 has associated nuclease activity that is believed to contribute to the processing of the double-strand break ends (81, 82). Once a single-stranded 3' overhang

is formed, it is protected from further nuclease activity by coating with the single-strand binding protein RPA.

In addition to ATM and MRN, another complex of proteins, the Fanconi Anemia complex (FANCA, C, E, F, G, L), is activated in response to DNA damage and aids in the initiation of the next step of homologous recombination, the loading of RAD51 onto the 3' overhangs (83-85). The activated Fanconi Anemia complex promotes the loading of BRCA2 onto the ends of the double-strand break (86, 87). Subsequently, BRCA2 displaces RPA from the single-stranded 3' ends and initiates the formation of the RAD51 nucleoprotein filament (88). The RAD51 nucleoprotein filament facilitates homology searching, invasion into duplex DNA, and strand exchange (Figure 1-3A, 2) (89). In addition to RAD51, many proteins including RAD52, RAD54, and the RAD51 paralogs (RAD51b, RAD51c, RAD51d, XRCC2, and XRCC3) stimulate homologous recombination, although their exact functions have not yet been elucidated (2).

Once a homologous sequence is located, DNA synthesis using the duplex DNA as a template is initiated to extend the 3' end of the invading strand beyond the original break site, restoring any missing sequence information at the double-strand break (Figure 1-3A, 3). The process of invasion and strand synthesis leads to the formation of a four-branched structure called a Holliday Junction (Figure 1-3A, 4). The steps described above (*i.e.*, 5' to 3' resection of the double-strand end to produce a 3' overhang, formation of a RAD51 nucleoprotein filament, homology searching, strand invasion, strand exchange, synthesis and Holliday Junction formation) are common to all homologous recombination events. However, the resolution of the Holliday Junction differs between proposed models of homologous recombination.

The predominant mechanism by which homologous recombination repairs two-ended double-strand breaks is believed to be Synthesis-Dependent Strand Annealing (SDSA) (12). In the Synthesis-Dependent Strand Annealing model, the Holliday Junction can move along the DNA via branch migration (90) (Figure 1-3A, 5), which is believed to be facilitated by a number of proteins including BLM, WRN, and RAD54 (41, 43, 91). Branch migration of the Holliday Junction in the direction of DNA synthesis can result in release of the invading DNA strand from the duplex DNA (Figure 1-3A, 6). The invasion and synthesis steps can occur independently for both ends of the double-strand break. If DNA synthesis of at least one invading strand extends past the break point, the two ends of the double-strand break will contain complementary sequences that can be used to anneal the two double-strand ends together (Figure 1-3A, 6), thus repairing the double-strand break with transfer of genetic sequence from the donor template to the site of the double-strand break.

Although Synthesis-Dependent Strand Annealing is thought to be the prevalent mechanism of double-strand break repair by homologous recombination, a second type of repair, Double-Strand Break Repair (DSBR), can occur if branch migration does not release the invading strand (92, 93). In this case, in order to separate the two DNA duplexes, the Holliday Junctions formed from both ends of the double-strand break must instead be cleaved (Figure 1-3B, 4). Depending on how these junctions are cleaved, flanking sequences may or may not be exchanged between the two DNA duplexes (crossover or non-crossover, respectively) (Figure 1-3B, 5). To note, analysis of resolution products from the repair of site-specific double-strand breaks by homologous recombination shows that crossover events are rare. Instead, gene conversion events

without crossover are the predominant outcome (94), suggesting that the Double-Strand Break Repair model may not be the predominant mechanism by which most recombinational repair of double-strand breaks occurs *in vivo*.

While two-ended double-strand breaks can be repaired by either nonhomologous end-joining or homologous recombination, the accurate repair of one-ended double-strand breaks created at broken replication forks can only be accomplished by homologous recombination (12). When a replication fork encounters a single-strand nick or gap in the DNA backbone (or a lesion that it cannot bypass), the replication fork breaks down and a double stranded end is released (Figure 1-1) (5-9, 95). In a process called break induced replication (BIR), homologous recombination uses homologous sequences present in the sister chromatid to reinsert the broken end into the DNA duplex to restore the broken replication fork (12). During break induced replication, reinsertion of the double stranded end proceeds by a mechanism that is essentially a one-ended invasion process analogous to Synthesis-Dependent Strand Annealing, *i.e.,* 5' to 3' resection to produce a 3' overhang, homology searching, strand invasion, strand exchange, synthesis and Holliday Junction formation (Figure 1-3C, 1-2). The Holliday Junction formed by the invading and template strands must be cleaved in order to resolve the two DNA duplexes (Figure 1-3C, 3) and restart the replication fork (Figure 1-3C, 4). Thus, homologous recombination provides the only pathway to accurately repair replication fork-associated double-strand breaks.

1.3 Using Fluorescent Yellow Direct Repeat Mice to Study Homologous Recombination *in vivo*

A number of mouse models have previously been used to detect mitotic homologous recombination events that occur *in vivo*, including pink-eyed unstable (p^{un}), $Aprt^{+/-}$ and $Tk^{+/-}$ mice. p^{un} mice carry a naturally occurring 70 kb duplication at the *p* locus, a gene responsible for normal melanin production (96-98). The duplication prevents normal melanin expression, resulting in mice with light gray fur and pink eyes. A homologous recombination event early during embryonic development that deletes one of the 70 kb duplications can restore normal *p* gene function and give rise to black spots on the gray fur and retinal epithelium of adult mice (96, 99, 100). p^{un} mice have been used extensively to study the effect of environmental agents and genetic conditions on homologous recombination (101-107). However, because clonal expansion of recombined cells is required to detect spots on both fur and retinal epithelium, p^{un} mice can only be used to study recombination events that occur during embryogenesis, and information about the repair of DNA lesions by homologous recombination in adult tissues cannot be gathered.

In contrast to the naturally occurring recombination substrate present in p^{un} mice, $Aprt^{+/-}$ and $Tk^{+/-}$ mice have been engineering to measure homologous recombination events indirectly by assessing loss of heterozygosity (108-111). In these mice, loss of heterozygosity events leading to loss of function of Aprt or Tk result in cells that exhibit a selectable phenotype. Although loss of heterozygosity can be caused by many different mechanisms, it has been shown that a significant fraction of the gene loss in $Aprt^{+/-}$ mice

is due to mitotic recombination events (110, 112-114). Thus, these mouse models have been used to indirectly measure the effects of age, environment, and genetics on homologous recombination (115-122). However, since cells must be cultured in the presence of toxic base analogs in order to detect loss of heterozygosity events, these mice can only be applied to study homologous recombination in cells that can be cultured *ex vivo*.

Given the limitations of these mouse models for the detection of homologous recombination events that occur *in vivo*, little was known about homologous recombination in many adult somatic tissues until the development of the Fluorescent Yellow Direct-Repeat (FYDR) mice (123), which enabled studies of homologous recombination in adult somatic cells. The FYDR mouse carries two truncated copies of the expression cassette for the enhanced yellow fluorescent protein (EYFP) arranged in tandem (Figure 1-4). The 3' eyfp cassette lacks the first 96 bp of coding sequence from the N-terminus, and the 5' eyfp lacks the final 42 bp from the C-terminus of the *EYFP* coding sequence. The truncation of essential coding sequences prevents expression of *EYFP*. A homologous recombination event between the two cassettes can restore the full length *EYFP* coding sequence, thus yielding a fluorescent cell. One advantage of using fluorescence as a marker for sequence changes is that cells harboring recombined DNA can potentially be detected within intact tissues. Thus, the FYDR mice can be applied to reveal the effects age, genetics, and exposure on homologous recombination in adult somatic tissues *in vivo*.

1.4 Pancreas and Pancreatic Cancer

Pancreas Anatomy and Physiology

Inherited mutations in proteins that modulate homologous recombination (*i.e.*, *BRCA1*, *BRCA2*, and *FANCC*) increase the risk of pancreatic cancer (24-26, 29-32). Although these findings suggest that homologous recombination is critical for maintaining genomic integrity in the pancreas, no studies had explored the activity of homologous recombination in mature pancreatic cells.

The pancreas is located posteriorly in the upper abdomen, attached to the duodenum and can be divided histologically into two parts, the exocrine and endocrine pancreas (124) (Figure 1-5). The exocrine pancreas makes up approximately 95% of the volume of the organ and consists of two main cell types, acinar (85%) and ductal epithelial (10%) cells (125). The secretory unit within the exocrine pancreas is a grape-like structure called an acinus, which contains ten to forty acinar cells that secrete enzymes into a system of epithelial ducts (126).The epithelial ducts carry these hydrolytic enzymes, such as trypsin, chemotrypsin, elastase, and amylases, into the duodenum where they aid in digestion (125).

The endocrine pancreas makes up the remaining 5% of the volume of the organ and consists of islets of Langerhans interspersed throughout the pancreas (Figure 1-5). Islets contain four major cell types: β (beta), α (alpha), δ (delta), and PP cells. The β cells (60-80% of the endocrine pancreas) secrete insulin, α cells (15-20% of the endocrine pancreas) secrete glucagon, δ cells (5-10% of the endocrine pancreas) produce

somatostatin, and PP (< 2% of the endocrine pancreas) cells secrete a pancreatic polypeptide that stimulates secretion of gastric and intestinal enzymes (127).

Although the development and function of pancreatic cells has been studied extensively, the identification of an adult pancreatic stem cell within either the exocrine or endocrine pancreas has eluded scientists (128, 129). Data shows that in the adult pancreas, both cellular compartments of the pancreas proliferate, although at low levels (~1% of cells are in S phase (130)). In addition, pancreatic cells persist for long periods of time (131, 132), with half-lives estimated to be ~70-132 days for acinar cells, ~40 days for ductal cells, and ~47-170 days for islet cells (133, 134). Although pancreatic cells are normally quiescent, in response to damage, such as pancreatectomy or chemical injury, proliferation of acinar, ductal, and islet cells is stimulated (135-137). Since homologous recombination is most active during S phase of the cell cycle (94) and since pancreatic cells normally exhibit low proliferation rates, the importance of homologous recombination on mutant cell frequency in the pancreas may only be revealed after long periods of time, for example, in pancreata of aged mice. By labeling recombinant cells with fluorescence, the FYDR mice enable the analysis of the cumulative effects of homologous recombination in pancreatic cells over time, thus potentially revealling the role of homologous recombination in tissues with low proliferation rates such as the pancreas.

Pancreatic Cancer

With a 5 year survival rate of less than 5%, pancreatic cancer remains one of the deadliest cancers in the United States (138, 139). The majority of pancreatic neoplasms

are ductal pancreatic adenocarcinomas (80-90%). Although most pancreatic tumors appear to have a ductal phenotype, there is debate over the actual origin of cells that appear in ductal andenocarcinomas. Some studies suggest that acinar cells are able to transdifferentiate to acquire the features of ductal cells. For example, multiple *in vitro* studies have been able to show transdifferentiation of primary acinar cells to ductal cells (140-144). In addition, *in vivo* studies show that mice expressing oncogenic K-ras or TGF-α targeted specifically to the acinar cell compartment developed pancreatic intraepithelial neoplasias and pancreatic ductal adenocarcinoma (145-150). Interestingly, expression of oncogenic K-ras under promoter specific for pancreatic ductal epithelial cells does not result in the development of neoplastic lesions (151). Together, these data suggest that acinar cells may play an important role in tumor formation.

Pancreatic cancer, like most other cancers, results from the accumulation of multiple genetic mutations. Over the last several years, mutations in specific tumor-suppressor and oncogenes (*i.e.*, *KRAS*, *CDKN2A/p16*, *p53*, *SMAD4/DPC4*, *HER2/neu*, and *BRCA2*) have been identified for progressive stages of pancreatic cancer, with more severe cancers exhibiting accumulation of mutations in multiple genes (139, 152). The underlying causes of changes in the expression of many of these genes have been analyzed and include translocations, amplifications, deletions, point mutations, and loss of heterozygosity (139). Given the importance of homologous recombination proteins both in the predisposition to (*i.e.*, inherited mutations in *BRCA1*, *BRCA2*, *FANCC*) and in the progression of pancreatic cancer, determining the effect of cancer risk factors on homologous recombination in pancreatic cells may be important to understanding the mechanisms of cellular transformation in the pancreas, and the FYDR mice provide a

unique tool for assessing the effect of these risk factors on homologous recombination *in vivo*.

1.5 Skin and Wound Healing

Skin Anatomy and Physiology

A number of studies have analyzed homologous recombination in skin cells *in vivo*. As mentioned above, p^{un} mice, in which the clonal expansion of embryonic recombination events appear as dark spots on the light gray skin, have been used to study the effects of multiple genetic and environmental factors on homologous recombination in skin cells (101-107). However, since this assay is limited to measuring recombination events that occur during embryogenesis, homologous recombination in adult skin cannot be analyzed. Using $Aprt^{+/-}$ mice, one study determined the effect of age on homologous recombination in ear fibroblasts and observed a small increase in the frequency of loss of heterozygosity events with age in cells from female mice (117). However, in order to determine mutant cell frequency in $Aprt^{+/-}$ mice, cells have to be cultured in the presence of a base analog *ex vivo*; thus, mutation events that occur *in vitro* versus *in vivo* cannot be differentiated. Therefore, although some studies have analyzed homologous recombination in the skin, little is known about the *in vivo* frequency of recombinant cells in adult skin.

The skin forms the largest organ of the body and performs vital roles both as a barrier against microorganisms and chemical insults and as a regulator of heat and water

loss from the body. The skin can be divided histologically into 3 distinct layers: the epidermis, dermis, and subcutaneous tissue (Figure 1-6). The epidermis is the outermost layer of skin, composed mostly of keratinocytes, with interspersed antigen presenting cells and pigment producing melanocytes. The keratinocytes located within the basal layer of the epidermis actively proliferate, giving rise to cells that differentiate as they move upward through the suprabasal layers until being shed at the surface of the skin (153), a process that takes ~10-14 days in the mouse skin (154). The next layer of skin just below the epidermis is the dermis. The main cell type located within the dermis is the fibroblast which secretes collagen, elastin and other proteins and cytokines into the extracellular matrix. In addition, the dermis contains immune cells and other cellular structures such as hair follicles, sebaceous glands, sweat glands, nerves and blood vessels. Finally, the innermost layer of the skin is the subcutaneous layer which contains adipose tissue that serves to insulate the body, store energy and provide protection as a cushion against physical shock (155).

Although the existence of adult stem cells is questioned in the pancreas, studies in the skin have revealed multiple potential stem cells within both the epidermal and dermal layers. Within the epidermis, the stem cells are hypothesized to reside in the basal cell layer, accounting for ~10-12% of the cells within this layer (156). Each stem cell is part of an epidermal proliferating unit and gives rise to keratinocyte transit cells that populate the column of mature cells from the basal cell layer to the surface of the skin (156-161). In addition to those found in the epidermis, stem cells are also believed to reside in the bulge of the hair follicle. During the hair cycle, these cells are activated to proliferate and differentiate into the many cells of the follicle. In addition, in response to injury, the stem

cells located in the hair follicle bugle have also been shown to function as multipotent stem cells, giving rise to cells in the epidermis, sebaceous gland and hair follicle (160, 162-166).

For the dermis, the stem cells that repopulate the fibroblast pool are most likely different from those found in the epidermis and hair follicle. Since fibroblasts remain fairly quiescent and are stimulated to proliferate only in response to injury (167), stem cells may only be activated to proliferate during wound healing. One study aimed at identifying fibroblast stem cells showed that both bone marrow-derived cells and dermal mesenchymal cells can reconstitute the dermal fibroblast population in response to wound healing (168). Thus, in addition to differences in the normal proliferation rates of skin cells, these data suggest that each layer of the skin may have distinct stem cells that repopulate the tissue. To study the role of homologous recombination within skin cells, the FYDR mice can be used to measure the frequency of recombinant cells that arise in adult tissue *in vivo*. In addition, because recombinant cells are labeled with fluorescence, the cumulative effects of homologous recombination can be monitored over time, potentially revealing the role of homologous recombination in skin cells with different proliferation rates.

Wound Healing

Given the essential role of the skin as a protective barrier against external insults, it is critical that the skin be able to quickly and effectively repair tissue damage. Wound healing involves three major overlapping phases: inflammation, proliferation, and tissue remodeling (167). The inflammatory phase of wound healing is characterized by platelet

aggregation and immune cell infiltration into the wound site. The primary immune cells at the wound are leukocytes, which are responsible for both fighting pathogens and stimulating the subsequent stages of wound healing (169). During the proliferation stage of wound healing, mitogenic cytokines secreted by activated leukocytes induce the proliferation of many cell types at the site of the wound, including keratinocytes and fibroblasts (170). In addition to cell proliferation, this stage is also characterized by wound contraction, reconstitution of the cutaneous barrier and stimulation of angiogenesis. The final tissue remodeling phase of wound healing involves the production of collagen into the extracellular matrix and its remodeling, the regression of the newly formed blood vessels and mature scar formation (167).

Dermal fibroblasts play a critical role in wound healing of the skin. In response to injury, fibroblasts migrate to the wound site, initiate proliferation, and facilitate wound contraction. In addition, fibroblasts secrete extracellular matrix components, such as collagen, that are essential for tissue remodeling and restoration of tissue strength (170, 171). Indeed, the importance of fibroblasts to wound healing is highlighted by the fact that a reduction in the number and activity of fibroblasts with age is believed to contribute to the age-associated decrease in tissue repair capacity (167).

In addition to their ability to proliferate *in vivo*, fibroblasts have also been shown to proliferate *in vitro* for ~50 population doublings (172). This significant *in vitro* proliferative capacity combined with the relative ease with which they are cultured has enabled fibroblasts to be used extensively for *in vitro* studies. The ability to induce of fibroblast proliferation *in vivo* and *in vitro* makes them an interesting cell type in which to study homologous recombination. Using FYDR mice, the role of homologous

recombination can be measured in quiescent fibroblasts within non-injured tissue *in vivo*. Subsequently, the ability of fibroblasts to use homologous recombination during cell division can be measured by stimulating fibroblast proliferation either by culturing them *in vitro* or by inducing injury *in vivo*. Thus, differences in the utilization of homologous recombination as a double-strand break repair pathways can be probed as cells move from quiescence to proliferation.

1.6 Mutation Formation in Aging and Cancer

The accumulation of multiple mutations within cells is believed to cause both aging and cancer (173, 174). During the aging process, mutations accumulate within cells, causing diminished cell viability or capacity to carry out normal functions (173). Over time, the number of mutant cells within a tissue can increase, resulting in an overall reduction of tissue function.

If the mutations that arise with age occur in key tumor suppressor or oncogenes, they may confer cells with growth and survival advantages. Indeed, malignant transformation is believed to occur through successive rounds of clonal expansion and selection of cells with acquired mutations in key tumor suppressor or oncogenes (174-177). The importance of clonal expansion to tumor formation is highlighted by analyses of tumors which show that key mutations in tumor suppressors and oncogenes are often shared by most, if not all, malignant cells within the tumor (178-180). However, although the clonal expansion of pre-malignant cells is an important precursor to the development

of cancer, no methods have been developed to study clonal expansion within intact histologically normal tissue.

To study the effect of age on mutant cell frequency in various tissues, a number of mouse models, including Big Blue (181, 182), Muta Mouse (183), and LacZ (184), have been used. Intriguingly, the effect of age on mutation frequency appears to be strongly tissue-dependent, and differences in mutation accumulation do not correlate with proliferation of cells within the tissue. For example, for tissues with low proliferation rates, an increase in mutant cell frequency with age is seen in the heart (183, 184) but not in the brain (181-183, 185). In addition, for tissues that exhibit high proliferation rates, mutant cell frequency increases in the small intestine (184, 186) but not in the testis (181, 183, 187). Thus, the accumulation of mutations differs among tissues, and factors in addition to cell proliferation have a large impact on the accumulation of mutant cells with age.

Most mouse models used for measuring mutations generally require tissue disaggregation followed by analysis of either cultured primary cells or genomic DNA (188-191); thus, information about the clonal relationship among mutant cells 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 (184, 185, 187, 192) or completely remove the contribution of clonal expansion by specifically analyzing only independent mutation events (181, 182). However, given the importance of clonal expansion to tumor

formation, analyzing clonal expansion may be important for understanding the mechanisms of cellular transformation. By using fluorescence as a marker for sequence changes, the FYDR mice potentially enable the detection of cells harboring recombined DNA within intact tissue, providing a tool to determine the contribution of clonal expansion to changes in recombinant cell frequency with age.

1.7 Specific Aims

Homologous recombination has been established as an important modulator of genomic stability. It is essential for the repair of potentially cytotoxic or mutagenic DNA damage, such as double-strand breaks and broken replication forks (12). Germline mutations that result in an impaired ability to repair double-strand breaks by homologous recombination are either embryonic lethal mice (18-23, 193, 194) or tumor-promoting (24-28). However, since recombination events are associated with the non-reciprocal transfer of genetic information from a donor template to the locus of the double-strand break, every recombination event carries some risk of acquiring deleterious sequence changes. Indeed, recombination between misaligned sequences can result in insertions, deletions, translocations, and loss of heterozygosity, sequences changes that can promote genomic instability (35). Consequently, increased levels of homologous recombination also lead to an increased risk of cancer (195).

Although homologous recombination may be critical for tumorigenesis, prior to the development of the FYDR mice (123), studies of recombination in adult tissues *in*

vivo had not been possible. The FYDR mice carry a direct repeat recombination substrate in which a homologous recombination event can restore full-length EYFP coding sequence, resulting in a fluorescent cell (123). Thus recombination events can be detected in adult tissues *in vivo* simply by measuring the frequency of fluorescent cells. Through the work described here, we reveal the effects of cancer risk factors such as age, genetic predisposition, and exposure on homologous recombination *in vivo* using the FYDR mice.

Age is an important risk factor for pancreatic cancer (152), with approximately 60% of all cases occurring in patients over 65 years of age (196). In addition, inherited defects in homologous recombination proteins are known to increase pancreatic cancer risk (24-26), suggesting that homologous recombination is an important DNA repair pathway for maintaining genomic stability in pancreatic cells. However, prior to our work no studies had analyzed recombination in the pancreas. Here, we use FYDR mice to explore the activity of homologous recombination in the adult pancreas. We develop *in situ* imaging methodology for the rapid and sensitive detection of recombinant cells within intact pancreatic tissue. Using both *in situ* imaging and tissue disaggregation, we analyze the effect of exposure to two cancer chemotherapeutic agents, mitomycin-C and cisplatin, on the frequency of homologous recombination *in vivo* and demonstrate that *in situ* imaging of recombinant cells is more sensitive than tissue disaggregation for determining environmentally induced recombination events in adult tissues. Furthermore, we investigate the effects of aging on recombination in the pancreas and determine that a dramatic increase in recombinant cell frequency occurs with age as the result of both *de novo* recombination events and clonal expansion of previously existing recombinant cells.

Since the FYDR mice use fluorescence as a marker for homologous recombination, expression of the fluorescent protein can affect the apparent frequencies of recombinant cells. For example, if *EYFP* is expressed at lower levels, it will be more difficult to detect recombinant cells. Thus, differences in recombinant cell frequencies seen with age or with exposure to cancer chemotherapeutics may be due to differences in the expression levels of the FYDR transgene. To explore the effect of transgene expression on recombinant cell frequencies, we exploit positive control FYDR-Recombined mice that carry the full-length *EYFP* coding sequence in every cell. Because in positive control mice the *EYFP* coding sequence is expressed under an identical promoter and at the same locus as the FYDR recombination substrate in FYDR mice, it is likely that any differences observed in expression in positive control mice will be similarly reflected in FYDR mice. In this work, we show inter-mouse and gender-dependent variation in FYDR transgene expression, examine its impact on data interpretation, and discuss solutions to overcoming the effects of such variation. In addition, we analyze EYFP expression in several tissues in addition to the pancreas and show that some of these tissues may be amenable for studying homologous recombination using the FYDR mice.

Our studies of aging on recombinant cell frequency in the pancreas revealed the importance of both *de novo* recombination events and clonal expansion on the accumulation of recombinant cells with age. Given the importance of clonal expansion of mutant cells to cancer formation (174), we set out to develop a method to study clonal expansion within intact histologically normal tissue. In collaboration with the laboratory of Peter T. So, an integrated one- and two-photon microscopy imaging system was

created to rapidly identify very rare fluorescent cells within an entire mouse tissue and subsequently provide 3D images of each fluorescent cell. Using these techniques, we study the effect of age on clonal expansion of fluorescent cells in the pancreata of FYDR mice and show that there is a significant increase in the number of cells within fluorescent cell clusters.

Our analyses of positive control mice show that the pancreas is not the only tissue amenable for studying homologous recombination in FYDR mice. Thus, we set out to explore differences in the spontaneous recombinant cell frequency in two tissues, pancreas and skin. Since it has been shown that tissue-specific differences exist in the accumulation of mutations with age (186), we also investigate the effect of age on the accumulation of recombinant cells in pancreas and skin. We found that while recombinant cells accumulate in the pancreas of FYDR mice, no difference is seen in the skin with age. To investigate the reasons for the differences in age-dependent accumulation of recombinant cells in pancreas and skin, we study homologous recombination in primary fibroblasts from FYDR mice *in vitro*. We conclude that neither a decreased rate of homologous recombination, a decrease in spontaneous damage levels nor a decrease in the ability to cope with exogenous DNA damage contribute to the lack of accumulation of recombinant skin cells with age *in vivo*. Rather, we propose that differences in the accumulation of recombinant cells with age may result from differences in the ability of recombinant cells to persist and clonally expand within the tissue.

In addition to age and environmental exposures, certain genetic conditions increase the risk of cancer formation. One gene that appears to be especially critical for maintaining genomic stability is p53 (197). Indeed, acquired mutations in p53 are seen in

approximately half of all human tumors (198, 199), and inherited mutations in p53 lead to increased cancer risk (200, 201). A number of studies have analyzed the effect of p53 on homologous recombination *in vitro* and *in vivo*; however, these studies show conflicting results (103, 107, 202-210). Thus, to determine the effect of p53 on homologous recombination *in vivo*, we analyze the frequency of recombinant cells and the number of recombination foci in pancreata from $FYDR^{y/+};p53^{+/+}$ and $FYDR^{y/+};p53^{-/-}$ mice. In addition, to determine if differences in p53 status may affect homologous recombination *in vitro*, we measure the rate of homologous recombination in primary fibroblasts derived from $FYDR^{y/+};p53^{+/+}$ and $FYDR^{y/+};p53^{-/-}$ mice. These results show that in FYDR mice p53 status does not significantly affect homologous recombination in pancreatic cells *in vivo* or in ear fibroblasts *in vitro*.

Through the work described here, we aim to understand the role of homologous recombination in multiple tissues *in vivo*. By creating techniques to specifically detect and quantify recombinant cells within intact tissues, we can analyze the impact of exposure to cancer risk factors on not only *de novo* recombination events but also clonal expansion of previously existing recombinant cells. The assays developed here can be further applied to study homologous recombination in other tissues and by other tumor-promoting genetic conditions and environmental exposures. Ultimately, these studies may increase our understanding of the interplay between DNA damage, homologous recombination, and cancer formation.

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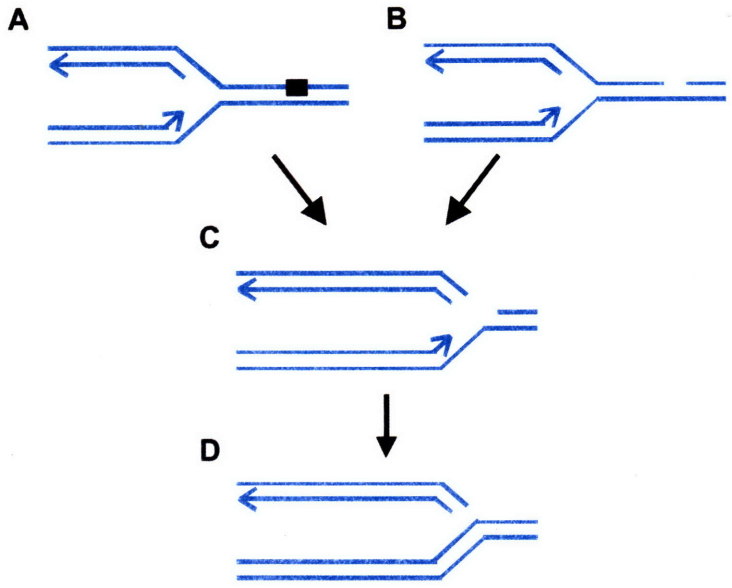

Figure 1-1. Replication fork breakdown leading to the formation of a one-ended double-strand break. Replication fork encounter with a (A) blocking DNA lesion or (B) single-strand gap can lead to (C) replication fork breakdown and the formation of a (D) one-ended double strand end.

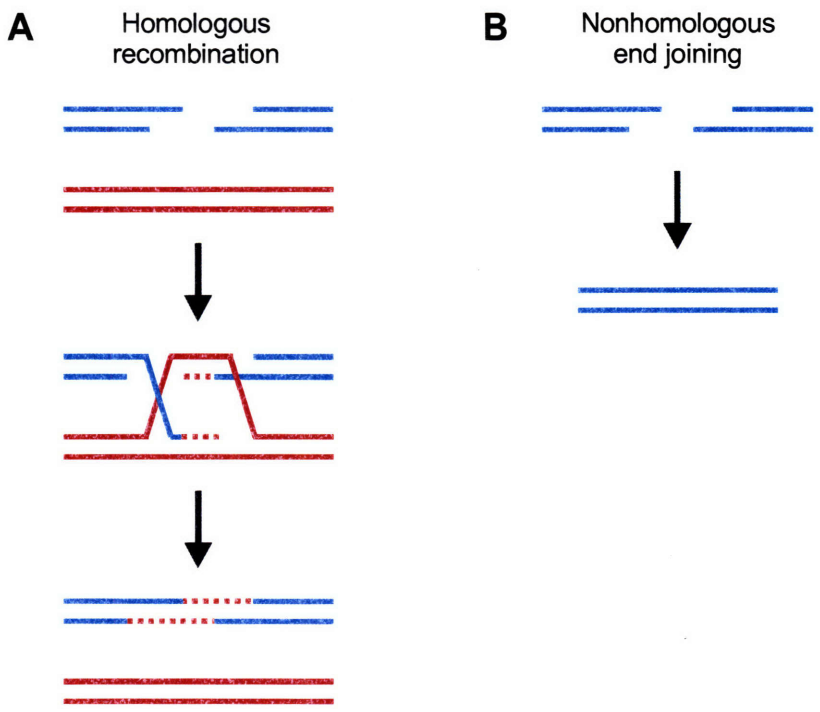

Figure 1-2. Basic mechanisms of double-strand break repair. (A) Homologous recombination uses homologous sequences present on the sister chromatid or homologous chromosome as temaplates for double-strand break repair. (B) nonhomologous end-joining directly rejoins two double-strand ends regardless of sequence.

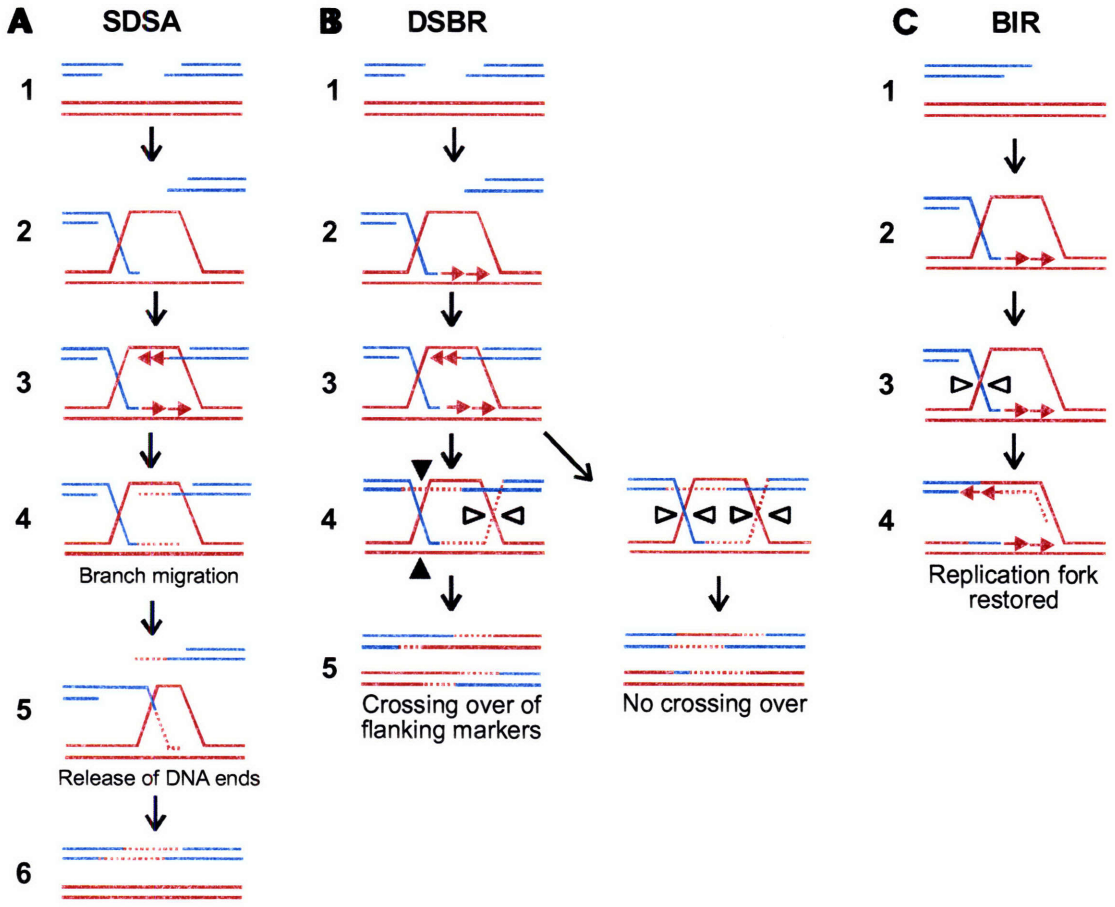

Figure 1-3. Mechanisms of double-strand break repair by homologous recombination. (A) Synthesis-Dependent Strand Annealing: 1. Resection of the double-strand end to produce a 3' overhang, 2. Homology searching, strand invasion and strand exchange, 3-4. DNA synthesis and Holliday Junction formation, 5. Branch migration, 6. Release and annealing of DNA ends to repair the double-strand break. (B) Double Strand Break Repair: 1. Resection of the double-strand end to produce a 3' overhang, 2. Homology searching, strand invasion, strand exchange, and DNA synthesis for one DNA end, 3. Homology searching, strand invasion, strand exchange, and DNA synthesis for second DNA end, 4. Cleavage of Holliday Junctions, 5. Formation of crossover or non-crossover products. (C) Break induced replication: 1. Resection of the double-strand end to produce a 3' overhang, 2. Homology searching, strand invasion, strand exchange, DNA synthesis and Holliday Junction formation, 3. Cleavage of the Holliday Junction, 4. Restoration of the broken replication fork.

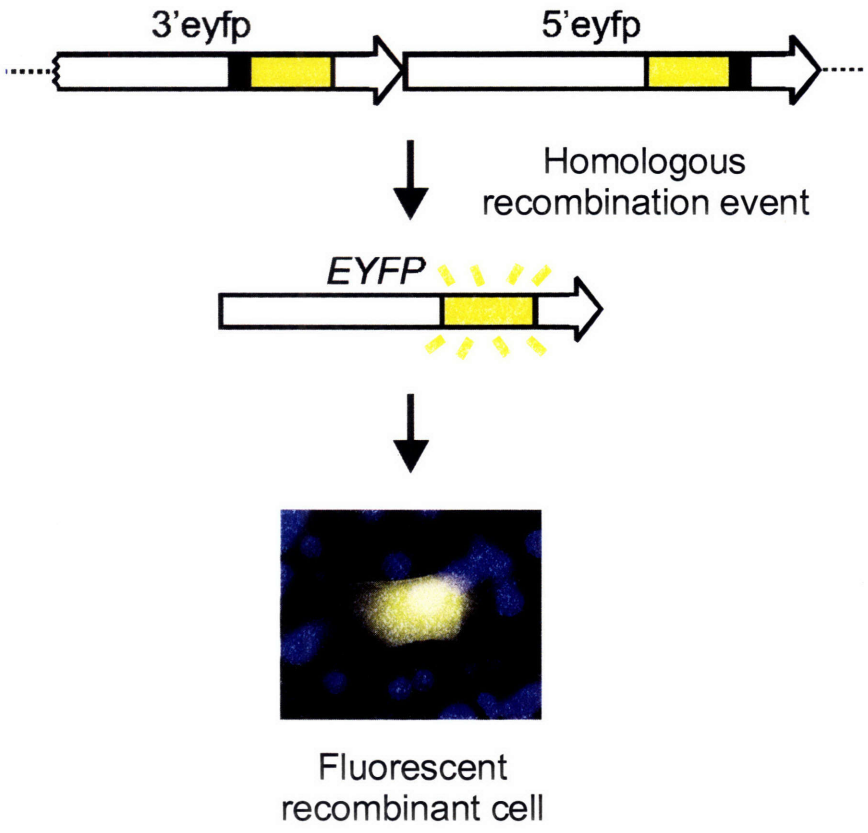

Figure 1-4. Arrangement of the FYDR recombination substrate: large arrows indicate expression cassettes; yellow boxes show coding sequences; black boxes show positions of deleted sequences (deletion sizes not to scale). A homologous recombination event between the two expression cassettes can restore full-length *EYFP* coding sequence, resulting in a fluorescent cell. Image depicts recombinant pancreatic cell within freshly excised tissue.

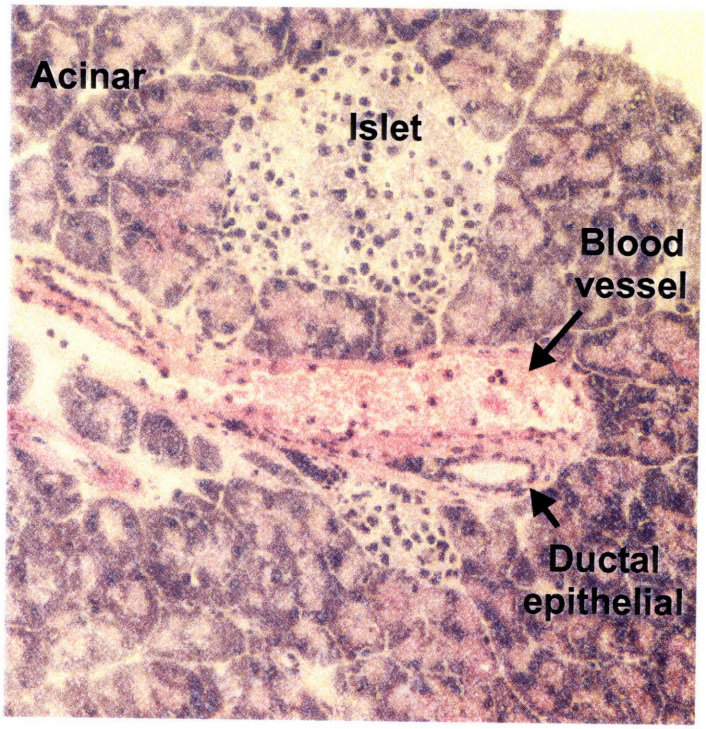

Figure 1-5. Histology of a mouse pancreas. The exocrine pancreas contains acinar cells that secrete hydrolytic enzymes into a series of ducts lined with ductal epithelial cells. The endocrine pancreas is composed of islets of Langerhans interspersed throughout the exocrine pancreas. Finally, a system of blood vessels is present within the pancreas to carry nutrients and signaling molecules to and from the pancreas.

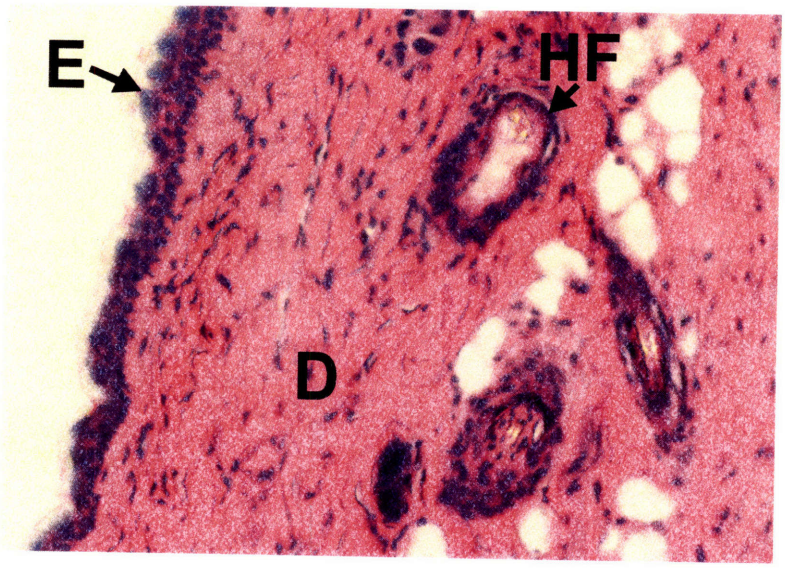

Figure 1-6. Histology of the mouse skin. The epidermis (E) is the outer most layer of skin, with a thickness of just a few cell layers. The dermis (D) contains a number of different cell types including fibroblasts and many cellular structures such as hair follicles (HF).

Chapter II

Age Dependent Accumulation of Recombinant Cells in the Mouse Pancreas Revealed by *in situ* Fluorescence Imaging

Chapter 2

2.1 Abstract

Mitotic homologous recombination (HR) is critical for the repair of double-strand breaks, and conditions that stimulate HR are associated with an increased risk of deleterious sequence rearrangements that can promote cancer. Because of the difficulty of assessing HR in mammals, little is known about HR activity in mammalian tissues or about the effects of cancer risk factors on HR *in vivo*. To study HR *in vivo*, we have used Fluorescent Yellow Direct Repeat mice, in which an HR event at a transgene yields a fluorescent phenotype. Results show that HR is an active pathway in the pancreas throughout life, that HR is induced *in vivo* by exposure to a cancer chemotherapeutic agent, and that recombinant cells accumulate with age in pancreatic tissue. Furthermore, we developed an *in situ* imaging approach that reveals an increase in both the frequency and the sizes of isolated recombinant cell clusters with age, indicating that both *de novo* recombination events and clonal expansion contribute to the accumulation of recombinant cells with age. This work demonstrates that aging and exposure to a cancer chemotherapeutic agent increase the frequency of recombinant cells in the pancreas, and it also provides a rapid method for revealing additional factors that modulate HR and clonal expansion *in vivo*.

2.2 Introduction

Cells are constantly exposed to endogenous and exogenous DNA-damaging agents that can lead to double-strand breaks, either by causing breaks in both strands of DNA or by causing replication fork breakdown (1). Homologous recombination (HR) is critical for repairing double-strand breaks in mammalian cells. By using homologous DNA sequences present on the sister chromatid or homologous chromosome, damage can be repaired accurately without loss of sequence information (2, 3). Thus, the frequency of HR reflects both the levels of double-strand breaks and the ability of cells to use HR during DNA repair.

Although HR is generally error-free, recombination between misaligned sequences can cause insertions, deletions, and translocations. Furthermore, recombination between homologous chromosomes can lead to loss of heterozygosity (4), and HR has been estimated to be the underlying cause of loss of heterozygosity 25-80% of the time in mammalian cells (*e.g.*, see (5)). Germ-line mutations in genes that modulate the frequency of HR are associated with an increased risk of cancer. For example, inherited mutations in the HR helicases BLM and WRN lead to increased rates of HR (6, 7) and increase the risk of cancer (8).

Whereas too much HR can be problematic, too little HR can also destabilize the genome, possibly as a result of nonhomologous end-joining of DNA ends created at broken replication forks (4, 9). In the pancreas, inherited mutations in *BRCA1* (8), *BRCA2* (10), and *FANCC* (11) increase the risk of pancreatic cancer, and loss of function of these genes suppresses HR (12-14), causing an increased frequency of tumorigenic sequence rearrangements (15, 16). Although these findings suggest that HR is critical for maintaining genomic integrity in the pancreas, it had

not been shown that HR is an active pathway in mature pancreatic cells and no studies had explored the effects of cancer risk factors on potentially mutagenic HR events in the pancreas.

With a 5 year survival rate of less than 5%, pancreatic cancer remains one of the deadliest cancers in the United States (17, 18). One important risk factor for pancreatic cancer is aging (19). To our knowledge, mutation frequency has not been reported in the pancreas (20), so the effect of age on pancreatic mutation frequency was not known. However, a number of studies have investigated the impact of age on mutation frequency in other cell types. For example, the frequency of loss of heterozygosity increases by ~10-fold with increasing age in lymphocytes (21, 22). Furthermore, a significant fraction of these loss of heterozygosity events are because of mitotic recombination, suggesting that HR contributes to gene inactivation during aging (21-23).

In this study, we have explored the effects of aging and exposure to a cancer chemotherapeutic agent on the frequency of HR in the mouse pancreas by exploiting mice in which HR at an integrated transgene yields a fluorescent phenotype (24). Furthermore, we describe novel methodology for rapid and sensitive detection of recombinant cells within intact pancreata.

2.3 Materials and Methods

Animals

C57BL/6 FYDR mice have been described in ref. (24). Positive control FYDR-recombined (FYDR-Rec) mice arose spontaneously from an HR event in a FYDR parental gamete, and all cells within these mice carry the full-length *EYFP* coding sequence. FYDR

cohorts had an ~1:1 ratio of males to females (preliminary data suggests that there may be a difference in *EYFP* expression levels among males and females). Controls were sex- and age-matched, except the aged negative control C57BL/6, which were 47-85 weeks old, and the aged positive control FYDR-Recombined mice, which were 52 weeks old.

Flow Cytometry

Pancreata were isolated and placed in ice-cold PBS containing 0.01% soybean trypsin inhibitor (Sigma). Almost all samples were analyzed by flow cytometry after imaging. Pancreata were minced and divided into two samples. Samples were shaken (150 cycles per minute) in 5 ml of 2 mg/ml collagenase V (Sigma) in Hanks' buffered salt solution (Invitrogen) at 37°C for 20 min. Triturated tissue was filtered (70 μm), and 10 ml of DMEM-F12 (Sigma) supplemented with 20% FBS (Atlanta Biologicals, Lawrenceville, GA) was added. Cells were pelleted, resuspended in 400 μl of OptiMEM (Invitrogen), filtered (35 μm), and analyzed by flow cytometry as described in ref. (24). On average, ~1 million cells were analyzed per sample.

Fluorescence Intensity Measurements

Geometric mean of fluorescence intensity of pancreatic cells in the R2 region of juvenile and aged mice was calculated by CellQuest acquisition and analysis software on the Becton Dickinson FACScan flow cytometer.

Imaging

Pancreata were isolated as described. Nuclei were stained with 50 μg/ml Hoechst 33342 (Sigma). Whole pancreata pressed between glass slides separated by 0.5 mm spacers were

imaged on a Nikon E600 microscope with a SPOT RT camera (Diagnostic Instruments, Sterling Heights, MI) with a Nikon x1 objective. Images were manually compiled to cover the entire visible surface area. SPOT Advanced (Diagnostic Instruments) was used to colorize black and white images. Filters included: visible light; UV (excitation, 330–380 nm; emission, 420 nm); red (excitation, 540/25 nm; emission, 605/55 nm); and EYFP (excitation, 460–500 nm; emission, 510–560 nm). Images were collected by using a fixed aperture time. For foci counting, Adobe Photoshop 5.0 (Adobe Systems, San Jose, CA) was used to optimize brightness and contrast. For images collected with the EYFP filter, identical adjustments were made for all images. Similarly, for images collected with the red filter, brightness was optimized and identical adjustments were made for all red fluorescence images. For pancreata with foci in the red channel, images taken under the red filter were inverted to create negatives that were merged with images taken under the EYFP filter. Foci were counted manually. Similar results were obtained in a subset of blinded samples. The area of compiled pancreata images was determined by using Scion Image Beta 4.02 Win (Scion, Frederick, MD) by manually tracing the pancreas edge

Mitomycin-C treatment

Five- to 6-week-old FYDR mice were i.p. injected with 2 mg/kg of body weight of 0.5 mg/ml MMC (Sigma) in PBS. Mock-treated controls were injected with equal volumes of PBS. Mice were analyzed 3.5 weeks after injection.

Histology

Frozen sections (5 μm) were imaged at x40 with an EYFP filter (excitation, 460–500 nm; emission, 515 nm) on a Nikon E600 microscope. SPOT Advanced (Diagnostic Instruments) was

used to colorize images. Sections were subsequently stained with H&E, imaged, and manually overlaid. Histology was assessed by Arlin Rogers (Massachusetts Institute of Technology)..

Statistics

Recombinant cell frequency follows a non-normal distribution (Fig. 2-1C). Therefore, frequencies among cohorts were compared using a one-tailed Mann-Whitney test (for both flow cytometry and foci data). When comparing the frequency of fluorescent cells among positive control cohorts, a two tailed Student's *t* test was performed.

2.4 Results

Flow Cytometry Analysis of Homologous Recombination Events in Adult Pancreatic Cells

The fluorescent yellow direct repeat (FYDR) mice carry a direct repeat recombination substrate in which an HR event can restore full-length enhanced yellow fluorescent protein (EYFP) coding sequence (24) (Fig. 2-1A). Because HR had not been studied in primary pancreatic cells, we first set out to determine whether HR is an active process in the pancreas. To establish flow cytometry parameters, we compared fluorescence intensities in disaggregated whole pancreata from negative control and positive control mice (Fig. 2-1B). To quantify fluorescent recombinant cells, a region (R2) was created that excludes negative control cells (Fig. 2-1B). We analyzed >34 million cells from 33 negative control pancreata by flow cytometry, and only one cell appeared in the R2 region, indicating an extremely low background. Analysis of 100 mice aged 4-10 weeks shows the median frequency of recombinant pancreatic cells is five

per million (Fig. 2-1C). In addition, the recombinant cell frequency is highly variable among mice (Fig. 2-1C), which is consistent with the possibility that recombinant cells that arise at different times during growth can clonally expand.

In Situ Detection of Recombinant Pancreatic Cells

To learn more about the timing of HR events, and to reveal the cell types in which they occur, methodology was developed for direct detection of recombinant cells *in situ*. Under an epifluorescent microscope, negative control pancreata are essentially nonfluorescent when viewed with an EYFP filter, whereas Hoechst 33342-stained nuclei fluoresce under UV (Fig. 2-2A). In contrast, in positive control pancreata, much of the tissue is brightly fluorescent (Fig. 2-2B). In FYDR pancreata, yellow fluorescent foci are readily apparent (Fig. 2-2C). At a higher magnification, it becomes clear that these fluorescent foci are actually single isolated cells and small clusters of cells (Fig. 2-2D), indicating that recombinant cells can be directly detected within intact tissue via microscopic examination of intact pancreata.

To explore which cell types recombine, frozen sections of pancreata were analyzed. By overlaying fluorescence and H&E-stained images, histological analysis of pancreata from positive control mice revealed that acinar, islet, and ductal cells can fluoresce (data not shown). In pancreata of FYDR mice, EYFP fluorescence is confined within cell boundaries and recombinant cells are acinar cells (no islet or ductal cells were detected among 43 independent foci) (Fig. 2-2E). Therefore, the fluorescent foci seen within the pancreas of FYDR mice are most often due to fluorescent acinar cells.

To standardize conditions for foci quantification, pancreata were uniformly compressed to a thickness of 0.5 mm, and composite images that cover one side of the pancreatic surface area

were created (Fig. 2-3A). Comparison of both sides of the same pancreas showed that similar frequencies of recombinant cells were detected on both sides (data not shown; note that most foci detected on one side could not be detected on the other side because of sample thickness). In negative control mice, background fluorescence was occasionally observed and generally emitted under multiple filters. To reduce background fluorescence, images collected under a red filter were inverted, and these negatives were then merged with images taken with an EYFP filter (Fig. 2-3B). Using this subtraction methodology, we analyzed 24 negative control animals, and no foci were detected. These methods make it possible to specifically detect EYFP fluorescence and thus provide a means for rapid quantification of recombinant cells *in situ*.

DNA-damage Induced Recombination in Pancreatic Cells

To determine whether HR can be induced in postnatal pancreatic cells, 5- to 6-week-old FYDR mice were injected with the recombinogenic interstrand cross-linking agent mitomycin-C (MMC). The median frequencies of recombinant cells by flow cytometry (Fig. 2-4A) and of recombinant foci by *in situ* imaging (Fig. 2-4B) were higher among the MMC-treated mice (note the logarithmic scale). It is formally possible that the increased frequency of recombinant cells after MMC treatment is because of increased *EYFP* expression. To explore this possibility, we exploited positive control animals in which all cells carry the recombined substrate (full-length *EYFP*). Flow cytometry of pancreatic cells from mock- and MMC-treated positive control mice revealed that there was no statistically significant difference in *EYFP* expression between the cohorts (data not shown). Thus, we conclude that the increase in recombinant cell frequency after MMC treatment is the result of an induction of recombinant cells, indicating that the FYDR

model specifically detects HR events, and that HR is an active repair process in the postnatal pancreas

Interestingly, MMC induction is statistically significant only when analyzed by *in situ* imaging. It is noteworthy that mice with a similar number of recombinant foci can show a broad range of recombinant cell frequencies when analyzed by flow cytometry, possibly because a single focus may contain many recombinant cells. Thus, when studying the effects of an environmental exposure by flow cytometry, it should be noted that large foci can potentially mask the induction of multiple smaller foci. Although both flow cytometry and *in situ* imaging detect recombinant cells, *in situ* imaging may be a more sensitive method for detecting exposure-induced recombinant cells (e.g., independent HR events).

Effect of Aging on Recombinant Cell Frequency

Age is an important risk factor for cancer. To explore the effects of aging, recombinant foci were quantified in three different age groups: juvenile (4 weeks old), adult (9 weeks old), and aged (67–74 weeks old). Whereas the number of foci detected by *in situ* imaging varied among individual animals within each age group, the median clearly increased with age (Fig. 2-5B). This increase was especially evident when examining images of pancreata with the highest frequency of recombinant foci from each age group (Fig. 2-5A). Relative to juvenile mice, the median number of recombinant foci increased ~4- and ~16-fold in adult and aged mice, respectively (Fig. 2-5B), demonstrating that recombinant cells accumulate with age.

During aging, the mouse pancreas continues to grow (Fig. 2-5C) (25, 26), which raises the possibility that the increase in the number of foci is because of the increase in surface area of pancreata (Fig. 2-5C). After correcting for the total surface area, the median frequencies of

recombinant foci per cm^2 in adult and aged cohorts are still significantly higher than juvenile mice (~2 and ~9 fold higher, respectively; Fig. 2-5D), suggesting that new recombination events contribute to the accumulation of recombinant cells with age.

As an alternative approach for analyzing the effects of aging on HR, disaggregated pancreatic cells were quantified by flow cytometry (Fig. 2-5E). Similar trends in the frequency of recombinant cells were observed with age. Relative to juvenile mice, the median frequency of fluorescent cells per million increased in adult and aged mice by ~8- and ~26-fold, respectively. A comparison of the mice with the highest frequencies from the young vs. the aged cohorts shows there are 14 vs. 914 recombinant cells per million, respectively (note the logarithmic scale). Together, these data indicate that as mice age, the frequency of cells harboring DNA sequence rearrangements increases significantly.

To determine whether the increased frequency of recombinant cells with age is because of an increase in *EYFP* expression, we analyzed pancreata from positive control mice. Although *EYFP* expression varies greatly among individual positive control mice (which undoubtedly contributes to variation in the frequency of fluorescent cells among the FYDR mice), there were no statistically significant differences in expression levels among the young, adult, and aged cohorts (data not shown). Because expression of the FYDR locus does not increase with age, we conclude that the increase in recombinant cell frequency with age is the result of the accumulation of recombinant cells in the pancreas.

Clonal Expansion of Recombinant Cells

Clonal expansion of mutant cells is an important precursor to the development of cancer (27). To explore the possibility that recombinant cells clonally expand with age, images from all

juvenile and aged animals were carefully examined to identify the five largest foci from within each cohort. Comparison of the foci images revealed that foci are clearly much larger in aged animals (Fig. 2-5F). Given that the geometric mean fluorescence intensity of recombinant cells is not significantly different among cohorts (data not shown), the observed increase in foci sizes cannot be due to increased brightness. It is formally possible that the increase in recombinant foci sizes is the result of multiple independent recombination events occurring in neighboring cells. However, because the frequency of recombinant cells in the pancreas is ~5 per million, the probability that two adjacent recombinant cells occurred from independent events is ~1 in 10^{10} (assuming that each cell touches ~10 neighbors), making it virtually impossible that multiple adjacent recombinant cells in large foci occurred independently. These data indicate that recombinant cells can clonally expand during aging, which suggests that there is clonal expansion throughout the pancreas that can be visualized in cases where the progenitor cell is fluorescent.

2.5 Discussion

With a 5 year survival rate of less than 5% (17, 18), pancreatic cancer remains a fatal disease. Pancreatic cancer is caused by the accumulation of genetic mutations in a single cell lineage (*e.g.*, activation of *K-ras* and inactivation of *p16*, *p53*, *Smad4*, and *BRCA2*) (28). The probability that multiple mutations occur in the same lineage depends upon both the mutation rate and the total number of cells that harbor tumorigenic mutations (27, 29). Therefore, increasing either the mutation rate or the number of mutant cells (by clonal expansion)

concomitantly increases the risk of acquiring subsequent, and possibly transforming, mutations in the same cell lineage.

Among >2000 rodent experimental records in which mutation frequencies have been assessed using transgenic animals, none describe the mutation frequency in the pancreas (20). Here, we have used FYDR mice to study one important class of mutations, HR events. When both *in situ* imaging and flow cytometry are used, results show that the number of recombinant cells increases with age, and a comparison of the sizes of recombinant foci in juvenile and aged mice shows that pancreata of aged mice have larger foci. Therefore, as mice age, both *de novo* HR events and clonal expansion contribute to the overall increase in the number of pancreatic cells harboring rearranged DNA.

Within all cohorts, a wide range of recombinant cell frequencies is observed. This intermouse variation may result from differences in recombination rates, fluctuations in foci sizes caused by clonal expansion, or variation in expression of *EYFP*. Although we have not yet tested for differences in recombination rates among individual mice, it is clear that clonal expansion can contribute to variation in the total number of recombinant cells. In addition, the positive control mice indicate that variable levels of *EYFP* expression also contribute to intermouse variation. Although we do not yet know the cause for the variegated expression in the positive control mice, recombination can still be studied by ascertaining whether a variable of interest affects expression in a cohort of positive control animals. For example, in these studies, EYFP fluorescence does not increase with age, indicating that the increased frequency is due to HR. Indeed, expression may even decrease with age, so the effect of age on the accumulation of recombinant cells may actually be underestimated.

Histological analysis of ~40 foci revealed that all recombinant fluorescent cells are acinar cells. Although the majority (80-90%) of human pancreatic neoplasms are ductal pancreatic adenocarcinomas (18), there is debate over the actual origin of cells that lead to ductal adenocarcinomas. Interestingly, acinar cells can transdifferentiate into ductal cells both *in vitro* and *in vivo* (30-32). Furthermore, expression of activated *K-ras* in pancreatic acinar cells has been shown to induce preinvasive pancreatic neoplastic lesions (31). Therefore, genetic changes in acinar cells may contribute to tumor formation. Regardless of whether acinar cells are the precursors of ductal adenocarcinomas, genetic and environmental conditions that induce HR in acinar cells may do so in other pancreatic cell types as well. Therefore, detection of HR in acinar cells may be a gauge of genetic insult to the pancreas as a whole, making the FYDR mice potentially useful as sensors of pancreatic genotoxins.

Other mouse models, including Big Blue® (33, 34), Muta™Mouse (35), and LacZ (36), have been used to examine changes in the frequency of point mutations and small deletions with age. The effect of age on these classes of mutations appears to be strongly tissue-dependent. In certain tissues, such as brain and testis, the mutant frequency remains fairly constant (33-35). In contrast, for liver and bladder, mutant cell frequency increases with age similar to what has been observed for the pancreas in these studies (33-35). It is interesting to speculate that for tissues in which spontaneous mutations accumulate with age, mutagenic exposures during adult life may have a greater influence on cancer risk. Indeed, smoking is an important risk factor for liver, bladder, and pancreatic cancer, and has less of an effect on the risk of brain or testicular cancer (37).

HR events are an important class of mutations that are known to promote cancer (4, 38). Here we have shown that detection of recombinant cells in FYDR mice by *in situ* imaging and

flow cytometry can be used to monitor the effects of cancer risk factors on HR. Compared with analysis by flow cytometry, *in situ* detection improves the sensitivity for detecting new mutation formation (e.g., small foci) that can be masked by previously existing larger foci upon tissue disaggregation. Furthermore, because the accumulation of recombinant cells can be monitored over months and even years, long-term effects of both acute and chronic exposures relevant to cancer can also be studied. Although small differences in recombination rate may not be immediately reflected as differences in mutant cell frequency, these changes in recombination rate may result in large changes in mutant cell frequency over time. In summary, we have explored how a key risk factor for pancreatic cancer, aging, affects the frequency of cells harboring recombined DNA. The results of these studies demonstrate that HR is an active process in the adult pancreas, and that cells harboring sequence rearrangements can persist and clonally expand. Furthermore, the methodology used in these studies can now be applied to explore how additional genetic and environmental risk factors modulate double-strand break formation and repair by HR.

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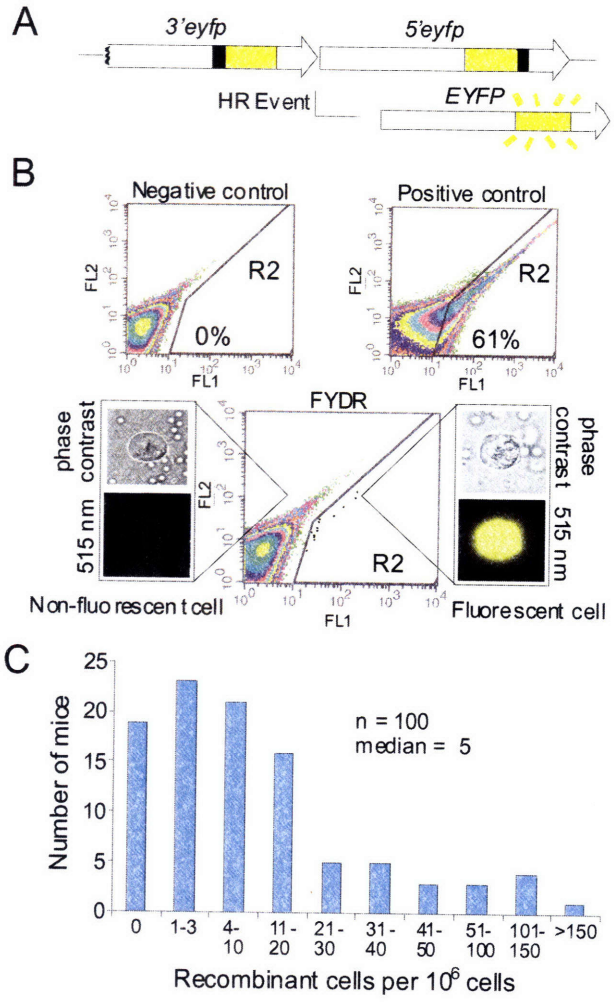

Figure 2-1. FYDR system and analysis of pancreatic cells by flow cytometry. (A) Arrangement of the FYDR recombination substrate. Large arrows indicate expression cassettes; yellow boxes show coding sequences; black boxes show positions of deleted sequences (deletion sizes not to scale). For a more complete description of HR mechanisms that can restore full-length *EYFP* coding sequences, see Jonnalagadda *et al.* (39). (B) Flow cytometry results of disaggregated pancreatic cells. Axes indicate relative fluorescence intensity at 515–545 nm (FL1) vs. 562–588 nm (FL2). R2 region delineates *EYFP*-positive cells. Representative data are shown for a negative control mouse, a positive control FYDR-recombined mouse, and an FYDR mouse. For clarity, data for individual cells (dots) have been darkened in the FYDR R2 region. Percentages of fluorescent cells identified in the R2 region are indicated for the positive and negative controls. Cell images are representative of disaggregated pancreatic cells from an FYDR mouse taken at x40 with phase contrast or an EYFP filter (515 nm). Image of a fluorescent cell under an EYFP filter is colorized. (C) Spontaneous frequency of recombinant pancreatic cells per 10^6 as determined by flow cytometry for 100 4- to 10-week-old FYDR mice. *n* = number of independent samples.

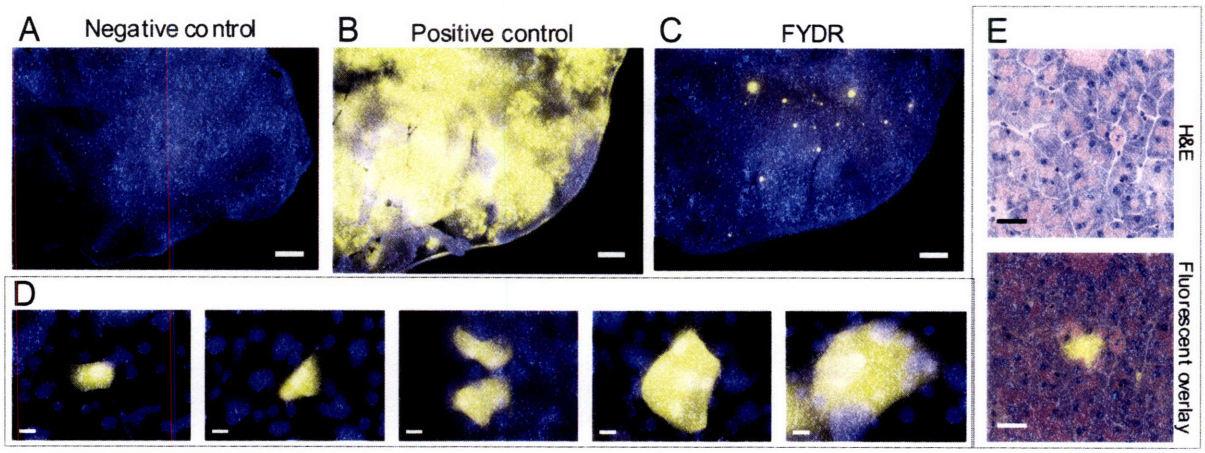

Figure 2-2. Analysis of fluorescent foci in mouse pancreata. For analysis of freshly excised tissue, images show overlays of EYFP- (510–560 nm) and UV- (420 nm) filtered images. Nuclei are stained with Hoechst 33342. (A-C) Portions of negative control (A), positive control (B), and FYDR (C) mouse pancreata imaged at x1. (Scale bar, 1 mm.) Brightness and contrast for UV-filtered images were adjusted equivalently. For EYFP images, brightness and contrast for negative control and FYDR (5-s exposure) images were adjusted equivalently. To avoid overexposure of the positive control, a shorter exposure time was used (1 s) and brightness and contrast were not adjusted. (D) Images of fluorescent recombinant cells in freshly excised tissue at x40. (Scale bar, 10 μm.) Brightness and contrast were optimized for each image. (E) Histological images were collected at x40. (Scale bar, 30 μm.) (*Upper*) H&E-stained section. (*Lower*) Overlay of H&E and fluorescence imaged under an EYFP filter (515 nm). Brightness and contrast of fluorescence were optimized. EYFP fluorescence is colorized.

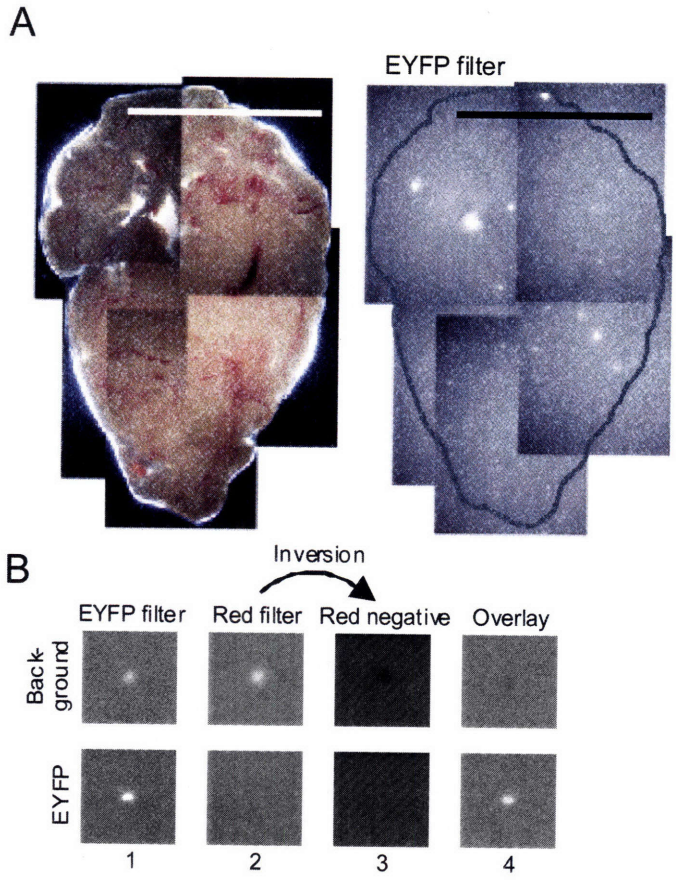

Figure 2-3. Imaging methodology for quantifying recombinant cells in whole pancreata. (A) Compiled image of 9-week-old FYDR pancreas at x1 taken under visible light (*Left*) and an EYFP filter (510–560 nm) (*Right*). (Scale bar, 1 cm.) The edge of the pancreatic tissue is outlined. (B) Method for reducing background fluorescence. (*Upper*) Background fluorescence appears brightly under EYFP (510–560 nm, column 1) and red (605/55 nm, column 2) filters. The red-filtered image is inverted (column 3) and merged with the EYFP-filtered image (column 4). (*Lower*) Similar analysis of an EYFP focus.

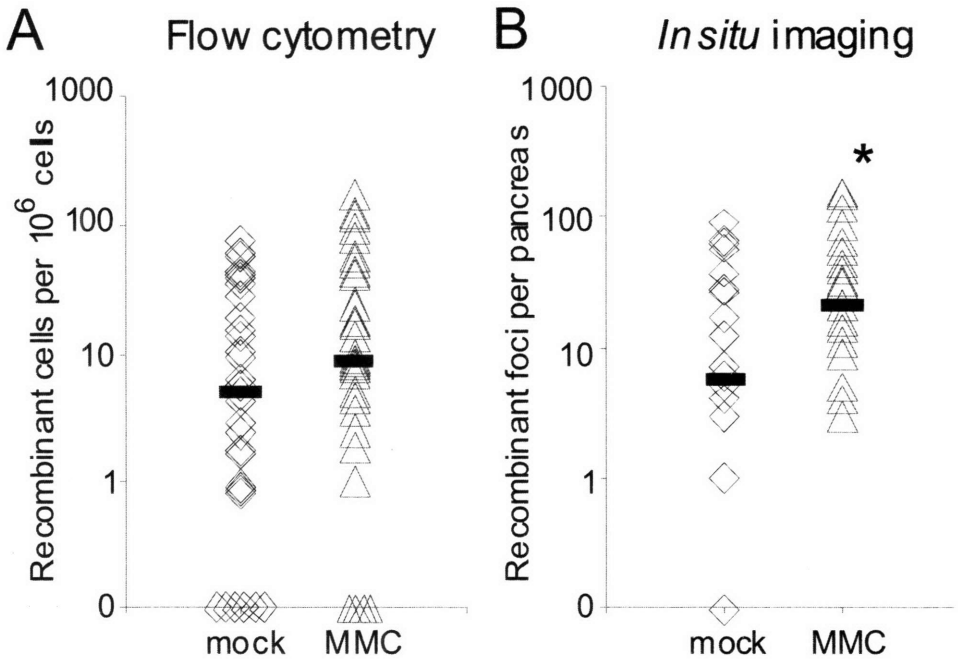

Figure 2-4. MMC-induced HR in mouse pancreata. Medians are indicated by black bars. Points on the *x* axis indicate individual mice with zero recombinant cells. (A) Frequency of recombinant cells per million as determined by flow cytometry for mock-treated (n = 35) and MMC-treated (n = 34) FYDR mice (P = 0.06). (B) Recombinant foci per pancreas detected by *in situ* image analysis for mock-treated (n = 24) and MMC-treated (n = 23) FYDR mice. * MMC-treated cohort is statistically significantly higher than mock-treated cohort ($P < 0.05$).

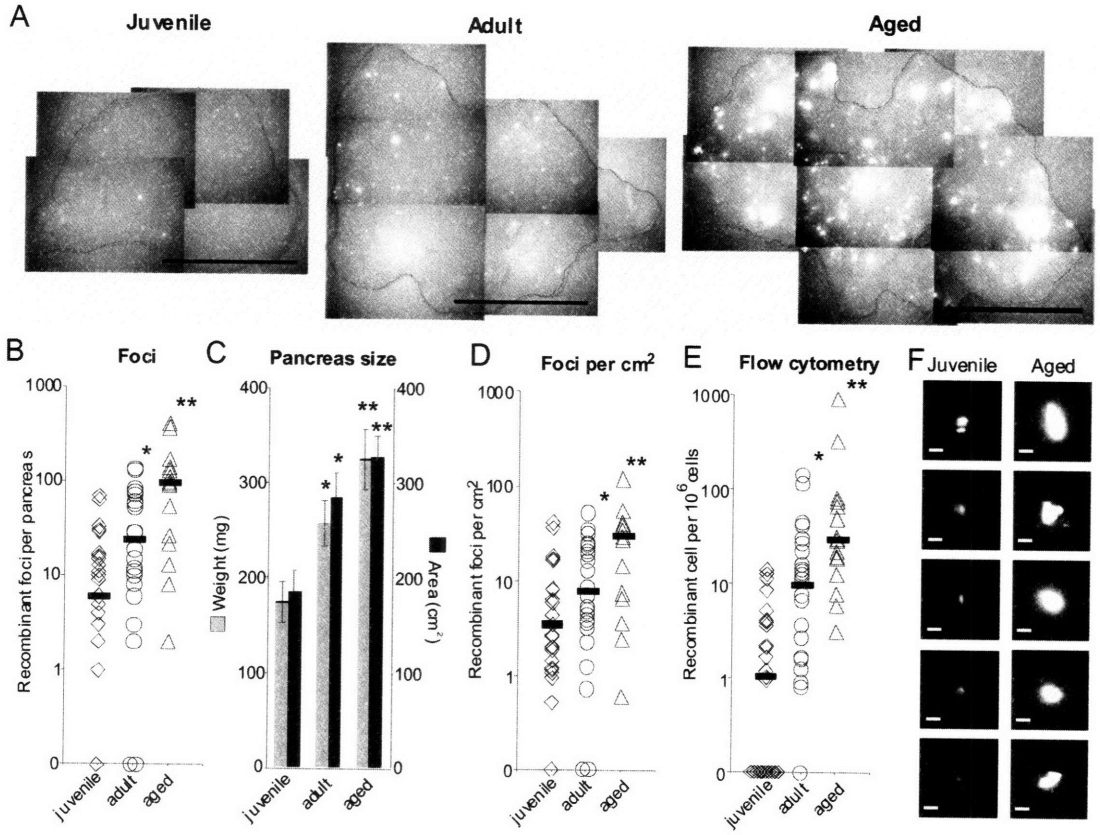

Figure 2-5. Effects of aging on the frequency of recombinant cells in the pancreas. (A) Compiled images for juvenile (4 weeks old) (*Left*), adult (9 weeks old) (*Center*), and aged (64–72 weeks old) (*Right*) FYDR mice. Examples of pancreata with the highest number of recombinant foci for each cohort are shown. Images were collected at x1 with an EYFP filter (510–560 nm). (Scale bar, 1 cm.) The edge of the pancreatic tissue is outlined. (B) Recombinant foci per pancreas detected by *in situ* image analysis for juvenile (n = 25), adult (n = 24), and aged (n = 16) mice. (C) Weight (mg) and area (cm^2) of mouse pancreata for juvenile (n = 25), adult (n = 25), and aged (n = 17) cohorts. (D) Recombinant foci per squared centimeter for juvenile (n = 25), adult (n = 24), and aged (n = 16) mice. (E) Frequency of recombinant cells per million as determined by flow cytometry for juvenile (n = 24), adult (n = 24), and aged (n = 16) mice. (B-E) * Adult cohort is significantly higher statistically than juvenile cohort ($P < 0.05$). ** Aged cohort is significantly higher statistically than juvenile and adult ($P < 0.05$) cohorts. Medians are indicated by black bars. Points on the x axis indicate individual mice with zero recombinant cells detected. (F) Images of the five largest foci among all juvenile (*Left*) and all aged (*Right*) mice taken under an EYFP filter (510–560 nm) at x1. (Scale bar, 100 µm.) Brightness and contrast were not adjusted.

Chapter III

Applications of Fluorescence for Detecting Rare Sequence Rearrangements *in vivo*

Chapter 3

3.1 Abstract

Homologous recombination (HR) is an important pathway for the accurate repair of potentially cytotoxic or mutagenic double strand breaks (DSBs), as well as double strand ends that arise due to replication fork breakdown. Thus, measuring HR events can provide information on conditions that induce DSB formation and replicative stress. To study HR events in vivo, we previously developed Fluorescent Yellow Direct Repeat (FYDR) mice in which a recombination event at an integrated transgene yields a fluorescent signal. Recently, we published an application of these mice demonstrating that fluorescent recombinant cells can be directly detected within intact pancreatic tissue. Here, we show that *in situ* imaging is a more sensitive method for detecting exposure-induced recombinant cells, yielding statistical significance with smaller cohorts. In addition, we show inter-mouse and gender-dependent variation in transgene expression, examine its impact on data interpretation, and discuss solutions to overcoming the effects of such variation. Finally, we also present data on enhanced yellow fluorescent protein (EYFP) expression, showing that several tissues, in addition to the pancreas, may be amenable for *in situ* detection of recombinant cells in the FYDR mice. The FYDR mice provide a unique tool for identifying genetic conditions and environmental exposures that induce genotoxic stress in a variety of tissues.

3.2 Introduction

Mitotic homologous recombination (HR) is critical for the repair of genome destabilizing DNA damage, including double strand breaks (DSBs) and interstrand cross-links. These DNA lesions can be caused by environmental exposures, such as radiation or chemotherapy, and by endogenous cellular metabolites, such as reactive oxygen species (1-4). In addition, DSBs can occur as the result of DNA processing. For example, replication fork encounter with a DNA lesion or a single strand gap can cause replication fork breakdown, creating a double strand end (5-9). HR is the only DNA repair pathway that can accurately repair replication fork associated double strand ends (1, 2). With its critical role in the repair of replication-dependent and –independent DSBs, it is not surprising that mitotic HR is critical to human health (10-14).

In addition to HR, cells use another DNA repair pathway, nonhomologous end-joining (NHEJ), to recognize and repair DSBs. In contrast to HR which uses homologous sequences as templates for repair, NHEJ directly rejoins DSBs, regardless of sequence (15, 16). In order to maintain genomic stability, cells maintain an appropriate balance between NHEJ and HR in the repair of DSBs. NHEJ is the preferred pathway during G_0/G_1 phases of the cell cycle, whereas HR is critical during late S/G_2 (17). Specifically, HR repairs broken replication forks that arise during S phase by reinserting broken ends into their respective sister chromatids. If the double strand ends were instead acted upon by NHEJ, the mis-joining of broken ends from independent replication forks at two different loci will inevitably lead to large scale sequence rearrangements that may be tumorigenic or cytotoxic. On the other hand, since a significant portion of the human genome is composed of repetitive

elements, the repair of DSBs during G_0/G_1 phases by HR could lead to exchanges between misaligned sequences that promote tumorigenic aberrations (18). Thus, many inherited mutations in genes that modulate HR, whether up or down, are associated with an increased risk of cancer, (13, 14, 19) and defects in HR proteins are also often seen in sporadic cancers (20-23).

Ironically, although DNA damage that inhibits replication can induce tumor-promoting sequence rearrangements, many agents used to treat cancer are in fact DNA damaging-agents that inhibit replication fork progression. Thus many chemotherapeutic agents are highly recombinogenic (24-29). Interestingly, elevated levels of HR proteins within tumor cells are associated with an increased resistance to many cancer chemotherapeutics (30-35) and a poor prognosis for cancer survival (36). In contrast, cancer cells deficient in HR (e.g., breast tumors with BRCA2 mutations (37, 38)) are rendered sensitive to chemotherapeutic agents that induce replication fork breakdown (39-42). Therefore, knowledge about HR capacity is relevant both to one's risk of developing cancer (13, 14, 19) and to the response of a tumor to chemotherapy (39-43).

Although HR is an important pathway for overcoming the potential lethality and mutagenicity of replicative stress, studying HR has been difficult since many key HR proteins are either essential for viability (44-49) or possibly sufficiently redundant that their absence does not fully reveal the role of this pathway *in vivo*. An alternative approach for studying HR is to measure DNA sequence rearrangements that result from HR events. Toward this end, we have developed Fluorescent Yellow Direct Repeat (FYDR) mice that enable the detection of HR events *in vivo*.(50) FYDR mice carry a direct repeat recombination substrate that contains 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. 3-1A). One exciting advantage of using fluorescence as a marker for sequence changes is that cells harboring recombined DNA can potentially be detected within intact tissues.

3.3 Results

Advantages of *In Situ* Detection

Recently, we have shown that fluorescent recombinant cells are directly detectable within pancreatic tissue (51). Examination of aged mice by in situ imaging revealed a dramatic increase in not only the number but also the size of recombinant cell clusters (Fig. 3-1B), indicating that both *de novo* events as well as clonal expansion contribute to the overall increase in the frequency of recombinant cells with age. The advantage of being able to detect the accumulation of *de novo* recombination events is that it enables the study of the cumulative effects of long term exposures on recombination. In addition, the ability to detect clonal expansion enables the identification of clonal cell populations in normal tissues (52). Both the formation of new genetic changes and clonal expansion of cells with preexisting genetic changes contribute to cancer development. Therefore, being able to study conditions that induce new genetic changes as well as stimulate clonal outgrowth will likely yield interesting insights into the earliest steps of cancer formation.

Although the FYDR mice have been used to study the accumulation of environmentally induced recombinant cells in adult mice (51), one limitation to the

sensitivity of this assay is the variation in the frequency of spontaneous recombinant cells among mice. When the tissue is disaggregated and subsequently analyzed by flow cytometry, large recombinant cell clusters can lead to a high frequency of fluorescent recombinant cells, which could mask the induction of *de novo* exposure-induced recombinant cells. Therefore, the detection of discrete recombinant cell clusters by *in situ* imaging may be a more sensitive method for detecting *de novo* recombination events. We tested this hypothesis by comparing our ability to detect HR events after treatment with the DNA cross-linking agent mitomycin-C (MMC). Analysis by both *in situ* imaging and flow cytometry show an induction in recombinant cells *in vivo*. However, with ~24 mice per cohort, the induction was statistically significant only when foci were analyzed *in situ* (Fig. 3-2A). Statistical significance by flow cytometry was observed when approximately twice as many (~47) mice per cohort were analyzed (Fig. 3-2B). These data show that because independent mutation events can be distinguished, *in situ* imaging provides a more sensitive method for determining environmentally induced mutations in adult tissues. Interestingly, many previously published mutation studies have been done by analyzing DNA from disaggregated tissue (e.g., *Aprt*, *Tk*, Big Blue, Muta Mouse, *GptΔ*) (53-55), an approach that limits information about the clonal relationship among mutant cells. The observation that *in situ* analysis is more sensitive than tissue disaggregation for detecting environmentally induced mutations raises the possibility that previous studies of disaggregated tissues may underestimate the impact of some environmental exposures. It is noteworthy that the techniques described here are not limited to HR and could potentially be applied to studies of other types of mutations.

Controlling For Variation in Transgene Expression Levels

In the course of studying MMC-induced recombination, we noticed an apparent difference in foci frequencies amongst males and females. Indeed, closer examination of recombinant foci in pancreata of FYDR mice reveals a statistically significant difference in the number of recombinant foci between males and females (Fig. 3-3A), even without exposure to exogenous DNA damaging agents. Although this difference in recombinant foci may be the result of differences in HR, it is also possible that this difference results from differences in the ability to detect fluorescent cells (e.g., differences in expression levels of *EYFP*). To explore the possibility that the higher number of recombinant foci in female pancreata may be due to higher levels of FYDR transgene expression, we exploited positive control FYDR-Recombined (FYDR-Rec) mice. FYDR-Rec mice arose spontaneously from an HR event in a FYDR parental gamete. Thus, all cells in FYDR-Rec mice carry the full-length *EYFP* coding sequence under the identical promoter and locus as the FYDR mice and have the potential to express *EYFP*. Therefore, the FYDR-Rec mice are the perfect positive control for FYDR transgene expression, and expression of the FYDR transgene can be analyzed simply by looking at expression of *EYFP*. We have measured the percentage of fluorescent cells from over 50 disaggregated positive control FYDR-Rec pancreata (Fig. 3-3B). It is noteworthy that regardless of sex, pancreatic cells show a range of *EYFP* expression levels. However, the average percentage of cells expressing *EYFP* was statistically significantly higher in female mice. Therefore, the male-female difference in recombinant foci is at least partially due to differences in expression.

In addition to gender, exposures may also affect expression of the FYDR transgene. Thus, in addition to its effect on recombination, the effect of any condition on transgene

expression should be determined concurrently in a cohort of FYDR-Rec mice. For example, to determine the effect of MMC treatment on FYDR transgene expression, we included mock- and MMC-treated FYDR-Rec cohorts in our study. Analysis of disaggregated FYDR-Rec pancreata showed that MMC treatment did not lead to any statistically significant change in the percentage of fluorescent cells (Fig. 3-3C). These studies indicate that the increase in recombinant cells after MMC treatment in FYDR mice is indeed due to HR and not to an increase in expression of the FYDR transgene.

In the FYDR mice, expression of *EYFP* is controlled by the cytomegalovirus enhancer/chicken beta actin (CAG) promoter. Although this promoter has previously been shown to be ubiquitously expressed in all cell and tissue types (56), this is not the case in our particular model. Histological analysis shows significant variation in expression levels even among the same cell type within a tissue. The variability in expression may be the result of the locus of integration and the number of integrated transgene copies (note that in these mice, a single copy of EYFP is expressed (50)). Such expression variability is unlikely to be unique to this particular mouse model. Transgene expression variability within a single tissue type can have a tremendous impact on experimental design and data interpretation, resulting in the need to explore and control for inter-mouse variation in transgene expression. In our system, when comparing HR among different genetic conditions or environmental exposures, inter-mouse variation in transgene expression can be overcome by using appropriate FYDR cohort sizes and by using the positive control FYDR-Rec mice to determine the effect of a condition on transgene expression. Thus, the FYDR and FYDR-Rec mice together provide a unique system for determining the effect of any genetic condition or environmental exposure on HR.

Future Applications: Studies of Homologous Recombination in Multiple Tissues

In our studies, we have focused on analyzing recombination in the pancreas, cutaneous tissue, and cultured primary ear fibroblasts (50, 51, 57). In addition to the pancreas and skin, recombination in other tissues of the FYDR mouse can potentially be studied. However, one limitation of using fluorescence as a measure of recombination is that *EYFP* is not expressed equally in all cell types. In order to detect rare recombinant cells (~1-5/10^6), both a high signal to noise ratio and a large number of analyzable cells is required. To learn more about which tissue types are amenable for analysis, we examined the fluorescence signal to noise ratio in a variety of tissues from FYDR-Rec and negative control C57Bl/6 mice. These studies revealed that *EYFP* is detectable in multiple tissue types, but that the percentage of cells expressing *EYFP* and the brightness of *EYFP* expression are highly variable within each tissue (Fig. 3-4) and among individual mice (data not shown). In the pancreas (Fig. 3-4A), *EYFP* expressing cells are generally extremely bright. Similar to the pancreas, expression of *EYFP* in skeletal muscle is uniform and comparable in brightness (data not shown). In contrast, *EYFP* expression is seen sporadically in renal tubular epithelial cells (Fig. 3-4B), in biliary epithelial cells and in hepatocytes within the liver (data not shown), and in alveoli and bronchiolar epithelial cells within the lung (Fig. 3-4C). Within these tissues, however, expression is generally dimmer than in the pancreas. Within the brain, *EYFP* expression is dim and limited to cells consistent with Purkinje cells (data not shown), although more experiments are needed to determine cell type. Lastly, in addition to tissue sections, flow cytometry analysis of cells from blood and bone marrow show that certain cell types within these tissues also express *EYFP* (data not shown). Since the ability to study recombination in certain tissues of FYDR mice depends upon the brightness of fluorescence,

the fraction of cells expressing the FYDR transgene, and the total number of cells that can be analyzed from a given tissue, it may be more difficult to detect rare recombinant cells in tissues where *EYFP* expression is either dim or sporadic. However, given the sensitivity of epifluorescence for being able to detect as few as one recombinant cell within an entire pancreas (~$3x10^7$ cells (58)) these preliminary studies suggest that with appropriate imaging techniques it may be possible to use the FYDR mice to study HR in a variety of tissues.

3.4 Discussion

Using fluorescent proteins to mark cells that have undergone an HR event makes it possible to detect rare recombinant cells that arise *in vivo* and to learn about the underlying cell types that have undergone a recombination event. Furthermore, *in situ* detection enables differentiation between *de novo* and clonally expanded recombinant cells. By combining both *in situ* imaging and flow cytometry of disaggregated tissue, the effects of environmental exposures on both new mutation formation and clonal expansion can be revealed. One application of these approaches is to use the FYDR mice to study the recombinogenic effects of chemotherapeutic regimens, yielding valuable information about tissue specificity as well as potential long term effects of such exposures. Given that HR is a critical process for maintaining genomic integrity and for preventing DNA damage-induced cell death, *in situ* detection of fluorescent recombinant cells provides a sensitive approach for studying the potential genotoxic effects of pharmaceuticals and environmental exposures.

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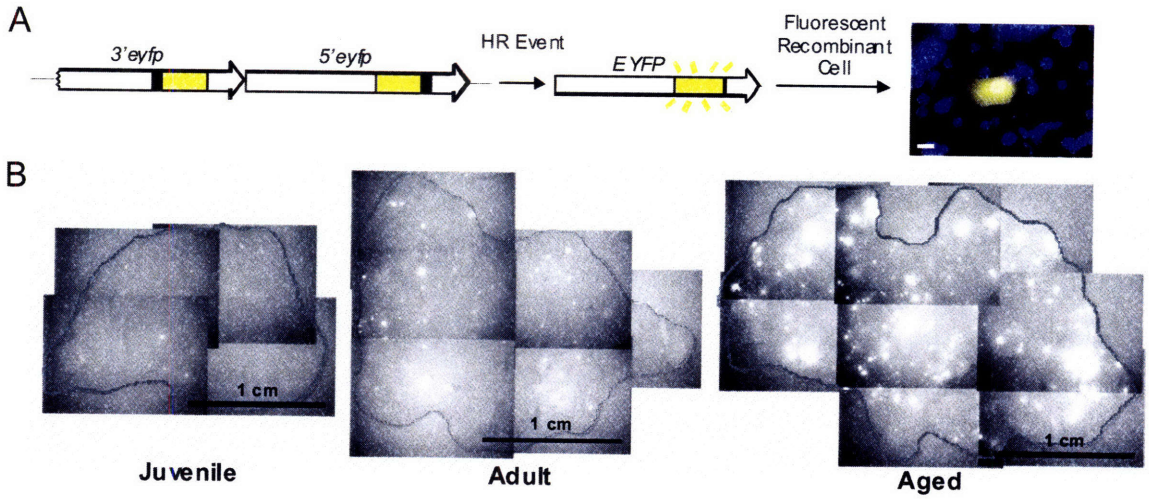

Figure 3-1. FYDR system and analysis of pancreatic cells by *in situ* imaging. (A) Arrangement of the FYDR recombination substrate: large arrows indicate expression cassettes; yellow boxes show coding sequences; black boxes show positions of deleted sequences (deletion sizes not to scale). An HR event between the two expression cassettes can restore full-length *EYFP* coding sequence, resulting in a fluorescent cell. Image depicts recombinant pancreatic cell within freshly excised tissue; bar, 10 μm. Not that the EYFP emission spectra largely overlaps with that of EGFP; this image has been pseudocolored yellow. (B) Effects of aging on the frequency of recombinant cells in the pancreas. Compiled images for juvenile (4 weeks old), adult (9 weeks old), and aged (64-72 weeks old) FYDR mice. Examples of pancreata with the highest number of recombinant foci for each cohort are shown. Images were collected at 1x (bar, 1 cm) using an EYFP filter (510-560 nm). The edge of the pancreatic tissue is outlined. This figure was adapted from Wiktor-Brown, *et al* (51).

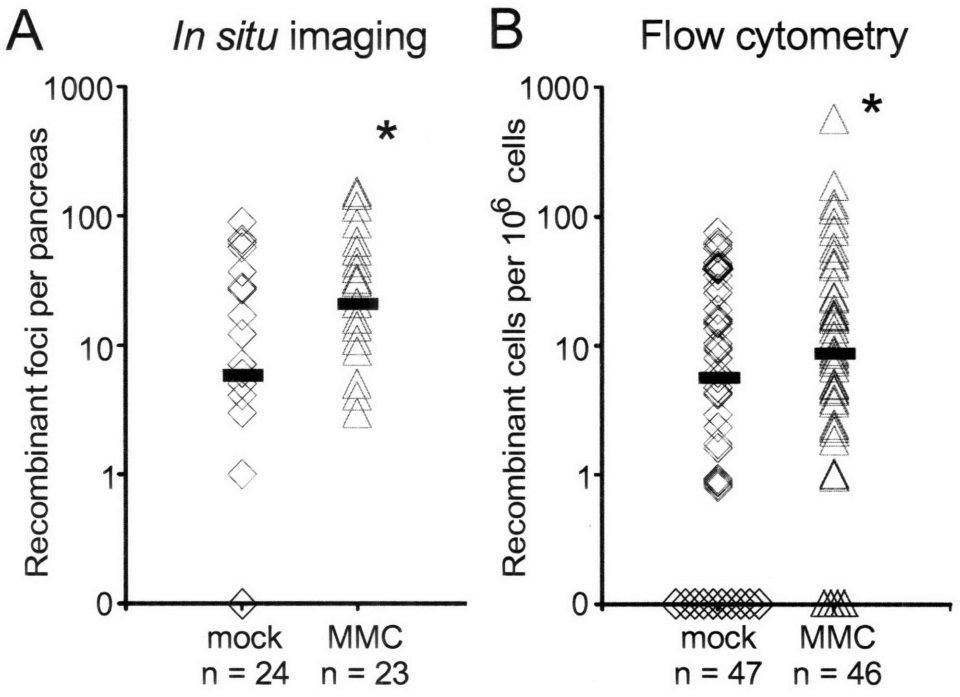

Figure 3-2. MMC induced HR in mouse pancreata. (A) Recombinant foci per pancreas detected by *in situ* image analysis for mock- and MMC-treated FYDR mice. (B) Frequency of recombinant cells per million as determined by flow cytometry for mock- and MMC-treated FYDR mice. * MMC-treated cohort is statistically significantly higher than mock treated cohort ($p < 0.05$, Mann-Whitney). Medians are indicated by black bars. Points on the x-axis indicate individual mice with no detectable recombinant cells. For methods see Wiktor-Brown, *et al* (51).

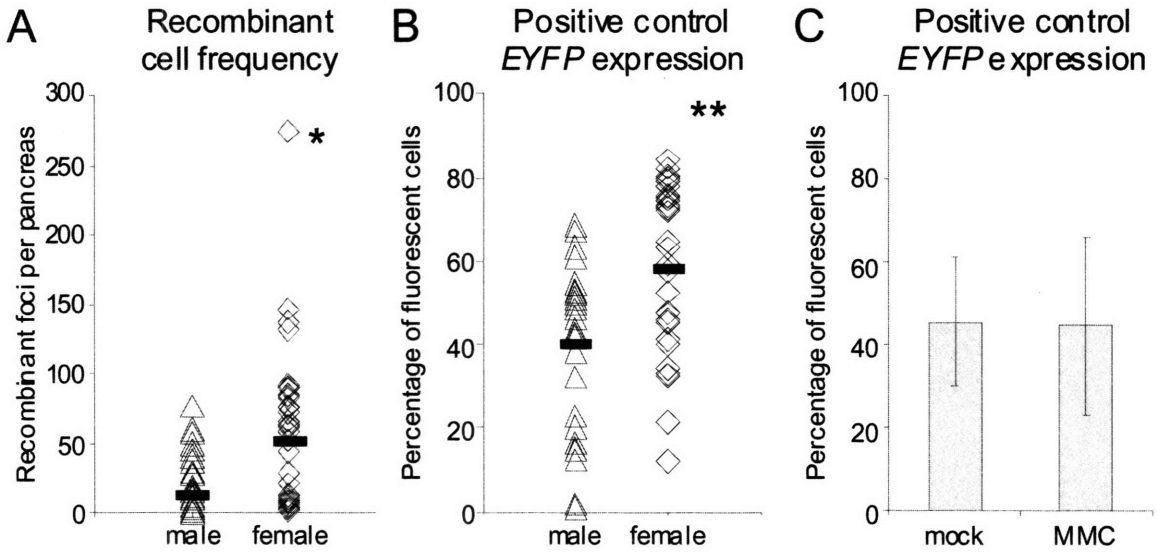

Figure 3-3. Inter-mouse variation in recombination and expression. (A) Recombinant foci per pancreas detected by *in situ* image analysis in 9 week old male (n = 35) and female (n =37) FYDR mice. Data combined from cohorts of untreated and mock-treated mice. Medians are indicated by black bars. * Female cohort is statistically significantly higher than male cohort ($p < 0.05$, Mann-Whitney). (B) Percentage of fluorescent pancreatic cells as determined by flow cytometry from 9 week old male (n = 26) and female (n = 28) FYDR-Rec mice. Data combined from cohorts of untreated and mock-treated mice. Means are indicated by black bars. ** Female cohort is statistically significantly higher than male cohort ($p < 0.05$, Student's t-test). (C) Average percentage of fluorescent pancreatic cells as determined by flow cytometry from mock- (n = 10) and MMC- treated (n = 10) FYDR-Rec mice (ratio of males to females is the same in mock- and MMC-treated cohorts). Error bars indicate 1 standard deviation. For methods see Wiktor-Brown, *et al* (51).

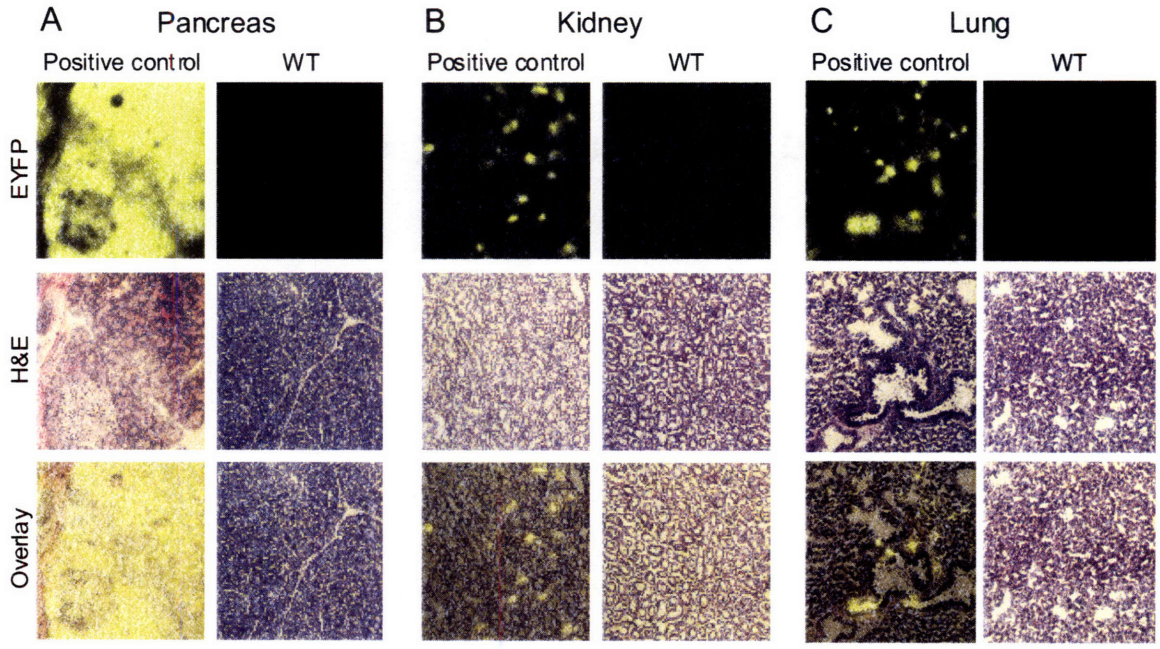

Figure 3-4. Expression of *EYFP* in various tissues of female positive control FYDR-Rec C57Bl/6 and negative control WT C57Bl/6 mice. (A) Pancreas (B) Kidney (C) Lung. Histological images were collected at 10x. Left column: Positive control FYDR-Rec tissue. Right column: Negative control WT C57Bl/6 tissue. Top row: Fluorescence image under EYFP filter (510-560 nm). Middle row: H&E stained section. Bottom row: Overlay of H&E and fluorescence images. Brightness/contrast for EYFP filtered images was optimized for each tissue and adjusted equivalently for positive control FYDR-Rec and negative control WT C57Bl/6 mice. EYFP fluorescence is pseudocolored.

Chapter IV

Novel Integrated One- and Two-Photon Imaging Platform Reveals Extent of Clonal Expansion *in situ*

Chapter 4

4.1 Abstract

The clonal expansion of cells with mutations in genes that provide growth and survival advantages is one of the pivotal first steps in cancer formation. To understand the earliest stages of cellular transformation, a method to identify and analyze these pre-malignant cells is needed. We have created a transgenic Fluorescent Yellow Direct Repeat (FYDR) mouse in which cells that have undergone sequence rearrangements (via a homologous recombination event) express a fluorescent protein, enabling the labeling of phenotypically normal cells. In order to measure clonal expansion *in situ*, we have integrated one- and two-photon microscopy to create a sensitive imaging system that spans four orders of magnitude. This imaging platform rapidly identifies very rare fluorescent cells within an entire mouse tissue (at the cm scale) and subsequently provides 3D images of each fluorescent cell (at the micron scale). We applied these techniques to study the effect of age on clonal expansion of fluorescent cells in the pancreata of FYDR mice. Results show that as mice age, there is a significant increase in the number of cells within fluorescent cell clusters, indicating that pancreatic cells can clonally expand with age. This combination of mechanico-optical engineering technologies with genetically engineered FYDR mice can be applied to study the effects of genetic and environmental exposures on the risk of clonal expansion.

4.2 Introduction

Cancer is caused by the accumulation of mutations within a single cell lineage. This multi-step process occurs through successive rounds of clonal expansion and selection of cells that have acquired mutations that confer growth advantages (1-4). 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 (5-7). Although the clonal expansion of pre-malignant cells is an important precursor to the development of cancer, no methods had been developed to study clonal expansion within intact histologically normal tissue.

Mutations that drive cancer development are often caused by DNA damage. Human cells are subjected to thousands of DNA lesions each day (8), including a wide variety of base modifications, cross-links and strand breaks. Of these DNA lesions, double strand breaks (DSBs) are considered to be among the most dangerous, since a single unrepaired DSB can result in the permanent loss of over 100 million base pairs of genetic information (9), and misjoining of DSBs can lead to gross chromosomal rearrangements (10). Mitotic homologous recombination (HR) is a critical pathway for the accurate repair of potentially mutagenic DSBs (for review see (11)). During recombinational repair, homologous sequences present on the sister chromatid or homologous chromosome are used as templates for repair. In addition, HR provides the only pathway for the accurate repair of DSBs that arise as a result of broken replication forks (12-16).

Given its key role in the repair of DSBs and broken replication forks, HR is critical for maintaining genomic integrity. Indeed, inherited mutations in genes that modulate HR (*e.g.* BLM (17), WRN (18), BRCA1 (19), BRCA2 (20)), are associated with an increased risk of cancer (21, 22). In addition, environmental exposures to agents that induce recombination events (*e.g.* ionizing radiation (23), sunlight (24) and chemicals present in food (23)) also increase cancer risk (25-27), which is thought to be at least partially due to the associated increased risk of tumor-promoting errors during the recombinational repair of DNA damage. Thus, the frequency of HR events reflects both the demand for repairing DBSs and the ability of cells to use HR as a repair pathway.

In order to study HR *in vivo*, we have developed transgenic Fluorescent Yellow Direct Repeat (FYDR) mice, in which a HR event at an integrated transgene results in expression of a fluorescent protein (28). In FYDR mice, cells harboring recombined DNA can be detected within intact pancreata (29). Age is a key risk factor for pancreatic cancer (30); therefore, we studied the effect of aging on HR in the pancreas (29). Our previous work shows that recombinant cells accumulate in the pancreas of FYDR mice with age and that this accumulation results from both *de novo* recombination events as well as clonal expansion of previously existing recombinant cells. Although clonal expansion was estimated using traditional epifluorescence microscopy, it was not possible to determine the number of recombinant cells per focus using this approach. Thus, the extent of clonal expansion, an important precursor to tumor formation, could not be quantified.

Traditional epifluorescence microscopy uses one-photon for excitation of fluorophores. In a spatially uniform fluorescent sample, equal fluorescence intensities are

contributed from each z-section above and below the focal plane (31). Thus, within a thick sample, the boundaries of fluorescent structures are difficult to resolve when the emitted light is diffracted by tissue between the fluorescent object and the surface (*i.e.*, the light is scattered by out-of-focus tissue). To overcome this problem, Denk et al., developed two-photon microscopy wherein chromophores can be excited only at a region of high temporal and spatial concentration of photons (32). This is achieved by the simultaneous absorption of two-photons, each having half the energy needed for the excitation transition (32, 33). Thus, only molecules at the focal point are excited, reducing photobleaching and phototoxicity, and enabling higher resolution of fluorescent images. While two-photon microscopy offers increased resolution of images, it is most effective for imaging on micrometer length-scales. Consequently, analysis of significant tissue volumes (e.g., > 1 cm^3) is prohibitively slow, making this a suboptimal approach for studies of rare fluorescent objects within a tissue.

Here, we have combined the speed of traditional fluorescence microscopy with the resolution of two-photon microscopy to create a quantitative method for the analysis of fluorescent foci within intact tissue. Specifically, in these studies using FYDR mice, rapid two-dimensional fluorescence imaging of pancreata enables the identification and mapping of recombinant foci within the tissue. Subsequent optical sectioning of recombinant foci using two-photon microscopy enables the quantification of cells within each focus with minimal damage to the tissue and without distortion of cell-cell relationships. The integrated one- and two-photon imaging platforms were then applied to study the effect of aging on HR in the pancreas. Results reveal that as mice age, recombinant pancreatic cells undergo clonal expansion, in some cases to an unexpected

degree, resulting in a significant increase in the overall frequency of cells harboring DNA sequence rearrangements.

4.3 Materials and Methods

One-Photon Imaging

Female C57BL/6 FYDR mice (28) were IP injected with 0.04 mg/g of body weight of 5 mg/ml Hoechst 33342 (Sigma) in phosphate buffered saline. Twenty minutes after injection, pancreata were isolated and placed in ice-cold PBS containing 0.01% soybean trypsin inhibitor (Sigma). Whole pancreata were pressed between glass slides separated by 0.5 mm spacers. Sequential images were collected in black and white using a 1.25x objective, a 532 nm laser for EYFP excitation, and a fixed aperture time. Images were compiled to cover the entire visible surface area. Foci positions were mapped using an automated x-y stage positioner. At each x-y coordinate, sequential 2D two-photon images are collected.

Two-Photon Imaging

Procedure for collecting two-photon images is described elsewhere (Kwon *et al.*, in preparation)

4.4 Results

Development of Two-Photon Imaging for Analysis of Recombinant Foci

To study homologous recombination *in vivo*, we developed FYDR mice, which carry a direct repeat recombination substrate that contains two differently mutated copies of the coding sequence for enhanced yellow fluorescent protein (EYFP). An HR event can restore full-length *EYFP* coding sequence, resulting in the appearance of fluorescent cells (28) (Fig. 4-1A). To identify recombinant cells within intact pancreata of FYDR mice, previously we developed an *in situ* imaging method using traditional epifluorescence microscopy (29). Briefly, FYDR pancreata are imaged using filters specific for Hoechst-stained nuclei (Fig. 4-1B, left) and EYFP (Fig. 4-1B, middle). Overlaying these images shows that recombinant foci can be directly detected within intact pancreata (Fig. 4-1B, right).

Using epifluorescence microscopy, when a recombinant focus is present on the surface of the pancreas, the boundaries are distinct and it is possible to estimate the number of cells within the focus (Fig. 4-1C, left). However, for foci located below the surface, the scattering of emitted fluorescence prevents identification of specific cell boundaries, making it impossible to quantify the number of cells per focus (Fig. 4-1C, right). Due to the limitation of traditional epifluorescence microscopy to obtain high resolution images of fluorescent foci, a two-photon method to create images of cross-sections of recombinant foci was developed. To image recombinant foci by two-photon microscopy, a low energy laser is used to excite Hoechst stained nuclei and EYFP containing cytoplasm. By overlaying images taken under the two filters, the number of

individual cells (~20-30 μm in diameter) contained within a single cross-section of the recombinant focus can be quantified. These two-dimensional images show that two-photon microscopy can provide high resolution images of fluorescent recombinant foci at a depth of up to 70 μm within pancreata of FYDR mice.

To determine the extent to which one-photon microscopy distorts the true size of recombinant foci, a comparative analysis was performed for multiple foci collected by both epifluorescence and two-photon microscopy. A fluorescent focus that appears large by epifluorescence only contains a single cell (Fig. 4-2A), while a focus that appears small by epifluorescence contains multiple cells (Fig. 4-2B). The discrepancies between foci sizes as seen by epifluorescence and two-photon microscopies may be due to focus depth and brightness. First, the depth of a focus may affect its size when imaged using epifluorescence microscopy since the diffraction of light emanating from fluorescent objects located deep in a tissue can lead to the appearance of a larger size relative to the same object located on the surface. In addition, the intensity of fluorescence from cells may affect apparent focus size by epifluorescence microscopy with brighter foci having an apparently larger size than dimmer foci. Thus, while epifluorescence microscopy enables the rapid identification of recombinant foci within an entire pancreas, two-photon imaging provides a more accurate method for *in situ* quantification of the number of cells within a single recombinant focus.

Integrating One- and Two-Photon Imaging Platforms

While two-photon imaging is effective for measuring the number of cells per recombinant focus, analyzing the entire surface of a pancreas (~2.5 cm^2) would take at

least ~20 hours. Therefore, to study multiple mice under different conditions, we created a system that enables rapid data collection for both the frequency of recombinant foci and the number of cells within foci. For each sample, the pancreas is uniformly compressed to a thickness of 0.5 mm and images are taken using an epifluorescence microscopy with a low powered objective to cover the entire surface area. Fluorescent recombinant foci are identified and their x,y coordinates are mapped within the composite images. Subsequently, using two-photon microscopy, centering upon the coordinates of the previously mapped positions, multiple sequential z-planes are acquired through a maximum thickness of 70 μm for each focus. The images are then deconvoluted to produce a 3D reconstruction of the focus, differentiating Hoechst-stained nuclei and EYFP. Thus, the combination of epifluorescence and two-photon microscopy enables the identification and analysis of recombinant foci over the entire surface of a pancreas.

The Frequency and the Size of Fluorescent Foci Increase With Age

In order to study the effect of aging on recombinant cell frequency, we analyzed pancreata from two age groups, 'juvenile' (4-6 weeks old) and 'aged' (74-83 weeks old). Composite epifluorescence images show that both the frequency and the apparent sizes of the foci appear to increase with age, which is consistent with our previous studies (see Wiktor-Brown *et. al.* (29)). Further analysis of the foci using two-photon microscopy reveals that the number of cells within recombinant foci varies within each age cohort (Fig. 4-3). Of the foci analyzed in pancreata of juvenile mice, the largest recombinant focus contained five cells. In contrast, ~54% of recombinant foci in aged mice contain more than five cells, with one focus containing at least 68 cells. Overall, from juvenile to

aged mice, the median number of cells per focus increases from two to six cells. Given that expression of the FYDR substrate is not statistically significantly different between the two cohorts (data not shown), these data indicate that as mice age, recombinant pancreatic cells can clonally expand.

It is formally possible that the increase in the number of cells per recombinant focus is the result of multiple independent recombination events occurring in neighboring cells. However, since the frequency of recombinant cells in the pancreas is ~5 per million (29), the probability that two adjacent recombinant cells occurred from independent events is roughly $1/10^{10}$ (assuming that each cell touches ~10 neighbors), making it virtually impossible that multiple adjacent recombinant cells in large foci occurred independently. Therefore, recombinant foci containing multiple cells are most likely the result of clonal expansion and not independent recombination events in neighboring cells.

4.5 Discussion

For some tumors, it may take 20-40 years from the initial formation of a cancer progenitor cell to the appearance of a detectable tumor (34-37). During this time, the population of pre-cancerous cells can increase as the result of clonal expansion (3). However, these clonal cell populations can appear to be phenotypically normal, even though they have acquired mutations in tumor suppressors or oncogenes that may provide a survival and proliferative advantage. The ability to detect clonal expansion *in situ* within histologically normal tissue enables the identification of these clonal cell

populations, and studying these cells may provide insights into of the earliest stages of cellular transformation, prior to the clinical appearance of a tumor.

Two-dimensional epifluorescence microscopy enables the rapid, low-resolution identification of rare fluorescently labeled cells over the entire surface of an intact tissue. Three-dimensional two-photon microscopy provides high-resolution imaging on a micrometer scale to quantify the number of cells contained within fluorescent foci. By combining these approaches, rare fluorescent cells can be analyzed from the tissue (cm scale) to the single cell (μm scale). A number of imaging techniques are available for the gross analysis of tissues (*e.g.*, MRI, CT); however, these cannot provide resolution on the single cell level. Other methods that have been used to obtain high resolution 3D images of tissues (e.g., physical serial tissue sectioning (38) and confocal microscopy (Pawley 1995)) have multiple limitations including the length of time to collect data, distortion of cell morphology and phototoxicity. In contrast, optical sectioning by 3D two-photon microscopy enables cells to be studied within their normal physiological context, obtaining accurate information regarding cell-cell relationships in a relatively short amount of time. Thus, the integration of one- and two-photon microscopy allows the rapid collection of data for not only the total number of fluorescent cell clusters within an intact tissue but also the number of cells within each cluster, over a range of length-scales that span >4 orders of magnitude.

HR events are an important class of mutations that are known to promote cancer (11). Here, we have applied the combination of one- and two-photon imaging to study HR events in the pancreata of FYDR mice *in situ*. Most other techniques for studying mutations require tissue disaggregation followed by analysis of either cultured primary

cells or genomic DNA (*e.g.*, RMC, *Aprt*, *Tk*, Big Blue, Muta Mouse, *Gpt*Δ)(39-42), thus limiting information about the clonal relationship among mutant cells. In contrast, the techniques described here enable the quantification of cells within recombinant foci, providing information about the extent of clonal expansion. In addition, being able to detect a single fluorescent cell within an entire pancreas (~$3x10^7$ cells (43)), makes this imaging technique one of the most sensitive methods for detecting rare DNA sequence changes. Finally, we have previously shown that because independent mutation events (*i.e.*, independent recombinant foci) can be distinguished, *in situ* imaging is more sensitive than tissue disaggregation for determining environmentally induced mutations in adult tissues (44). Taken together, integrated one- and two-photon imaging provides a highly sensitive method for detecting and analyzing cells that contain small sequence rearrangements within intact tissues.

Inherited mutations in several genes known modulate HR (*i.e.*, BRCA1 (45), BRCA2 (46), and FANCC (47)) increase the risk of pancreatic cancer, suggesting that HR plays an important role in maintaining genomic integrity in the pancreas. Using the FYDR mice, we have previously shown that as mice age, recombinant cells accumulate in the pancreas in part due to *de novo* recombination events (29), suggesting that HR remains an active repair pathway in the pancreata of adult animals. Although HR is generally error-free, recombination between misaligned sequences can cause insertions, deletions, and translocations that promote cancer. In fact, the FYDR mice specifically detect sequence rearrangements that result from misalignments at a repeat sequence (for mechanisms, see (48)). Although the FYDR mice detect HR only at one specific locus, DNA damage and repair are not confined to this locus, and misalignments can occur

spontaneously throughout the genome at natural repeat sequences (~40% of the genome is comprised of repeat elements (49)). The susceptibility of cells to both new mutation formation and clonal expansion of pre-existing mutant cells are important in carcinogenesis. Here, results shows that the number of cells per recombinant focus increases with age, indicating that clonal expansion of recombinant cells within existing foci significantly contributes to the accumulation of recombinant cells within the pancreas. By applying this new imaging platform to the FYDR mice, it is now possible not only to detect the accumulation of cells harboring rare sequence rearrangements but also to study the extent to which conditions foster clonal expansion of such mutant cells.

Within the FYDR pancreas, recombinant cells are most likely acinar cells. Acinar cells are part of the exocrine pancreas and secrete hydrolytic enzymes into the duodenum that aid in digestion. The secretory unit within the exocrine pancreas is a grape-like structure called an acinus, which contains ten to forty acinar cells that secrete enzymes into a system of epithelial ducts (50). Acinar cells may play an important role in tumor formation. Evidence suggests that acinar to ductal transdifferentation can occur *in vivo* and *in vitro*, leading to the formation of pancreatic intraepithelial neoplasias and pancreatic ductal adenocarcinoma (51-57). In the adult pancreas, acinar cells exhibit extremely low levels of proliferation, with ~1% of cells in S phase (58). However, in response to damage, such as pancreatectomy or chemical injury, proliferation of acinar cells is stimulated (59, 60). Furthermore, the half-life of acinar cells in the mouse pancreas has been estimated to be at least 70 days (61-63), indicating that acinar cells persist within the pancreas for long periods of time. In this study, we observed one recombinant focus from an aged mouse that contained 68 cells, indicating that significant

clonal expansion (at least seven population doublings) can occur within the acinar cell compartment of the adult pancreas. These data provide new information regarding the proliferative capacity of adult acinar cells *in vivo*. It will be interesting to further apply these techniques to learn more about how injury affects clonal expansion of pancreatic cells.

Measuring HR events provides information on both the formation of DSBs and the ability of cells to use HR as a repair pathway. Here, we have shown that by combining 2D epifluorescence microscopy and 3D two-photon microscopy, recombinant cells can be detected and quantified within intact FYDR pancreata. By providing information regarding both the induction of *de novo* recombination events as well as the clonal expansion of recombinant cells, the integration of these techniques can be applied to study the long term effects of environmental exposures and genetic alterations. Given the importance of clonal expansion as a precursor to tumor formation, these techniques provide new avenues to learn about the earliest stages of cellular transformation. Finally, this new imaging platform can be applied to analyze any cells that can be labeled with fluorescence (*i.e.*, putative adult somatic stem cells), enabling the detection of these rare cells within intact tissue and the study of their propensity to clonally expand.

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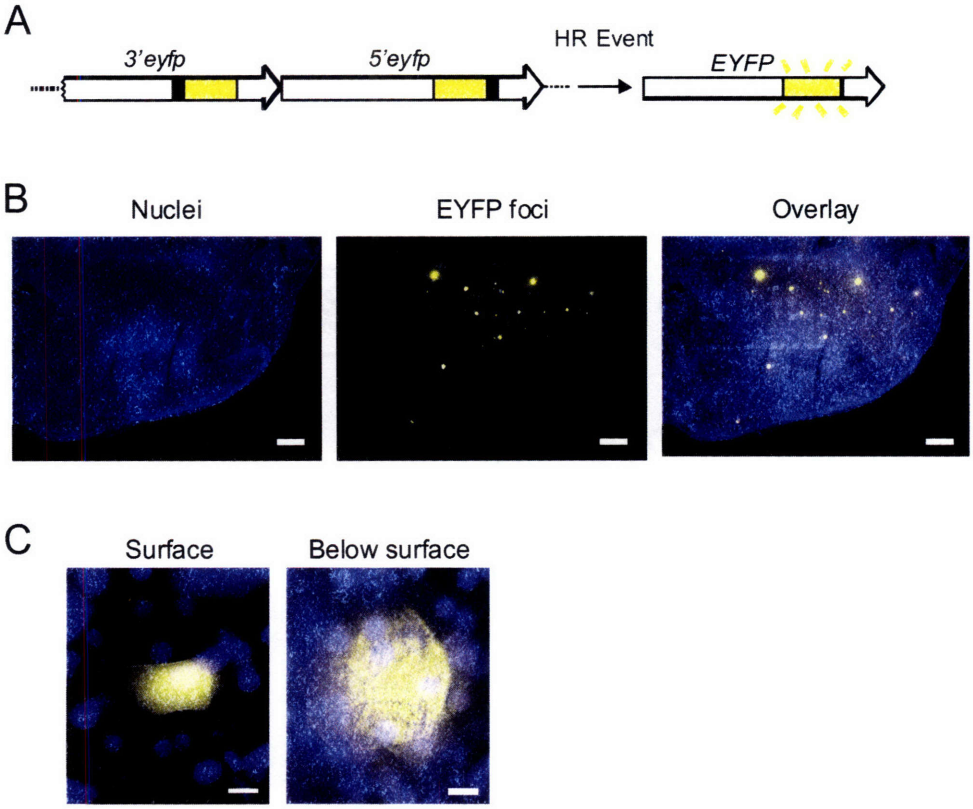

Figure 4-1. FYDR system and detection of recombinant cells by epifluorescence microscopy in mouse pancreata. (A) Arrangement of the FYDR recombination substrate: large arrows indicate expression cassettes; yellow boxes show coding sequences; black boxes show positions of deleted sequences (deletion sizes not to scale). An HR event between the two expression cassettes can restore full-length *EYFP* coding sequence, resulting in a fluorescent cell. (B) *In situ* images of a freshly excised FYDR pancreas taken at 1x (scale bar = 1 mm). Nuclei are stained with Hoechst 33324. Left: UV- (420 nm) filtered image to detect nuclei. Middle: EYFP- (510-560 nm) filtered image to detect recombinant foci. Overlay of UV- and EYFP-filtered images. (C) Distortion of recombinant cell boundaries by epifluorescence microscopy taken at 40x (scale bar = 10 μm). Left: recombinant focus located on the tissue surface. Right: recombinant focus located below the surface shows diffraction through cells.

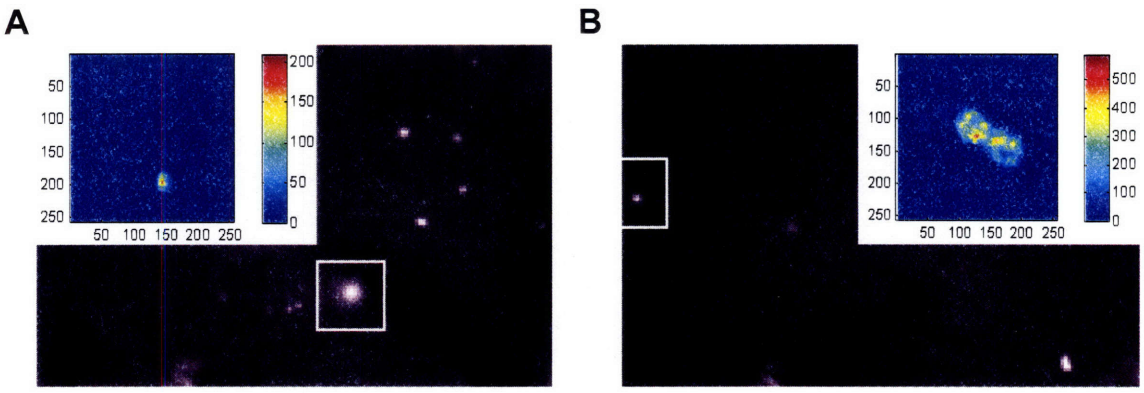

Figure 4-2. Comparison of apparent foci sizes by epifluorescence and two-photon microscopies. (A) A fluorescent focus that appears large by epifluorescence microscopy only contains a single cell as determined by two-photon microscopy (inset). (B) A fluorescent focus that appears small by epifluorescence contains multiple cells as determined by two-photon microscopy (inset).

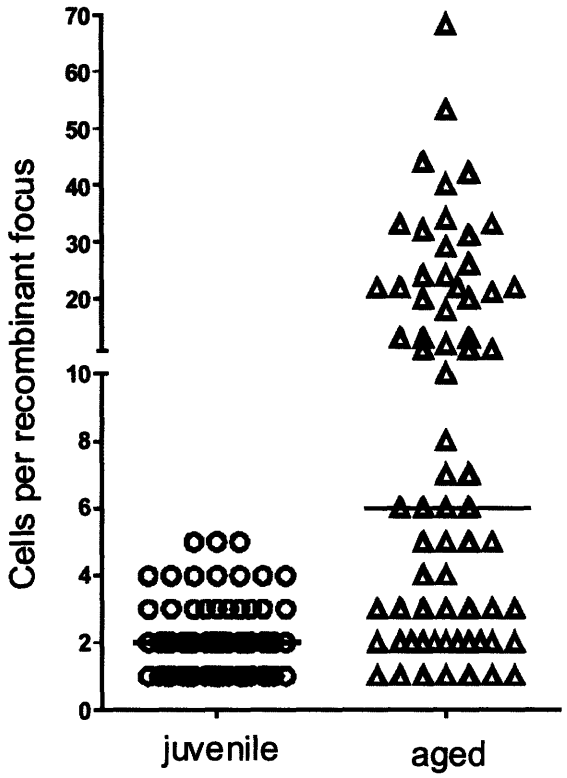

Figure 4-3. Effect of age on the number of cells within recomabinant foci. Number of cells per recombinant focus for (A) juvenile (n = 79 foci) and (B) aged (n = 68 foci) FYDR mice. Each point represents one focus. Medians are indicated by black bars.

Chapter V

Cells Harboring Sequence Rearrangements Accumulate with Age in the Pancreas but not in Skin

Chapter 5

5.1 Abstract

Mitotic homologous recombination (HR) is a critical pathway for the accurate repair of DNA double strand breaks (DSBs) and broken replication forks. The misrepair of DSBs can lead to large scale sequence rearrangements, and an increase in these types of mutations is often seen with age, suggesting that the inability to accurately repair DSBs is an important contributor to aging. To learn more about the effect of aging on the repair of DSBs by HR, 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. Pancreas shows a dramatic increase in recombinant cell frequency with age, whereas 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, suggesting that the lack of accumulation of recombinant cells 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 results from differences in the ability of recombinant cells to persist and clonally expand within the tissue.

5.2 Introduction

The accumulation of somatic mutations is considered to be a major cause of aging (1). Mutations are believed to accumulate with age due to a combination of increased levels of endogenous DNA damaging agents, such as reactive oxygen species (2), and decreased efficiency and fidelity of DNA repair (3-7). 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 (8, 9). Furthermore, the improper repair of DSBs can lead to large scale genomic sequence rearrangements, such as translocations, insertions, and deletions (10), and an increased frequency of such rearrangements is often observed in aged cells (11-15). Consistent with these findings, it has also been shown that deficiencies in the ability to repair DSBs cause accelerated aging (16). Together, these data suggest a model in which 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 (17, 18). 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 (10). NHEJ is the preferred pathway for the repair of DSBs during G_0/G_1 phases of the cell cycle, whereas HR is important during late S/G_2 (19). Although HR is generally error free, exchanges between misaligned sequences can lead to insertions, deletions, translocations,

and loss of heterozygosity (LOH). Since over 40% of the genome is comprised of repeated elements (20), during G_0/G_1, NHEJ is preferred to minimize deleterious rearrangements (21). 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 (22-26)). Indeed, HR is the only DNA repair pathway that can accurately reinsert the broken DNA end to restart the replication fork (10). If DSBs at broken forks are instead acted upon by NHEJ, ends from independent loci can 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 (27, 28). WRN is believed to play an important role in the resolution of HR intermediates (29, 30), and loss of function of WRN is associated with an increased frequency of deleterious recombination events (31-33). In addition, heritable mutations in ATM, a protein that plays a critical role in initiating DSB repair (34), result in ataxia telangiectasia, a disease that is associated with symptoms of premature aging (16). Interestingly, $ATM^{-/-}$ cells show an increased frequency of HR and are particularly susceptible to error-prone HR (35-38). Finally, mice with germline mutations in Ku80 (Ku86), an integral protein in NHEJ, exhibit an accelerated aging phenotype (39). Together, these results show that defects in DSB repair can promote aging.

Because of 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. To measure the accumulation of cells harboring recombined DNA during aging, LOH has been analyzed (40, 41). 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 significant fraction of LOH events in both lymphocytes and kidney cells that accumulate with increasing age (40, 41), suggesting that HR contributes significantly to DNA rearrangements that occur during aging. For technical reasons, 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 (42-45). Intriguingly, the effect of age on mutation frequency appears to be strongly tissue-dependent, and differences in mutation accumulation do not correlate with proliferation of cells within the tissue. For example, in tissues with low proliferation rates, an increase in mutant cell frequency with age is seen in the heart (44, 45) but not in the brain (42-44, 46). In addition, tissues that exhibit high proliferation rates, mutant cell frequency increases in the small intestine (45, 47) but not in the testis (42, 44, 48). Thus, although the accumulation of mutations differs among tissues, the reasons for these differences are not yet known.

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 an HR event at an integrated transgene yields a fluorescent cell (49). 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, 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 (50). 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 change 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 fibroblasts with age.

5.3 Materials and Methods

Animals

C57BL/6 FYDR were described previously (49). 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 (51). FYDR cohorts had equal ratios of males to females. Controls were sex and age matched, except

the aged negative control C57BL/6 were 47-85 weeks old and the aged positive control FYDR-Rec mice were 52-68 weeks old.

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% CO_2, cells were triturated, 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 to for *in vitro* studies.

Flow Cytometry

Pancreatic cells were disaggregated as described previously (50). 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.

Calculation of Recombination Cell Frequency and 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, ~10^4 cells were seeded into 24 independent cultures. Cultures were expanded and analyzed by flow cytometry once the density reached ~10^6 cells per well. The method of p_0 was used to determine the rate of recombination per cell division as described (52, 53). 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.

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^{++} and Mg^{++} free PBS and counted. 2x10^4 cells per slide were then suspended in 80 µl of 1% molten low melting point agarose and pipetted onto comet assay slides (Trevigen). After incubation at 4°C for 30 min slides were immersed in pre-chilled lysis solution (Trevigen) for 60min at 4°C, rinsed with ice cold PBS and transferred for a 40 min incubation in an alkaline solution (pH>13) at room temperature allowing DNA unwinding. Next, slides were subjected to electrophoresis at 30V for 30min followed by rinsing 5min 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.

SCE analysis

Primary ear fibroblasts from 5 juvenile and 5 aged FYDR mice were pooled and seeded at 2 x 10^5 cells per well. After 24 hours, 10μM BrdU was added to the culture media. Cells were harvested after undergoing two population doublings and analyzed for SCE frequency as previously described (54). SCEs were counted in a blinded fashion from 2 independent experiments with each experiment containing cells combined from 5 mice per cohort.

Quantification of DNA Damage-Induced Recombination

Primary ear fibroblasts from 5 juvenile and 5 aged FYDR mice were pooled and seeded at 0.5 x 10^6 cells per 100 mm dish. After 24 hours, triplicate or quadruplicate samples were exposed to 0.5 μg/ml mitomycin-C (MMC) 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.

5.4 Results

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. 5-1A). One method for determining the *in vivo* frequency of recombinant cells is to analyze disaggregated tissue by flow cytometry (50, 55). Briefly, we compared fluorescence intensities in disaggregated skin from negative control and positive control (FYDR-Recombined (51)) mice and established a region (R2) that excludes negative control cells (Fig. 5-1B). Over 21 million skin cells from 20 negative control mice were analyzed by flow cytometry and no cells appeared in the R2 region, indicating an extremely low background.

The distribution of spontaneous recombinant cell frequencies in pancreatic tissue from 100 mice aged 4-10 weeks was previously established (Fig. 5-1C and (50)). To compare homologous recombination between pancreas and skin, skin from 100 mice aged 4-10 weeks was similarly analyzed (Fig. 5-1C). 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 (five and one, respectively). 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 in 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 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 mice that carry the full-length *EYFP* coding sequence in every cell (51). *EYFP* expression is statistically significantly higher in pancreatic (~52%) versus skin (~30%) tissues (Fig. 5-1D). Because in positive control mice the *EYFP* coding sequence is expressed under an identical promoter and at the same locus as the FYDR recombination substrate, it is likely that there is a similar nearly 2-fold higher expression in pancreatic tissue of the FYDR mice as well. A 2-fold higher level of expression in the pancreas compared to skin partially explains the observation that there is a higher median frequency of recombinant pancreatic cells compared to skin cells. However, the difference in *EYFP* expression is not sufficient to account for the 5-fold higher median recombinant cell frequency in pancreatic compared to skin tissues, nor does it explain differences in the distribution of recombinant cell frequencies in each tissue type.

Tissue-Specific Effect of Aging on Recombinant Cell Frequency

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. To explore the effects of aging, recombinant cells were quantified in pancreas and skin from two different age groups: 'juvenile' (4 weeks old) and 'aged' (62-89 weeks old). For pancreatic tissue, the median frequency of recombinant pancreatic cells increased ~23 fold from juvenile to aged mice (Fig. 5-2A; (50)). Given that there is no statistically significant difference in *EYFP* expression with age in pancreatic cells (Fig. 5-2B), 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. 5-2C). Comparison of *EYFP* expression in skin with age shows no statistically significant difference between juvenile and aged mice (Fig. 5-2D). Thus, unlike in pancreatic tissue, recombinant fluorescent cells do not accumulate in cutaneous tissue with age.

Effect of Age on HR in Cultured Primary Fibroblasts

Previous studies (50) 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 decrease in the rate of HR with age. To test

this hypothesis, primary fibroblasts from juvenile and aged mice were cultured *in vitro*. Expansion of cells for ~7 population doublings shows that recombinant cell frequency in cultures of both juvenile and aged cells increases, suggesting that HR is an active repair process in dividing cells *in vitro* for both age groups (Fig 5-3A). Furthermore, we estimated the rate of recombination in juvenile versus aged primary fibroblasts using the p_0 method (52, 53). We found that there is no statistically significant difference in the rate of recombination between juvenile and aged cells (Fig. 5-3B). 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. These data suggest that juvenile and aged cells are comparable in their HR capacity.

The rate of HR can depend on the site of integration of a recombination substrate (56). To determine if the relationship between the rate of recombination in juvenile and aged cells is genome-wide, the frequency of HR events was measured using an independent method; namely, sister chromatid exchange (SCE) analysis. 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. 5-3C). Blinded analysis of SCEs from juvenile and aged cells shows no statistical difference in the frequency of SCEs (Fig. 5-3D). 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 accumulation of recombinant cells in the skin might be due to diminished pressure 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 under alkaline conditions. The average olive tail moment and percent tail DNA are two different ways to analyze the amount of DNA damage from Comet data. The averages for both olive tail moment (Fig. 5-3E) and percent tail DNA (Fig. 5-3F) 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.

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. 5-4A), 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. 5-4B). 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.

5.5 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 (16), 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 (1). 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 multiple 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 to recombinant 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 (40, 41). Here, using the FYDR mice, we have shown 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 (42, 43), Muta Mouse (44), and LacZ (45), have been used to examine changes in mutation frequency with age in various tissues. Consistent with our data, 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 gastro-intestinal tissues including the liver and small intestine (42-47). Interestingly, however, no tissues have shown such a dramatic increase in mutant cell frequency with age as we see in the pancreas. In terms of the skin, one study showed a slight increase (~1.5-fold) in mutant cell frequency with age, although the cell type examined was not described (44). Thus, 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 (44, 48), which contain highly proliferating cells (57). In contrast, in liver and heart, which are slow or non-proliferating tissues (57), there was a significant accumulation of mutations with age (43-46). 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 may have the greatest impact on the burden of mutant cell frequency with age.

Age is a risk factor for many diseases, including cancer (58). Similar to aging, the accumulation of multiple mutations within cells is believed to cause cancer (59). 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 (60-62). 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 (45, 46, 48, 63) or completely remove the contribution of clonal expansion by specifically analyzing only independent mutation events (42, 43). Our previous studies (50) combined with the results 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 at ~60% of the recombinant cells that accumulate with age 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.

In contrast to the pancreas, skin does not appear to accumulate recombinant cells with age. Previously, we have shown that not only clonal expansion but also *de novo* recombination events contribute to the increase in recombinant pancreatic cells with age (50). 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, suggesting that an inability to undergo *de novo* HR events with age does not cause the lack of accumulation of recombinant skin cells. These observations suggest that recombinant cells do not accumulate with age in the skin because recombinant cells are either short lived or do not clonally expand.

The ability of a cell to persist or clonally expand is cell type dependent. Thus, in order to analyze the contribution of clonal expansion and persistence, it is important to know the cell types of recombinant cells. Within the pancreas, recombinant cells are mostly likely acinar cells (50). Acinar cells are part of the exocrine pancreas and secrete hydrolytic enzymes into the duodenum that aid in digestion. The secretory unit within the exocrine pancreas is a grape-like structure called an acinus, which contains ten to forty acinar cells that secrete enzymes into a system of epithelial ducts (64). Interestingly, acinar cells may play an important role in tumor formation. Evidence suggests that acinar to ductal transdifferentation can occur *in vivo* and *in vitro*, leading to the formation of pancreatic intraepithelial neoplasias and pancreatic ductal adenocarcinoma (65-71). In the adult pancreas, acinar cells exhibit extremely low levels of proliferation (~1% of cells are in S phase (72)) and persist for long periods of time (the turnover time of mouse acinar

cells is estimated to be ~500 days (57)). Thus, the dramatic accumulation of recombinant acinar cells with age is most likely caused by a combination of *de novo* recombinant events, clonal expansion and long persistence of acinar cells within the tissue.

Within the skin, recombinant cells that are detected in our system are most likely fibroblasts. Fibroblasts are located within the dermal skin layer and secrete extracellular matrix components, such as collagen and elastin. In addition, fibroblasts appear to play a critical role in both wound healing and epithelial tumorigenesis (73-75). In response to skin injury, fibroblasts migrate to the site of the wound, initiate proliferation and secrete extracellular matrix components critical for restoration of skin structure and strength (73). In the adult skin, fibroblasts have a low proliferation rate (~1-2% (76)) and persist for long periods of time (the turnover time of fibroblasts is estimated to be 120-160 days (77)). Thus, both skin fibroblasts and pancreatic acinar cells exhibit low proliferation rates. Although the longer turnover time for acinar cells compared to skin fibroblasts may contribute to the differences in the accumulation of recombinant cells with age, fibroblasts do persist for significant periods of time within the skin. Thus, since fibroblasts are both able to undergo *de novo* recombination events and persist within the skin, an absence of clonal expansion contributes significantly to the complete lack of accumulation of recombinant cells in skin with age. One hypothesis resulting from this finding is that if fibroblasts are forced to clonally expand, for example in response to injury, there may be an accumulation of recombinant cells. Indeed, treatment of FYDR mice with an acute dose of high radiation induces an increase in recombinant cell frequency within FYDR skin (55).

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 (3-6, 78, 79). Here, we show that the rate of 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 (80); 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 (81-83) and others indicating an increase in SCE frequency with age (84-86). Finally, analysis of the male germline of *Drosophila* showed an increase in the frequency of HR events with age (87). 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.

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 in multiple tissues. Thus, the effect of not only

age but also genetics and environment on clonal expansion of recombinant cells can be examined. Determining the relative importance of clonal expansion to mutant cell frequency within different tissues may contribute to our understanding of mechanisms that modulate susceptibility of various tissues to aged associated degeneration and cancer.

5.6 Acknowledgements

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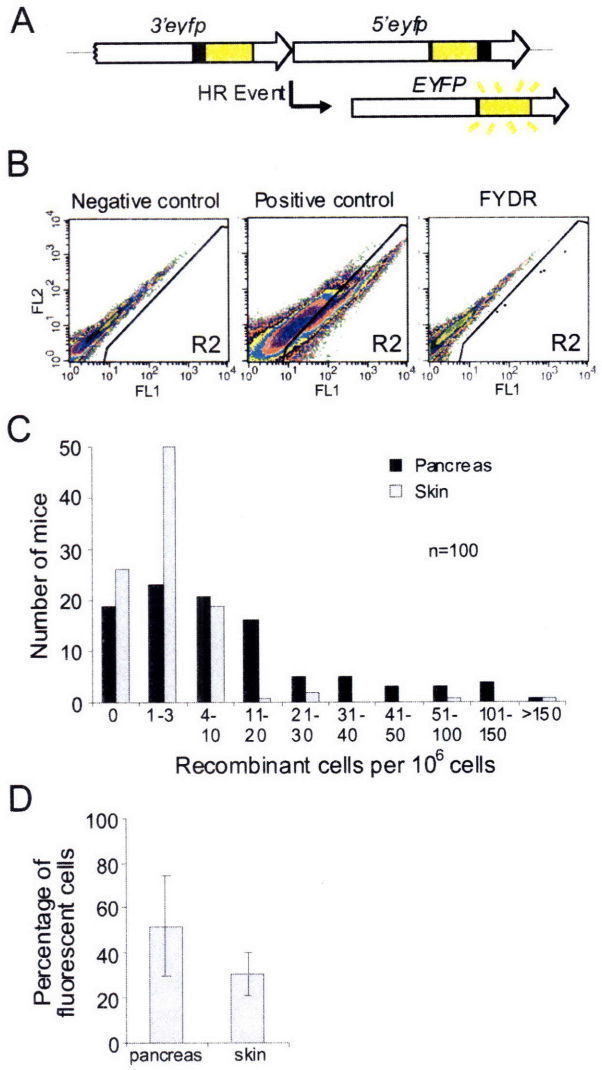

Figure 5-1. FYDR system and analysis of pancreatic and skin cells by flow cytometry. (A) Arrangement of the FYDR recombination substrate: large arrows indicate expression cassettes; yellow boxes show coding sequences; black boxes show positions of deleted sequences (deletion sizes not to scale). (B) Flow cytometry results 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 mouse, a positive control 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) 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) 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. Error bars indicate 1 standard deviation.

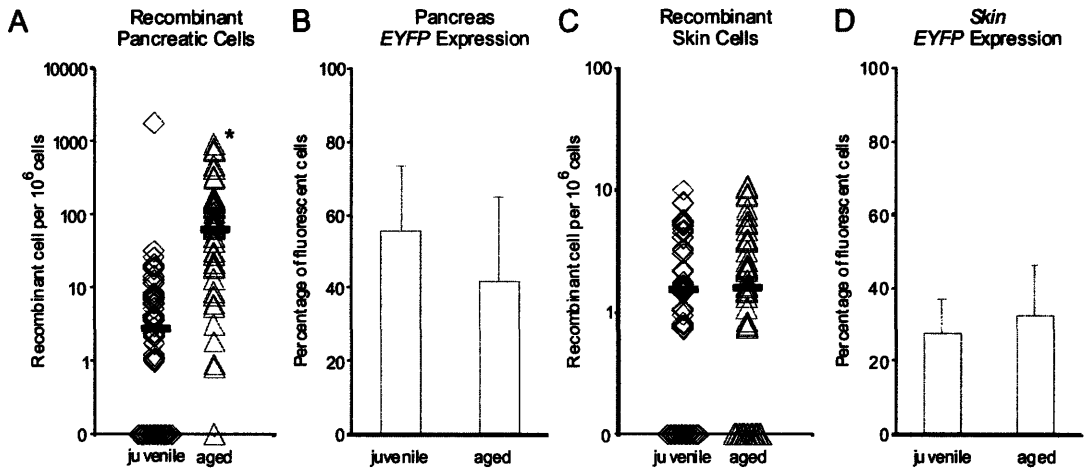

Figure 5-2. Effects of aging on the frequency of recombinant cells and expression of the FYDR substrate in the pancreas and skin. (A) Frequency of recombinant pancreatic cells per million as determined by flow cytometry for juvenile (n=49) and aged (n=41) mice. Previously, pancreata from a subset of juvenile and aged mice were analyzed (50). 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 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.

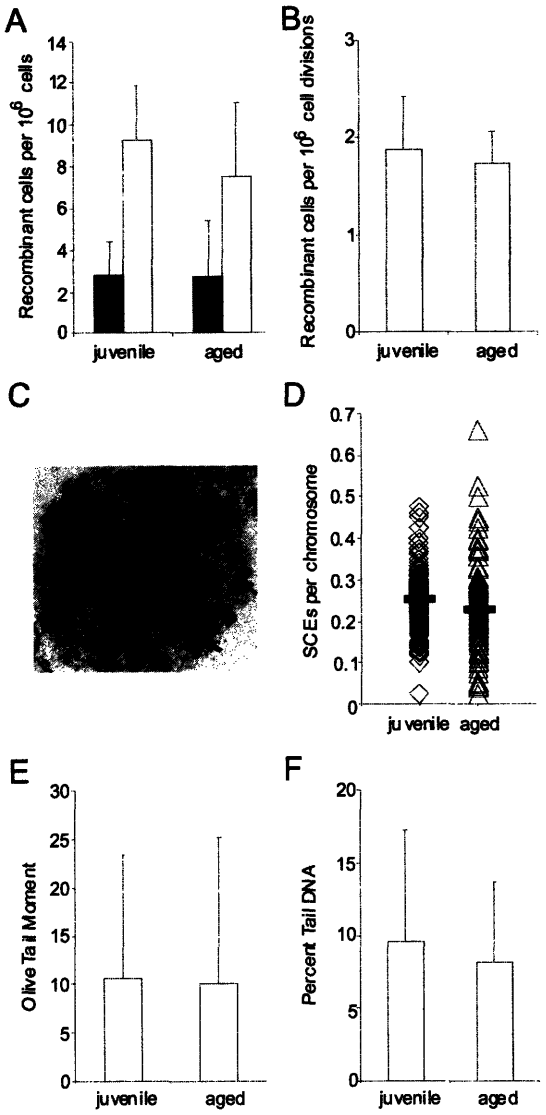

Figure 5-3. Effects of aging on spontaneous levels of HR and DNA damage in primary fibroblasts *in vitro*. (A) Frequency of recombinant cells per million at the time of initial plating (black bars) and after ~7 population doublings in culture (grey bars) of primary fibroblasts from juvenile and aged mice as determined by flow cytometry. Error bars indicate 1 standard deviation. (B) Average rate of HR in juvenile and aged cells as determined by the method of p_0 (52, 53). Error bars indicate 1 standard deviation. (C) Example metaphase spread. Arrows indicate SCEs. (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 (n=198) and aged (n=198) cells. Error bars indicate 1 standard deviation.

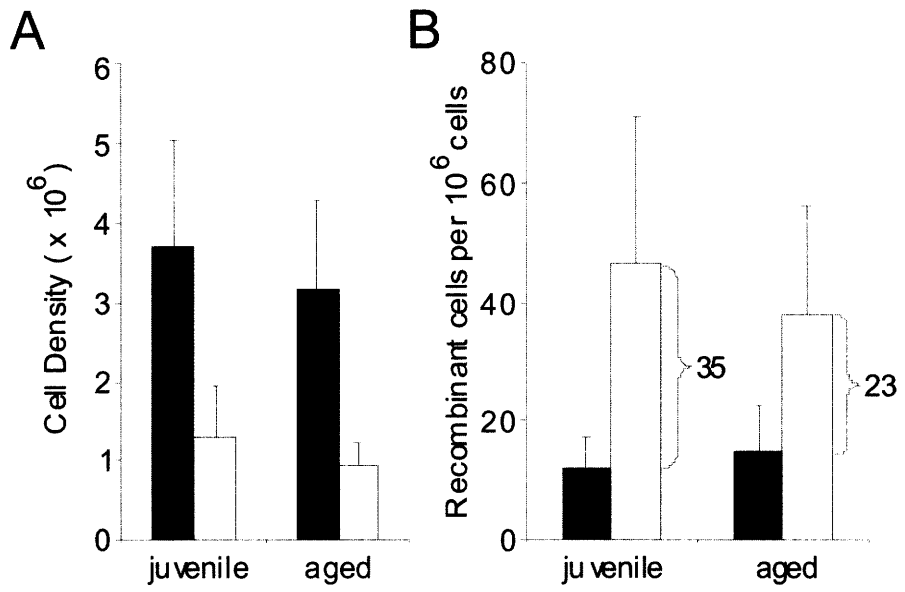

Figure 5-4. Effects of aging on spontaneous MMC induced HR in primary fibroblasts *in vitro*. (A) Average cell density per million for mock- (black bars) and MMC-treated (grey bars) primary fibroblasts from juvenile and aged mice. Error bars indicate 1 standard deviation. (B) Average frequency of recombinant cell per million for mock- (black bars) and MMC-treated (grey bars) primary fibroblasts from juvenile and aged mice. Error bars indicate 1 standard deviation. Numbers represent average difference between MMC- and mock-treated recombinant cell frequencies.

Chapter VI

Loss of p53 Does Not Affect the Frequency or Rate of Homologous Recombination *in vivo*

Chapter 6

6.1 Abstract

The tumor suppressor p53 is transcription factor whose function is critical for maintaining genomic stability in mammalian cells. In response to DNA damage, p53 initiates a signaling cascade that results in cell cycle arrest, DNA repair or, if the damage is too severe, programmed cell death. In addition, p53 directly modulates the repair of DNA by interacting with a number of DNA repair proteins, including those involved in homologous recombination. Mitotic homologous recombination (HR) plays an essential role in the repair of particularly deleterious DNA lesions, such as double-strand breaks (DSBs) and broken replication forks. Loss of function of either p53 or HR leads to an increased risk of cancer. Given the importance of both p53 and HR in maintaining genomic integrity, we analyzed the effect of p53 on HR *in vivo* using Fluorescent Yellow Direct Repeat (FYDR) mice. FYDR mice carry a transgene in which an HR event yields a fluorescent phenotype. Here we show that p53 status does not significantly affect homologous recombination in adult pancreatic cells *in vivo*. Furthermore, *in vitro* studies analyzing the effect of p53 on HR in primary fibroblasts show no statistically significant difference in the spontaneous rate of HR in p53 wild-type versus null cells. Thus, our results indicate that p53 status does not significantly affect the spontaneous frequency or rate of HR in the pancreas or in cultured fibroblasts.

6.2 Introduction

p53 is a transcription factor that plays an essential role in maintaining genomic stability. Indeed, greater than half of all tumors have lost p53 function either by mutation (1, 2) or epigenetic silencing (3-5). Inherited mutations in p53 cause Li Fraumeni syndrome, a genetic disorder characterized by a high and early incidence of cancer (6, 7), and transgenic mice carrying germline deletions in p53 develop tumors at an accelerated rate compared to p53 heterozygous and wild-type mice (8). Together, these data indicate that p53 is a key inhibitor of tumor formation, and loss of p53 function provides cells with critical selective advantages required for tumor formation.

Normally, p53 serves as the mediator of cell cycle arrest and/or apoptosis in response to genotoxic stress, such as DNA damage. Cellular DNA is constantly exposed to a plethora of endogenous and exogenous agents that can damage DNA (9). Of the DNA lesions that form, DNA double-strand breaks (DSBs) are considered to be among the most cytotoxic and mutagenic, since a single unrepaired DSB can result in the permanent loss of over 100 million base pairs of genetic information (10), and misjoining of DSBs can lead to gross chromosomal rearrangements (11). Mitotic homologous recombination (HR) provides a critical pathway for the accurate repair of potentially cytotoxic or mutagenic DSBs. By using homologous DNA sequences present on the sister chromatid or homologous chromosome as templates, HR can repair DSBs with minimal loss of sequence information (12). In addition, HR provides the only pathway for the accurate repair of DSBs that arise as a result of broken replication forks. Thus, HR

is a critical pathway for preventing tumor-promoting sequence rearrangements that can result from DSBs.

Similar to inherited mutation in p53, germline mutations in genes that modulate HR are associated with an increased risk of cancer (11, 13-16). Although HR is generally error free, since over 40% of the genome is comprised of repeated elements (17), exchanges between misaligned sequences that lead to insertions, deletions, translocations, and loss of heterozygosity (LOH) can occur. It is not surprising that inherited mutations in proteins that increase the rate of HR (*i.e.,* WRN (18), BLM (19)) are associated with an increased risk of cancer (16). While too much HR can be problematic, too little HR can also lead to genomic instability. In the absence of HR, DSBs at broken replication forks cannot be accurately reinserted to restart the replication fork (12). Instead, DSBs from independent replication forks can be misjoined, leading to large scale sequence rearrangements. Indeed, germline mutations in proteins that result in a suppression of HR (*i.e.,* BRCA1 (20), BRCA2 (21), FANCC (22)) also lead to an increased risk of cancer (23-25). Thus, maintaining the proper level of HR is critical for preventing tumor formation.

Given the importance of both p53 and HR in maintaining genomic stability, a number of studies have analyzed the effect of p53 status on the repair of DSBs by HR. Some *in vitro* studies suggest that p53 suppresses HR (26-31), potentially by interacting with and regulating the transcription of Rad51 (32), a protein essential for HR. Interestingly, however, a number of studies contradict these findings with results showing that p53 status has no effect on HR (33-35). In addition, studies measuring the effect of p53 on HR *in vivo* also show conflicting results. For example, using pink-eyed unstable

(p^{un}) mice, in which a HR event during embryonic development can give rise to black spots on the gray fur and retinal epithelium of adult mice (36, 37), p53 has been found in one study to have a suppressive effect (38) and in another to have no effect (39) on the spontaneous frequency of HR events. For technical reasons, the assays used in previous studies can only detect HR events that occur during embryogenesis or in cultured cells *in vitro*. Thus, the effect of p53 on HR in adult tissues *in vivo* had not been previously evaluated.

Here, we investigate the effects of p53 on the frequency of HR events *in vivo*. To study recombination *in vivo*, we applied the Fluorescent Yellow Direct Repeat (FYDR) mice, in which a HR event at an integrated transgene yields a fluorescent cell (40). Analysis of pancreata from $FYDR^{y/+};p53^{+/+}$ and $FYDR^{y/+};p53^{-/-}$ mice shows that neither the number of recombination events nor the frequency of recombinant cells is affected by p53 status. Since we have shown in previous studies that HR is an active repair process within adult pancreata of FYDR mice (41), these data indicate that p53 does not affect HR in adult pancreatic cells *in vivo*. To determine if p53 status affects the rate of HR, we analyzed primary fibroblasts from FYDR mice *in vitro*. Although we observed an increase in the average spontaneous rate of HR in $p53^{-/-}$ fibroblasts, the difference was not statistically significant. Taken together, studies of HR both *in vivo* and *in vitro* indicate that p53 status does not significantly affect the spontaneous frequency or rate of HR in the pancreas or in cultured fibroblasts.

6. 3 Materials and Methods

Animals

FYDR (40) and p53 mice (8) were described previously. 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 (42). 9 week old $FYDR^{y/+}$;$p53^{+/+}$ and $FYDR^{y/+}$;$p53^{-/-}$ cohorts had equal ratios of males to females.

Flow Cytometry

Pancreatic cells were disaggregated as described previously (41). Almost all samples were analyzed by flow cytometry after imaging (see below). Disaggregated pancreatic 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.

Imaging

Pancreatic cells were imaged as described previously (41). Briefly, nuclei were stained with 50 µg/ml Hoechst 33342 (Sigma). Whole pancreata were pressed between glass slides separated by 0.5 mm spacers. Sequential images were collected in black and white using a 1x objective. The images were manually compiled to cover the entire visible surface area. Filters included: visible light; UV (Ex:330-380 nm, Em:420 nm); Red (Ex:540/25 nm, Em:605/55 nm); and EYFP (Ex:460-500 nm, Em:510-560 nm).

Images were collected using a fixed aperture time. Foci were counted manually in blinded samples. The area of compiled pancreata images was determined using Scion Image Beta 4.02 Win (Scion Corporation) by manually tracing the pancreas edge.

Isolation of Ear Fibroblasts

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% CO_2, cells were triturated, filtered (70-µm mesh; Falcon), and seeded into dishes.

Calculation of Rate in Primary Fibroblasts

Primary ear fibroblasts were isolated from $FYDR^{y/+};p53^{+/+}$ and $FYDR^{y/+};p53^{-/-}$ littermates and rate experiments were performed in parallel. For each rate experiment, $\sim 10^4$ 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 MSS Maximum Likelihood Method was used to determine the rate of recombination per cell division as described (43). Rate experiments were repeated with fibroblasts from different pairs of mice 3 times for males and 4 times for females.

6.4 Results

Effect of p53 Status on Spontaneous Frequency of Recombinant Pancreatic Cells

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. 6-1A) (40). One method for measuring the *in vivo* frequency of recombinant cells is to analyze disaggregated tissue by flow cytometry (41). We compared fluorescence intensities in disaggregated pancreata from negative control and positive control (FYDR-Recombined (42)) mice and established a region (R2) that excludes negative control cells (Fig. 6-1B).

To determine the effect of p53 status on HR *in vivo*, we analyzed recombinant cells in pancreata of 9 week old $FYDR^{y/+};p53^{+/+}$ and $FYDR^{y/+};p53^{-/-}$ mice (8, 40). Analysis of recombinant cell frequency by flow cytometry reveals the total fraction of fluorescent recombinant cells within a pancreas regardless of whether they arise as a result of independent recombination events or clonal expansion (41, 42). Comparison of pancreata from $FYDR^{y/+};p53^{+/+}$ and $FYDR^{y/+};p53^{-/-}$ mice shows that the frequency of recombinant cells varies among individual animals within each cohort (Fig. 6-1C). However, the median frequency of recombinant pancreatic cells is not statistically different between the two cohorts (Fig. 6-1C), suggesting that p53 does not affect the *in vivo* spontaneous frequency of recombinant cells in FYDR pancreata.

Analysis of FYDR pancreata by flow cytometry requires tissue disaggregation; thus, the contribution of clonal expansion versus independent HR events on recombinant

cell frequency cannot be determined. To quantify independent recombinant events, we have previously developed *in situ* imaging techniques that enable the direct detection of recombinant foci within intact pancreata (41). Briefly, we compared fluorescence intensities under an EYFP-specific filter for negative control (Fig. 6-1D, left) and positive control pancreata (Fig. 1D, center), and established imaging conditions that specifically detect EYFP expressing cells in FYDR pancreata (Fig. 6-1D, right). To quantify recombinant foci, FYDR pancreata are uniformly compressed to a thickness of 0.5 mm and composite images that cover one side of the pancreatic surface area are created (Fig. 6-1E).

Recombinant cells within pancreata of FYDR mice are most likely acinar cells (41). In the pancreas, acinar cells are arranged into grape-like structures called acini, which contain ten to forty acinar cells that secrete enzymes into a system of epithelial ducts (44). Given the low levels of proliferation of pancreatic acinar cells (45) and the low probability of acinar cell migration among acini in adult tissue, each recombinant focus within FYDR pancreata is most likely an independent recombination event. Thus, the effect of p53 status on the ability of pancreatic cells to undergo recombination events can be quantified by analyzing recombinant foci using *in situ* imaging. While the number of recombinant foci detected by *in situ* imaging varied among individual animals within $FYDR^{y/+};p53^{+/+}$ and $FYDR^{y/+};p53^{-/-}$ cohorts (Fig. 6-1F), the median number of recombinant foci is not statistically different between the two cohorts (Fig. 6-1F). Additionally, because the sizes of pancreata vary among mice within each cohort (data not shown), we analyzed the number of foci per unit surface area for both $FYDR^{y/+};p53^{+/+}$ and $FYDR^{y/+};p53^{-/-}$ mice, and found that the median frequencies of recombinant foci per

cm^2 are not statistically different between the two cohorts (Fig. 6-1G). Since HR is an active repair process in the adult pancreas (41, 42), these data suggest that p53 status does not affect the ability of adult pancreatic cells to undergo HR *in vivo*.

Effect of p53 Status on Spontaneous Frequency of Recombinant Pancreatic Cells in Males Versus Females

Differences in susceptibility to certain diseases such as cancer differ between males and females. To determine if the effect of p53 status on HR is modulated by sex, we compared the frequency of recombinant cells and number of recombinant foci in male and female mice. For both male (Fig. 6-2A) and female (Fig. 6-2B) mice, a trend towards an increase in the median recombinant cell frequency and foci number in $FYDR^{y/+};p53^{-/-}$ compared to $FYDR^{y/+};p53^{+/+}$ mice is observed. However, these differences are not statistically significant, suggesting that sex does not modulate the effect of p53 status on HR in FYDR pancreata.

Interestingly, for both $FYDR^{y/+};p53^{+/+}$ and $FYDR^{y/+};p53^{-/-}$ mice, female FYDR mice have overall higher frequencies of recombinant cells than male mice (compare Fig. 6-2A and 6-2B). Previous studies of positive control FYDR-Recombined mice show that female mice have higher *EYFP* expression levels than male mice (42). Since positive control mice express the *EYFP* coding sequence under an identical promoter and at the same locus as the FYDR recombination substrate, it is likely that similar differences in expression between males and females are present in pancreatic tissue of the FYDR mice as well. Thus, the apparent higher frequencies of recombinant cells in female as compared to male $FYDR^{y/+};p53^{+/+}$ and $FYDR^{y/+};p53^{-/-}$ mice can at least partially be

explained by differences in the level of *EYFP* expression. Together these data indicate that p53 status does not affect HR in pancreatic cells of both male and female mice *in vivo*.

Effect of p53 sStatus on HR in Primary Fibroblasts *in vitro*

With its critical role in the repair of broken replication forks, HR is used preferentially during late S/G_2 phases of the cell cycle to repair DSBs (46). To explore the effect of p53 status on the rate of HR in dividing cells, primary fibroblasts from $FYDR^{y/+};p53^{+/+}$ and $FYDR^{y/+};p53^{-/-}$ mice were cultured *in vitro*. The average rate of HR as determined using the MSS Maximum Likelihood Method (43) appears to be higher in $FYDR^{y/+};p53^{-/-}$ compared to $FYDR^{y/+};p53^{+/+}$ fibroblasts (Fig. 6-3A), although this difference is not statistically significant. To determine if the effect of p53 status on the rate of HR is modulated by sex, we compared the rates in female versus male $FYDR^{y/+};p53^{+/+}$ and $FYDR^{y/+};p53^{-/-}$ fibroblasts separately. A trend towards increased rates of HR are seen in $FYDR^{y/+};p53^{-/-}$ compared to $FYDR^{y/+};p53^{+/+}$ fibroblasts for both sexes (Fig. 3B), although again these differences are not statistically significant.

To determine if differences in the *EYFP* expression levels exist, fibroblasts from male and female positive control mice were cultured in parallel with rate experiments. Results show that there is no significant difference in the levels of detectable *EYFP* expression between sexes *in vitro* (data not shown), indicating that differences in *EYFP* expression do not affect the apparent rates of HR in both female and male fibroblasts. Together these data suggest that $FYDR^{y/+};p53^{-/-}$ cells may have a higher rate of HR

compared to FYDR$^{y/+}$;p53$^{+/+}$, although the trend is not sufficient to render the results statistically significant.

6.5 Discussion

p53 is the guardian of genomic integrity. Indeed, loss of p53 function results in an early onset and increased frequency of many types of cancers (6-8). In response to DNA damage, p53 is activated and by regulating gene transcription initiates a signal cascade that results in either DNA repair or cell death. Initially, p53 promotes the repair of DNA damage by inducing cell cycle arrest and activating DNA repair proteins. However, in the presence of extensive DNA damage, p53 signals for the elimination of damaged cells through apoptosis, senescence or differentiation (for review see (47)). Because of the critical role p53 plays in preventing tumorigenesis, we set out to understand the mechanisms through which loss of p53 promotes genomic instability in pancreatic cells and fibroblasts.

Mitotic HR is an important pathway for the accurate repair of DNA DSBs. Although HR is generally error-free, recombination between misaligned sequences can result in tumor promoting sequence rearrangements. Thus, maintaining the proper level of HR is critical for maintaining genomic integrity. A number of studies have shown an interaction between p53 and proteins involved in HR including RPA (48), Rad51 (49, 50), BLM and WRN (51-53). These reports suggest that p53 interaction inhibits the function of HR proteins. However, using FYDR mice we show that p53 status has no effect on HR

in vivo in pancreatic cells and *in vitro* in primary fibroblasts, supporting a model in which loss of p53 function does not significantly alter the spontaneous activity of HR. Previous studies analyzing HR *in vivo* using p^{un} mice show conflicting results (38, 39). Using the fur spot assay, an initial study determined that p53 had no effect on HR *in vivo* (39). However, a later study using the more sensitive eye-spot assay showed that loss of p53 increased HR events and specifically HR events that occurred during early embryonic development (38). Because clonal expansion of recombined cells is required to detect spots on both fur and retinal epithelium, p^{un} mice can only be used to detect recombination events that occur during embryogenesis. In contrast, FYDR mice can be used to detect recombination events that occur in adult tissues *in vivo*. Therefore, the differences in the results between our studies and those done with the p^{un} mice suggest a difference in the importance of p53 in modulating HR during embryogenesis versus adult life. Specifically, p53 may be less important in suppressing HR in adult versus embryonic tissues. In addition, the effect of p53 on HR may be cell type specific with retinal epithelial cells being more sensitive to loss of p53 function than pancreatic cells or fibroblasts.

In addition to its role in modulating HR, p53 has been shown to modulate many proteins involved in other DNA repair pathways either through transcriptional regulation or direct protein interaction (47). Since efficient and accurate DNA repair plays a critical role in maintaining genomic stability, it is not surprising that loss of p53 function has been shown to cause defects in a number of DNA repair pathways including nucleotide excision repair (54) and base excision repair (55). In addition, p53-null cells show increased levels of error-prone DNA repair pathways such as non-homologous end

joining (56). Thus, the misregulation of DNA repair pathways in the absence of p53 may contribute to genomic instability, even if no dramatic change in the baseline rate of HR is present.

Germline mutations in p53 cause an increased frequency of tumor formation. Our data suggests that the increased cancer incidence in $p53^{-/-}$ mice may not be the result of misregulation of HR since $FYDR^{y/+};p53^{-/-}$ mice do not shown an increase in recombinant cells compared to their p53 wild-type littermates. The combination of flow cytometry and *in situ* imaging can provide information regarding the importance of both *de novo* recombination events and clonal expansion to the frequency of recombinant cells within the pancreas. In addition to genetic conditions, the effect of environmental exposures on HR can also be studied using FYDR mice (41, 42). Although p53 status does not appear to affect the spontaneous frequency of HR, given the importance of p53 in responding to DNA damage, treatment of $FYDR^{y/+};p53^{+/+}$ and $FYDR^{y/+};p53^{-/-}$ mice or cells with exogenous DNA damaging agents may result in a differential susceptibility to damage induced recombination. Thus, FYDR mice provide a unique tool to study the individual and combined effects of cancer risk factors, such as genetic conditions, environment exposures and aging, on the repair of DSBs by HR *in vivo*.

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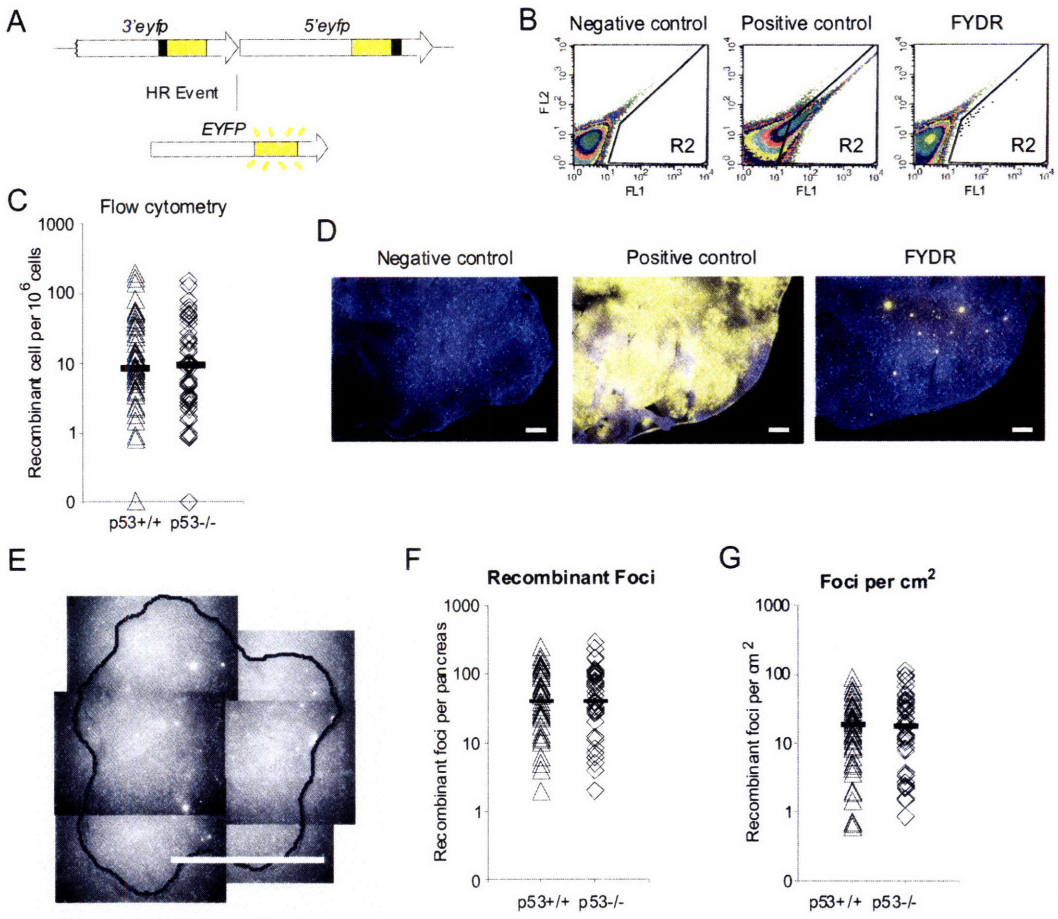

Figure 6-1. FYDR system and analysis of pancreatic cells by flow cytometry and *in situ* imaging. (A) Arrangement of the FYDR recombination substrate: large arrows indicate expression cassettes; yellow boxes show coding sequences; black boxes show positions of deleted sequences (deletion sizes not to scale). (B) Flow cytometry results of disaggregated skin cells. Images adapted from Wiktor-Brown, *et al* (41). 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 mouse, a positive control 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 cells per million as determined by flow cytometry for $FYDR^{y/+}$;$p53^{+/+}$ (n=48) and $FYDR^{y/+}$;$p53^{-/-}$ (n=47) mice. Medians are indicated by black bars. Points on the x-axis indicate individual mice with 0 recombinant cells. (D) Analysis of fluorescent foci in mouse pancreata. Images adapted from Wiktor-Brown, *et al* (41). For analysis of freshly excised tissue, images show overlays of EYFP- (510–560 nm) and UV- (420 nm) filtered images. Nuclei are stained with Hoechst 33342. Portions of negative control (left), positive control (middle) and FYDR (right) mouse pancreata imaged at 1x (scale bar, 1 mm). Brightness and contrast for UV-filtered images were adjusted equivalently. For EYFP images, brightness and contrast for negative control and FYDR (5-s exposure) images were adjusted equivalently. To avoid overexposure of the

positive control, a shorter exposure time was used (1 s) and brightness and contrast were not adjusted. (E) Compiled image for representative FYDR pancreata. Image was collected at 1x (bar, 1 cm) using an EYFP filter (510-560 nm). The edge of the pancreatic tissue is outlined. (E) Spontaneous recombinant foci per pancreas detected by *in situ* image analysis for $FYDR^{y/+};p53^{+/+}$ (n=49) and $FYDR^{y/+};p53^{-/-}$ (n=47) mice. Medians are indicated by black bars. (F) Spontaneous recombinant foci per cm^2 detected by *in situ* image analysis for $FYDR^{y/+};p53^{+/+}$ (n=49) and $FYDR^{y/+};p53^{-/-}$ (n=47) mice. Medians are indicated by black bars.

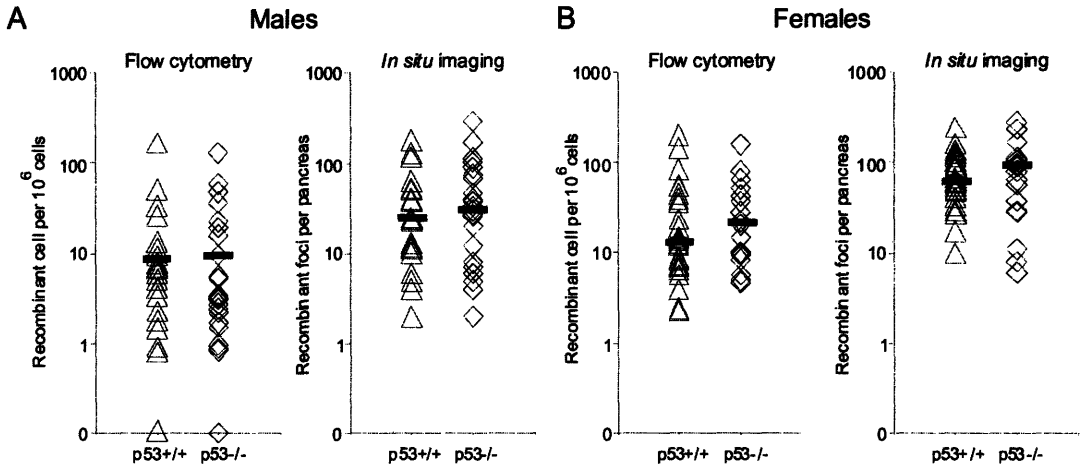

Figure 6-2. The effect of p53 status on HR in males versus female. (A) Male mice: spontaneous frequency of recombinant pancreatic cells per million as determined by flow cytometry (left, p=0.75, 2-tailed Mann-Whitney) and spontaneous recombinant foci per pancreas detected by *in situ* image analysis (right, p=0.42, 2-tailed Mann-Whitney) for $FYDR^{y/+};p53^{+/+}$ (n=25) and $FYDR^{y/+};p53^{-/-}$ (n=27) mice. (B) Female mice: spontaneous frequency of recombinant pancreatic cells per million as determined by flow cytometry (left, p=0.45, 2-tailed Mann-Whitney) and spontaneous recombinant foci per pancreas detected by *in situ* image analysis (right, p=0.48, 2-tailed Mann-Whitney) for $FYDR^{y/+};p53^{+/+}$ (n=24) and $FYDR^{y/+};p53^{-/-}$ (n=20) mice. Medians are indicated by black bars. Points on the x-axis indicate individual mice with 0 recombinant cells.

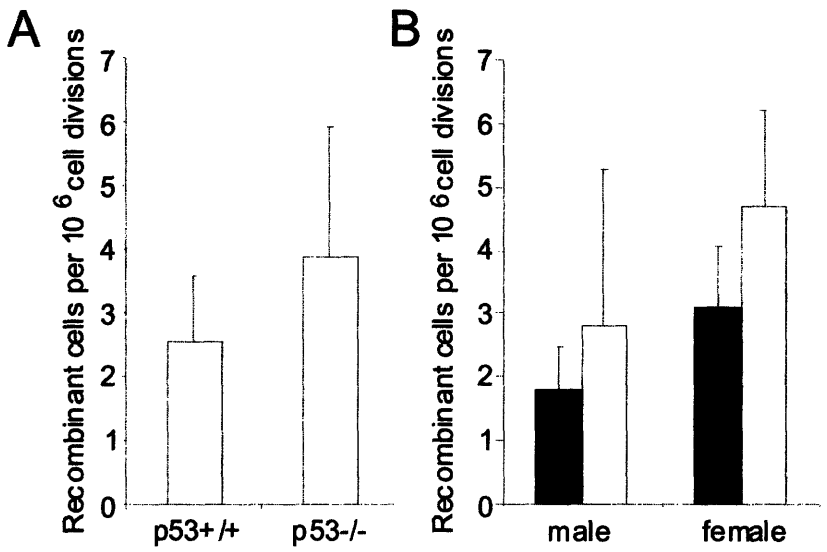

Figure 6-3. Effects of p53 status on spontaneous levels of HR as determined by the method of p_0 (43, 57). (A) Average rate of HR in $FYDR^{y/+};p53^{+/+}$ (n=7) and $FYDR^{y/+};p53^{-/-}$ (n=7) primary fibroblasts (p=0.16, 2-tailed Student's *t* test). Error bars indicate 1 standard deviation. (B) Average rate of HR in $FYDR^{y/+};p53^{+/+}$ (black bars) and $FYDR^{y/+};p53^{-/-}$ (grey bars) primary fibroblasts from male (p=0.56, 2-tailed Student's *t* test) and female (p=0.14, 2-tailed Student's *t* test). Error bars indicate 1 standard deviation.

Chapter VII

Conclusions and Future Work

With its essential role in the repair of double-strand breaks and broken replication forks, homologous recombination is an important modulator of genomic stability. Deregulation of homologous recombination, whether up or down, can lead to an increased risk of cancer. Here we have used Fluorescent Yellow Direct Repeat mice (FYDR) to explore the effects of certain cancer risk factors, such as age, exposure to exogenous DNA damaging agents, and p53 status on homologous recombination *in vivo*.

Given that homologous recombination appears to play an important role in increasing the risk of pancreatic cancer, we examined homologous recombination in pancreatic tissue of FYDR mice *in vivo*. We developed techniques to analyze recombinant cells both by flow cytometry of disaggregated tissue and by *in situ* imaging of intact pancreata. We first applied these techniques to determine if homologous recombination is an active repair process in the pancreas. Treatment of FYDR mice with the DNA damaging agent mitomycin-C or cisplatin (see Appendix I) shows an induction in both the frequency of recombinant cells and number of recombinant foci, indicating that homologous recombination is indeed an active repair process in adult pancreatic cells and that exposure to exogenous DNA damaging agents can induce recombination in the pancreas. Furthermore, we determined that *in situ* imaging is a more sensitive method than flow cytometry for detecting exposure-induced recombinant cells, yielding statistical significance with smaller cohorts. Thus, *in situ* imaging and flow cytometry can be used in combination to provide information about both independent recombination events and overall recombinant cell frequency.

Analysis of FYDR pancreata by both *in situ* imaging and flow cytometry revealed a large variation in recombinant pancreatic cell frequency among mice. While some of this variation may be due to differences in homologous recombination, the expression of the FYDR transgene may also contribute to inter-mouse variation in recombinant cell frequencies. To explore inter-mouse variation, we exploited positive control FYDR-Recombined mice. Our data showed that while there is variation in enhanced yellow fluorescent protein (EYFP) expression within male and female cohorts, on average females exhibit higher EYFP expression than males. Thus, we concluded that when comparing recombinant cell frequencies among different conditions equal ratios of males to females must be used. In addition, since expression of the FYDR transgene can affect the apparent frequency of recombinant cells, a cohort of positive control mice must be included in all experiments to determine the effect of any condition on EYFP expression.

The development of methods to detect recombinant pancreatic cells and the understanding of their constraints when analyzing homologous recombination enabled the application of these techniques to study the effect of additional cancer risk factors on homologous recombination in the pancreas. Given the importance of age as a risk factor for pancreatic cancer, we analyzed the changes in the frequency of recombinant cells and number of recombinant foci in pancreata of FYDR mice. We observed a dramatic accumulation of recombinant cells with age, including an ~23-fold increase in the frequency of recombinant cells and an ~9-fold increase in the number of recombinant foci per cm^2, suggesting that both *de novo* recombination events and clonal expansion contribute to the overall increase in the recombinant cell frequency with age. To further analyze the effect of clonal expansion on the accumulation of recombinant cells with age,

the laboratory of Peter T. So developed a two-photon imaging technique to quantify the number of cells per recombinant focus. Using two-photon imaging, we showed that the median number of cells per recombinant focus increased from two to six in juvenile and aged mice, respectively, indicating that recombinant cells can clonally expand with age.

The relative contribution of clonal expansion to the increase in recombinant cell frequency with age can be determined by analyzing the importance of *de novo* recombination events to the increase in recombinant cell frequency. The estimate of the contribution of clonal expansion to total recombinant cell number depends greatly on the total number of cells within the pancreas. Taga *et al* determined that a 4 week old mouse pancreas has ~27 million cells. In our studies, we have found that the mouse pancreas doubles in size from 4 weeks to 1 year; however, we do not know if this doubling in pancreas weight is due to a doubling in the number of pancreatic parenchymal cells, an increase in other cell types such as fibroblasts, inflammatory cells, adipocytes, a change in the concentration of extra cellular matrix components or a combination of these. If we assume no age-dependent increase in parenchymal pancreatic cells, we can calculate the minimum contribution of clonal expansion to recombinant cell accumulation with age.

The frequency of recombinant cells was ~$1/10^6$ and ~$30/10^6$ in juvenile and aged mice, respectively. Given that a pancreas contains ~27 million cells, we can calculate that juvenile mice have ~27 recombinant cells per pancreas and aged mice have ~810. A similar calculation can be made for the total number of recombinant foci per pancreas. If we assume that we are able to detect ~1/3 of all recombinant foci for both juvenile and aged mice, then we estimate that juvenile and aged mice have ~18 and ~282 recombinant foci per pancreas, respectively. Given that ~352 new recombination events and ~783 new

recombinant cells appear as the mice age, then ~60% of the increase in the number of recombinant cells with age is due to clonal expansion. Independently, we analyzed 68 recombinant foci from aged mice using two-photon microscopy and determined that the total number of recombinant cells contained within all foci was 898. Since 68 of these 898 recombinant cells were due to *de novo* recombination events, then ~90% of the increase in recombinant cells with age is due to clonal expansion. Together these data show that clonal expansion contributes ~60-90% of the overall increase in the number of pancreatic cells harboring DNA sequence rearrangements with age.

In addition to examining homologous recombination in pancreatic cells, we carried out similar analyses for skin tissue. Comparison of spontaneous recombinant cell frequencies in pancreatic and skin tissues from young FYDR mice shows that pancreatic tissue often contains more recombinant cells. This higher frequency of recombinant pancreatic compared to skin cells cannot be explained by expression of the FYDR transgene alone, suggesting that fluorescent recombinant cells within the pancreas may be more likely to clonally expand than those contained within the skin. The difference in recombinant cell frequency between pancreatic and skin tissues is further increased with age, as pancreatic tissue shows a dramatic accumulation of recombinant cell frequency and skin shows no accumulation. *In vitro* analyses of the rate of homologous recombination, spontaneous levels of DNA damage, and damage-induced recombinant cell frequency in juvenile versus aged primary fibroblasts show that homologous recombination is not suppressed with age, suggesting that the lack of accumulation of recombinant cells in the skin is most likely not due to an inability to undergo *de novo* homologous recombination events. Since we have shown that the accumulation of

recombinant cells in aged pancreata results from both *de novo* recombination events and clonal expansion of existing recombinant cells, the lack of accumulation of recombinant is most likely due to the absence of extensive clonal expansion in skin fibroblasts with age.

Finally, in addition to age and exposure to exogenous agents, genetic conditions can also affect susceptibility to cancer. Here we analyzed the effect of the key modulator of genomic stability, p53, on homologous recombination in FYDR mice. Results indicate that p53 status does not significantly affect the spontaneous frequency of recombinant cells in the pancreas *in vivo* or the spontaneous rate of homologous recombination in cultured fibroblasts *in vitro*. However, given the importance of p53 in responding to DNA damage, treatment of $FYDR^{y/+};p53^{+/+}$ and $FYDR^{y/+};p53^{-/-}$ mice or cells with exogenous DNA damaging agents may result in a differential susceptibility to damage induced recombination.

In this work, we have shown that FYDR mice provide a powerful tool for studying the effects of age, environmental exposure and genetic conditions on homologous recombination *in vivo*. These are the first series of studies to analyze homologous recombination in pancreatic cells and the first to demonstrate the significant contribution of clonal expansion to the accumulation of recombinant cells with age. The assays that we have developed to measure the frequency of recombinant cells, the number of independent recombination events and the contribution of clonal expansion can be applied to explore how additional genetic and environmental risk factors modulate double-strand break formation and repair by homologous recombination.

Because the accumulation of recombinant cells can be monitored over months and even years, long-term effects of both acute and chronic exposures relevant to cancer can also be studied. Although we see an accumulation of recombinant pancreatic cells with age, an important risk factor for pancreatic cancer, the effect of neoplastic transformation on homologous recombination in the pancreas is not yet known. Currently, FYDR mice are being crossed with mice that are predisposed to pancreatic intraepithelial neoplasias. The changes in the frequency of recombinant cells and the number of recombinant foci with cellular transformation can be assessed to determine if changes in the number of cells harboring DNA sequence are seen with progressive stages of cancer. In addition, since chronic infection is a risk factor for many types of cancer, FYDR mice are also being infected with gastro-intestinal bacteria to determine the effect of chronic inflammation on homologous recombination in pancreatic cells. Finally, FYDR mice can also be used to determine the combined effects of multiple risk factors on tumor formation *in vivo*.

In addition to analyzing the effects of cancer risk factors on homologous recombination pancreas and skin, this work can be extended to other tissues. We have shown using positive control FYDR-Rec mice that in addition to pancreas and skin, homologous recombination in kidney, lung, and other tissues can potentially be studied using FYDR mice. Similar techniques to the ones described here can be developed to measure independent recombination events, overall recombinant cell frequency and clonal expansion in other tissues. Thus, the FYDR mice can be used to elucidate the role of double-strand break formation and repair by homologous recombination in tumor formation for multiple tissues.

Appendix I

Cisplatin Treatment Induces Homologous Recombination in Adult Pancreatic Cells *in vivo*

A1.1 Introduction

Mitotic homologous recombination is critical for repairing double-strand breaks and interstrand cross-links. In addition, homologous recombination provides the only pathway for the accurate repair of double-strand ends that arise when a replication fork is broken upon encounter with a blocking DNA lesion or a single strand gap (1-5). If not repaired properly, DNA double-strand breaks and replication fork-associated double strand ends can lead to tumor-promoting DNA sequence rearrangements (6).

Ironically, although DNA damage that inhibits replication can induce tumorigenic sequence rearrangements, many agents used to treat cancer are in fact DNA damaging-agents that inhibit replication fork progression. Thus many chemotherapeutic agents are highly recombinogenic (7-12). Interestingly, elevated levels of homologous recombination proteins within tumor cells are associated with an increased resistance to many cancer chemotherapeutics (13-18) and a poor prognosis for cancer survival (19). In contrast, cancer cells deficient in homologous recombination (e.g., breast tumors with BRCA2 mutations (20, 21)) are rendered sensitive to chemotherapeutic agents that induce replication fork breakdown (22-25). Therefore, knowledge about the effect of a cancer chemotherapeutic on homologous recombination *in vivo* and the homologous recombination capacity of cells is critical for designing effective cancer treatments for different tumors.

Cisplatin is a common cancer chemotherapeutic that is used to treat many types of cancers including small cell lung, testicular, bladder, ovarian, head and neck and others. Depending on the type and stage of cancer, cisplatin is given in various doses ranging

from 20 to 100 mg/m^2, and usually in conjunction with other chemotherapeutic agents (26-30). For mice, the LD50 of cisplatin is estimated to be ~8.0 mg/kg for a single intraperitoneal injection (31), which would be an equivalent dose of ~309 mg/m^2 for a 150 lb human.

A1.2 Materials and Methods

Animals

C57BL/6 Fluorescent Yellow Direct Repeat (FYDR) mice have been described previously (32). 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 (33). Cisplatin- and mock-treated FYDR cohorts had equal ratios of males to females.

Cisplatin Treatment

Cisplatin (*cis*-Diamminedichloroplatinum(II), Sigma) was prepared fresh daily. To determine the molar concentration of cisplatin solution, the absorbance of the cisplatin solution at 300 nm was divided by the extinction coefficient (131). For treatment of mice, the cisplatin concentration was adjusted to 0.5 mg/ml. Five- to 6-week old mice were IP injected with 5 mg/kg of body weight of 0.5 mg/ml cisplatin. Mock-treated controls were injected with equal volumes of PBS. Mice were analyzed 3.5 weeks after injection.

Flow Cytometry

Pancreatic cells were disaggregated as described previously (34). Almost all samples were analyzed by flow cytometry after imaging (see below). Disaggregated pancreatic 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.

Imaging

Pancreatic cells were imaged as described previously (34). Briefly, nuclei were stained with 50 µg/ml Hoechst 33342 (Sigma). Whole pancreata were pressed between glass slides separated by 0.5 mm spacers. Sequential images were collected in black and white using a 1x objective. The images were manually compiled to cover the entire visible surface area. Filters included: visible light; UV (Ex:330-380 nm, Em:420 nm); Red (Ex:540/25 nm, Em:605/55 nm); and EYFP (Ex:460-500 nm, Em:510-560 nm). Images were collected using a fixed aperture time. Foci were counted manually in blinded samples. The area of compiled pancreata images was determined using Scion Image Beta 4.02 Win (Scion Corporation) by manually tracing the pancreas edge.

A1.3 Results

To determine the effect of cisplatin treatment on homologous recombination in pancreatic cells *in vivo*, 5- to 6-week-old FYDR mice were injected with cisplatin. The median frequencies of recombinant cells by flow cytometry (Fig. A1-1A) and of recombinant foci by *in situ* imaging (Fig. A1-1B) were higher among the cisplatin-treated mice, indicating that cisplatin induces homologous recombination events *in vivo*. However, cisplatin induction is statistically significant only when analyzed by *in situ* imaging, suggesting that although both flow cytometry and *in situ* imaging detect recombinant cells, *in situ* imaging may be a more sensitive method for detecting exposure-induced recombinant cells.

It is formally possible that the increased frequency of recombinant cells after cisplatin treatment is because of increased *EYFP* expression. To explore this possibility, we exploited positive control animals in which all cells carry the recombined substrate (full-length *EYFP*). Flow cytometry of pancreatic cells from mock- and cisplatin-treated positive control mice revealed that there was no statistically significant difference in *EYFP* expression between the cohorts (Figure A1-1C). Thus, we conclude that differences in *EYFP* expression do not affect the apparent frequencies of recombinant cells in cisplatin- and mock-treated mice, indicating that the increase in recombinant cell frequency after cisplatin treatment is the result of an induction of recombinant cells.

A1.4 Conclusions

The majority of cisplatin-induced lesions are intra-strand crosslinks (35), which are predominantly repaired by nucleotide excision repair (for review see (36)). However, if these lesions persist until replication, they can cause replication fork breakdown and the formation of a double-strand end, which can only be accurately repaired by homologous recombination (37). Treatment of mice with a dose of 5 mg/kg of body weight cisplatin is equivalent to ~190 mg/m^2 for a 150 lb human, which is ~2-fold higher than the highest single cisplatin dose (100 mg/m^2) given to patients during chemotherapy. However, since most chemotherapeutic regiments require multiple treatments, the total dose a patient receives may be higher than that give to the mice in this study.

The data presented here show that cisplatin is a potent recombinogen in adult pancreatic cells *in vivo*, inducing an ~4-fold increase in the frequency of recombinant cells and an ~3-fold increase in the number of recombinant foci. Thus, homologous recombination is an active repair pathway in adult pancreatic cells *in vivo* and is activated in response to cispatin-induced DNA lesions.

A1.5 References

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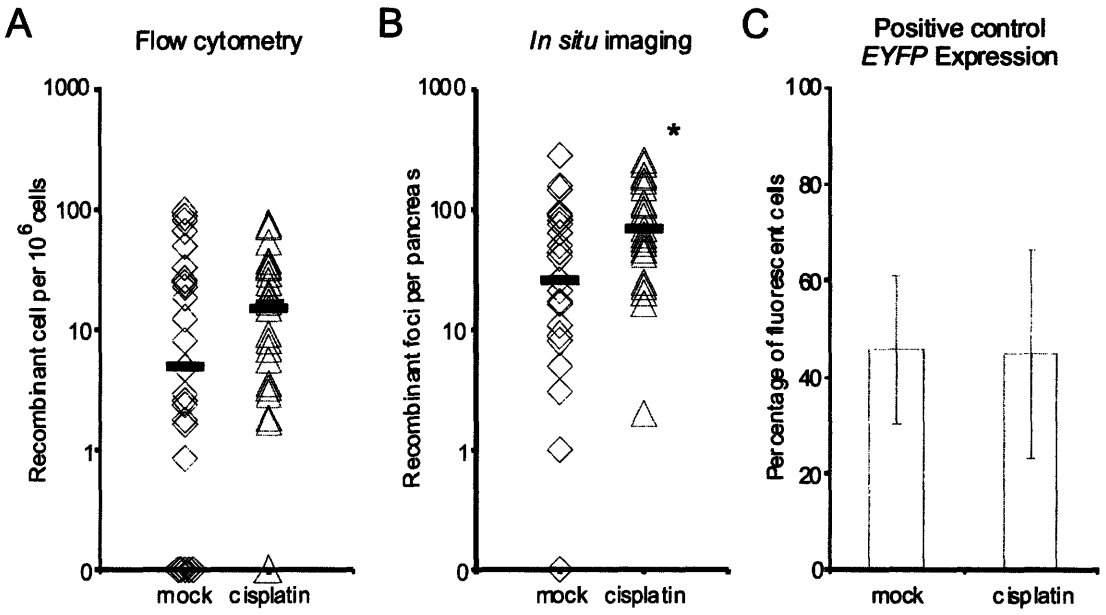

Figure A1-1. Cisplatin-induced homologous recombination in mouse pancreata. Medians are indicated by black bars. Points on the *x* axis indicate individual mice with zero recombinant cells. (A) Frequency of recombinant cells per million as determined by flow cytometry for mock-treated ($n = 25$) and cisplatin-treated ($n = 25$) FYDR mice ($p = 0.09$). (B) Recombinant foci per pancreas detected by *in situ* image analysis for mock-treated ($n = 25$) and cisplatin-treated ($n = 25$) FYDR mice. * Cisplatin-treated cohort is statistically significantly higher than mock-treated cohort ($p = 0.008$). (C) Average percentage of fluorescent pancreatic cells as determined by flow cytometry from mock- (n = 16) and cisplatin-treated (n = 17) positive control mice (ratio of males to females is the same in mock- and cisplatin-treated cohorts). Error bars indicate 1 standard deviation.

Appendix II

Comparing the Levels of mRNA and Protein Expression in Positive Control FYDR-Recombined Mice

A2.1 Introduction

The Fluorescent Yellow Direct Repeat (FYDR) mice carry a direct repeat recombination substrate in which a homologous recombination event can restore full-length enhanced yellow fluorescent protein (*EYFP*) coding sequence. The FYDR recombination substrate is integrated into chromosome 1 of the mouse genome in a region with no known genes (1). In the course of analyzing recombinant cells in pancreata of FYDR mice, we noticed a large variation in both the frequency of recombinant cells and the number of recombinant foci among mice. Although this inter-mouse variation in recombinant pancreatic cells may be the result of differences in homologous recombination, it is also possible that this variation results from differences in the ability to detect fluorescent cells (*e.g.*, differences in expression levels of *EYFP* from the FYDR transgene). For example, if *EYFP* is expressed at lower levels from the FYDR transgene, it will be more difficult to detect recombinant cells. Therefore, in order to study the effect of cancer risk factors on homologous recombination in the pancreas, the effect of those factors on expression of the FYDR transgene must also be determined.

One potential method for determining the level of FYDR transgene expression is to measure mRNA levels of the FYDR transgene. However, levels of mRNA transcript do not necessarily correlate with protein levels (2), and in our studies we are detecting EYFP protein expression after a recombination event in FYDR mice. In order to determine if there is a correlation between FYDR transgene mRNA levels and EYFP protein levels, we utilized positive control FYDR-Recombined mice. FYDR-Recombined mice arose spontaneously from a homologous recombination event in a FYDR parental

gamete. Thus, all cells in FYDR- Recombined mice carry the full-length *EYFP* coding sequence under the identical promoter and locus as the FYDR mice and have the potential to express EYFP. Because the FYDR- Recombined mice are the perfect positive control for FYDR transgene expression, they can be used to determine if FYDR transgene mRNA levels correlate with EYFP protein expression.

A2.2 Materials and Methods

Flow Cytometry

Pancreatic cells were disaggregated as described previously (3). Disaggregated pancreatic cells were pelleted and resuspended in 400 µl OptiMEM (Invitrogen) and filtered (35 µm). 100 µl of cell suspension post-filtration was removed for RT-PCR analysis. Remaining cell suspension (300 µl) was analyzed with a Becton Dickinson FACScan flow cytometer (excitation 488 nm, argon laser). Live cells were gated by using forward and side scatter.

RT-PCR

Pancreatic cells from the 100 µl sample removed for RT-PCR analysis were pelleted for 5 minutes in microcentrifuge at 3000 rpm, supernatant was removed and cells were immediately frozen in liquid nitrogen and stored at -80°C. Following RNA extraction using the RNeasy® Mini Kit animal cell isolation protocol (Quiagen), RNA concentration was determined by measuring absorbance at 260 nm. Following DNase I

(Invitrogen) digestion treatment, reverse transcription was performed using Superscript™ III First Strand Synthesis System (Invitrogen), and cDNA concentration was determined by measuring absorbance at 260 nm. RT-PCR was performed using SYBR Green PCR Kit (Quiagen) for GAPDH and FYDR genes with annealing temperatures of 60.5°C and 70°C, respectively. Primers: GAPDHfor: ACTGGCATGGCCTTCCG; GAPDHrev: CA GGCGGCACGTCAGATC; FYDRfor: AAGTTCATCTGCACCACCGGCAAGCTG; FYDRrev: TCGTGCTGCTTCATGTGGTCGGGGTAG. For each PCR run, samples including standers were run in triplicate. Quantification was based on standard curves made from serial dilutions of one sample, and average ratio of FYDR to GAPDH transcript was calculated for each sample.

A2.3 Results

To determine the correlation between FYDR transgene mRNA levels and EYFP protein expression, mRNA levels were measured using RT-PCR analysis, and EYFP protein expression was analyzed by flow cytometry. A fairly good correlation ($R^2 = 0.47$) between EYFP protein level and FYDR transgene mRNA level was observed (Figure A2-1), suggesting that FYDR transgene levels can be used to approximate expression of EYFP protein in FYDR- Recombined mice.

A2.4 Conclusions

Because in positive control mice the *EYFP* coding sequence is expressed under an identical promoter and at the same locus as the FYDR recombination substrate, it is likely that differences in expression of the FYDR transgene in FYDR- Recombined mice are also present in FYDR mice. Thus, the correlation between FYDR transgene mRNA level and EYFP protein expression observed for FYDR- Recombined pancreatic cells is most likely similar in FYDR pancreatic cells. Currently, in order to determine the effect of a genetic condition or environmental exposure on FYDR transgene expression, a cohort of FYDR- Recombined mice is included in all studies. However, given the correlation between FYDR transgene mRNA levels and EYFP protein expression, samples can instead be taken from disaggregated pancreata of FYDR mice for each study condition. RT-PCR analysis can be performed to compare FYDR transgene mRNA levels to determine if any apparent differences in the frequency of recombinant cells is due to differences in homologous recombination or differences in expression.

A2.5 References

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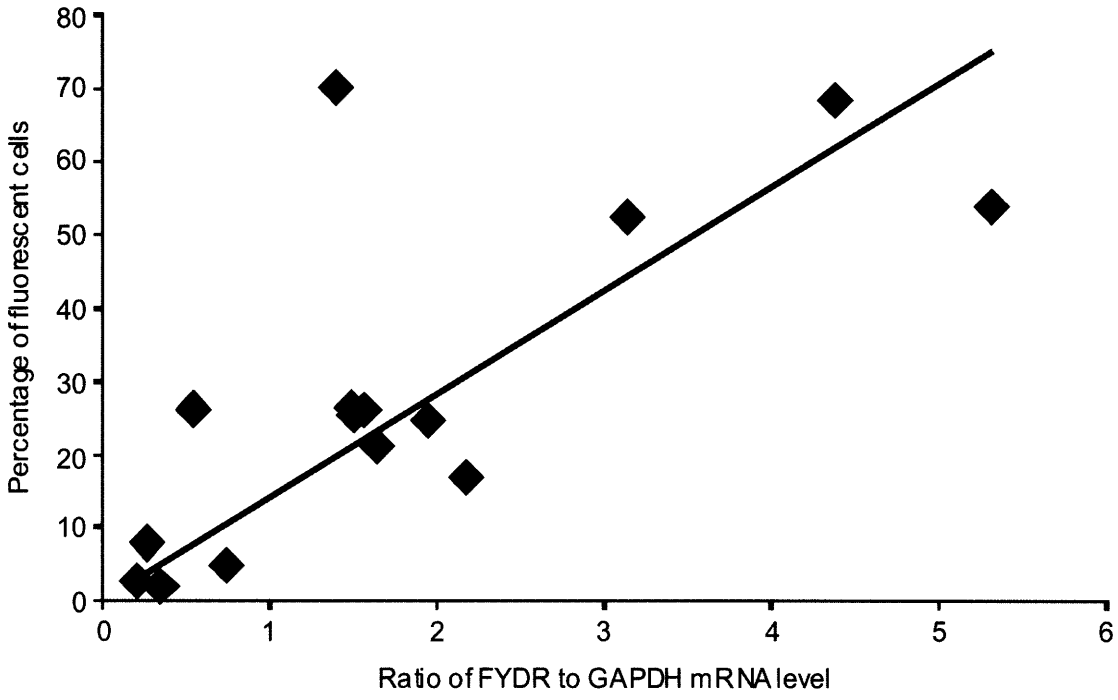

Figure A2-1. mRNA and EYFP protein expression in pancreatic cells from positive control FYDR-Recombined mice. Each point represents one mouse. $R^2 = 0.47$

Appendix III

Analysis of the Fraction of Recombinant Cells that can be Detected by *In Situ* Imaging on Both Sides of a Compressed Pancreatic Sample

A3.1 Introduction

To study homologous recombination in pancreata of Fluorescent Yellow Direct Repeat (FYDR) mice, an *in situ* imaging method was developed for detecting recombinant foci on the surface of intact pancreatic tissue (1). Briefly, pancreata are uniformly compressed to a thickness of 0.5 mm, and composite images that cover one side of the pancreatic surface area are created. Given the thickness of the pancreatic sample, all foci contained within the pancreas may not be detectable on the surface of the pancreas. In addition, recombinant foci within pancreatic samples may not be evenly distributed such that imaging only one side of the pancreas may not be representative of the recombinant foci contained within the entire pancreas.

A3.2 Results and Conclusions

To determine the percentage of recombinant foci that can be detected on both sides of a compressed pancreas and to determine the difference in the number of recombinant foci on the two sides of a compressed pancreas, composite images were taken of both sides of FYDR pancreata for five mice. Recombinant foci were manually counted on images of both sides of a pancreas. A comparison of the locations of foci on both sides was made to determine which foci could be seen on both sides of the pancreas. The results for the number of recombinant foci per pancreas side are shown in Table A3-1. Similar numbers of recombinant foci were detected on both sides of each pancreatic

sample. In addition, although the number of recombinant foci that could be detected on both sides of a pancreas varied, on average ~28% of foci could be detected on both sides of the pancreas sample. Together, these data indicate that analysis of one side of a pancreatic sample provides representative data for the number of recombinant foci located within the entire pancreas.

Table A3-1. Comparison of recombinant foci seen on two sides of a FYDR pancreas sample prepared for *in situ* imaging

Sample number	Number of foci Side 1	Number of foci Side 2	Number of foci common to both sides	Average percentage of foci seen
1	21	17	3	16.0
2	68	44	14	26.2
3	4	7	3	58.9
4	13	16	2	13.9
5	38	39	9	23.4
			Total average	**27.7**

A3.3 References

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