PROGRAMMING OF THE FETAL HEMATOPOIETIC STEM AND PROGENITOR CELL COMPARTMENT

By

Ashley N. Kamimae-Lanning

A DISSERTATION

Presented to the Department of Cell and Developmental Biology

and the Oregon Health & Science University

School of Medicine

in partial fulfillment of

the requirements for the degree of

Doctor of Philosophy

December 2013

School of Medicine

Oregon Health & Science University

CERTIFICATE OF APPROVAL

This is to certify that the PhD dissertation of

Ashley N. Kamimae-Lanning

has been approved

_____
Peter Kurre, MD; Mentor/Advisor

_____
Owen McCarty, PhD; Chair

_____
William H. Fleming, MD, PhD; Member

_____
David Farrell, PhD; Member

_____
Daniel Marks, MD, PhD; Member

TABLE OF CONTENTS

List of Figures

List of Abbreviations

1˚, primary
2˚, secondary
4-HNE, 4-hydroxynonenal
8-oxoG, 8-oxoguanine
AF488, Alexa Fluor 488
AGM, aorta-gonado-mesonephros
Aldh2, aldehyde dehydrogenase 2
AML, acute myeloid leukemia
ASL, $AA4.1^{+}$ $Sca\text{-}1^{+}$ $Lin^{low/-}$
ATP, adenosine triphosphate
BER, base excision repair
BM, bone marrow
CBC, complete blood count
CFU-C, colony forming unit cell
cGy, centigray
Co-IP, co-immunoprecipitation
CpG, cytosine-guanine bases
DIO, diet-induced obesity
DNA, deoxyribonucleic acid
DNA-PKcs, DNA-dependent protein kinase catalytic subunit
DOHaD, Developmental Origins of Health and Disease
dpc, days post coitum
DR, diet reversal
DSB, double strand break
ESC, embryonic stem cell
FA, Fanconi anemia
FAAP20, Fanconi anemia-associated protein of 20 kDa
FAAP24, Fanconi anemia-associated protein of 24 kDa
FAAP100, Fanconi anemia-associated protein of 100 kDa
FACS, fluorescence-activated cell sorting
FAN1, FANCD2/FANCI-associated nuclease 1
FANCA/Fanca, Fanconi anemia complementation group A
FANCB, Fanconi anemia complementation group B
FANCC/Fancc, Fanconi anemia complementation group C
FANCD1 (BRCA2), Fanconi anemia complementation group D1
FANCD2/Fancd2, Fanconi anemia complementation group D2
$FANCD2^{Ub}$, ubiquitinated Fanconi anemia complementation group D2
FANCE, Fanconi anemia complementation group E
FANCF, Fanconi anemia complementation group F
FANCG, Fanconi anemia complementation group G
FANCI, Fanconi anemia complementation group I
FANCJ, Fanconi anemia complementation group J
FANCL, Fanconi anemia complementation group L
FANCM, Fanconi anemia complementation group M

FANCN (PALB2), Fanconi anemia complementation group N
FANCO, Fanconi anemia complementation group O
FANCP (SLX4), Fanconi anemia complementation group P
FANCQ (ERCC4), Fanconi anemia complementation group Q
FAO, fatty acid oxidation
fcd-2, yeast homolog of FANCD2
FL, fetal liver
γH2AX, gamma-H2A histone family, member X
GAPDH, glyceraldehyde 3-phosphate dehydrogenase
hESC, human embryonic stem cell
HFD, high-fat diet
Hmga2, high-mobility group A2
HPC, hematopoietic progenitor cell
HR, homologous recombination
HSC, hematopoietic stem cell
HSPC, hematopoietic stem and progenitor cells
ICL, interstrand crosslink
KO, knockout
KS, c-Kit^{+} Sca-1^{+}
KSL, c-Kit^{+} Sca-1^{+} Lin$^{low/-}$ or Lin^{-}
LPS, lipopolysaccharide
LT-HSC, long-term hematopoietic stem cells
MDA, malondialdehyde
MDS, myelodysplastic syndrome
MFI, median fluorescence intensity
miRNA, microRNA
MM-C, mitomycin-C
mtDNA, mitochondrial DNA
NAC, *N*-acetyl cysteine
NADH, nicotinamide adenine dinucleotide (reduced form)
NER, nucleotide excision repair
NHEJ, non-homologous end-joining
Ogg1, 8-oxoguanine glycosylase
PAS, para-aortic splanchnopleure
PCR, polymerase chain reaction
PLT, platelet
PPARδ, peroxisome proliferator-activated receptor delta
qRT-PCR, quantitative real-time polymerase chain reaction
Rad51, Rad51 recombinase
RBC, red blood cell
ROS, reactive oxygen species
SEM, standard error of the mean
SLAM, signaling lymphocytic activation molecule markers (CD150^{+}CD48^{-}CD244^{-})
TLS, translesion synthesis
TNF-α, tumor necrosis factor-alpha
UBE2T, ubiquitin-conjugating enzyme E2T

UV, ultraviolet
V(D)J, Variable, Diverse, Joining (refers to gene segments)
WBC, white blood cell
WT, wild type

ACKNOWLEDGEMENTS

It is said that it takes a village to raise a child, and likewise, it takes an academic community to train a graduate student. Critical contributions from several people helped to make the work in this dissertation possible and support came from many. First and foremost, I thank my PI, Dr. Peter Kurre, to whom I am grateful beyond words for focused mentorship, invaluable training in critical thinking, grantsmanship, and scientific writing, and for taking us on all of those fantastic lab retreats. Many thanks to the current and former members of the Kurre lab, particularly Natalya Goloviznina for her dedication, countless long hours spent in the lab, excellent experimental support, and encouragement; much of the data contained herein was generated with her assistance. I am grateful to Dr. Kelsie Storm, who picked up much of the workload for our team and the mouse colony during the writing of this dissertation, and who inspires me with her compassion for her patients and ability to quickly integrate into the lab without previous experience. I thank Dr. Amy Skinner, who trained me, put up with my many inquiries in my early days as a student, and provided both technical and experimental support and encouragement. I am grateful to Katy Lawson, Lauren Uhde, Shannon Liudahl, Andrea McBeth, Kyle Lenz, and Tae Ha for assistance with fetal liver harvests and protocol troubleshooting. I also thank Noah Hornick for his friendship and consulting with PCR, and for being our in-house computer help whether he wanted to be or not. Thanks to Dr. Jianya Huan, Dr. Santhosh Chakkaramakkil Verghese, Dr. Shelton Viola, Rajani Kaimal, Dr. Lee O'Neill, Matthew Shurtleff, and Dr. Thomas Russell for helpful discussions and feedback.

I am indebted to Dr. Mitch Turker and his former research assistant, Adrian Chu for thorough training during my first lab rotation, particularly in tissue culture techniques and qRT-PCR. Thank you to Dr. Ginny Brooks and postdocs, Drs. Belinda Houghton-McCully and Priscilla Cassaglia, for generous training in acute surgeries during my winter lab rotation.

I am grateful to my thesis advisory committee members, Drs. Maureen Hoatlin and Michael Heinrich, to the members of my dissertation committee, Drs. Owen McCarty, David Farrell, and Daniel Marks, and to Dr. William H. Fleming, who served on both teams, for their guidance, helpful recommendations, and time.

Thanks also to Dr. Daniel Marks and his lab, Drs. Stephanie Krasnow and Peter Levasseur, as well as rotation student Quinn Roth-Carter, and Xinxia Zhu and Marek Szumowski for their valuable collaboration on the nutrition projects (Chapters 4 and 5 herein).

I thank the Pediatrics Department, Oregon Stem Cell Center, and Papé Pediatric Research Institute. Thanks to Dr. Markus Grompe and his lab, including Drs. Qingshuo Zhang, Nikki Paulk, Ray Hickey, Craig Dorrell, and Andy Duncan, as well as Sean Nygaard, Matthew Deater, Eric Benedetti, and Annelise Haft, for assistance with equipment and supplies and for providing us with the Fancd2 and

129Sv Fancc mice. Thanks to the Fleming Lab, particularly Drs. Devorah Goldman and Ronn Leon for technical support with antibodies and PCR. Thanks also to Qianyue Yang for support with equipment maintenance and repair, to Leslie Lublink, Kirsten Groener, and Elizabeth Perkins for administrative support, and to Dale Braden and Bill Kidd for grant assistance.

Thanks to Pamela Canaday and Mandy Boyd of the Flow Cytometry Core, Aurelie Snyder of the Advanced Light Microscopy Core, Rigo Perez from Department of Comparative Medicine for attentive mouse care, and to IACUC and DCM administrative staff for making the animal protocol process go smoothly.

I thank Drs. Amanda McCullough, Stephen Lloyd, and Anu Kumari for their consulting on DNA repair.

I am grateful to the Program in Molecular & Cellular Biosciences and the Cell & Developmental Biology Department, and to my funding sources: the Bill & Melinda Gates Millennium Scholars Foundation, the T32 Molecular Hematology training grant, N.L. Tartar Trust, and the American Society of Hematology for travel funds. I am also grateful to the Fanconi Anemia Research Fund for their Annual Symposium, which provided outstanding education, networking, and inspiration, and for their travel award to present my research at the 2011 Annual Meeting in Barcelona.

Finally, I thank my family and friends. Thanks to Damaris, Omega, Arial, Xin, Sharon, and friends, and to Becky, Tom, Ginger, Aunt Cherre, Papa Tom, Stephen and Kristin for love, support, and encouragement. Thank you, Mom and Dad, for believing in me so that I could grow up to believe in myself, for cultivating my curiosity and early love for science and school (so much in fact, that I took to being a student for 23 years!), and for your unconditional love and pride in me. Lastly, thanks to Jared, for your patience, encouragement, sense of humor, for being my relentless best friend, and for making me coffee in the mornings, without which, as we all know, scientists would be unable to function.

ABSTRACT

The role of prenatal development in programming adult cardiovascular and endocrine disease is increasingly well-characterized, and neurological programming has been a popular topic of research as well. However, despite rising pediatric hematological and immune disease rates, the potential contribution of developmental origins of disease in the blood system has been largely unexplored.

The most common cause of inherited bone marrow failure, usually diagnosed in childhood, is Fanconi anemia (FA), a recessive, multisystem syndrome. While the conventional assumption was that hematopoietic stem cells were lost from the bone marrow postnatally, precipitating bone marrow failure, evidence from *in vitro* studies suggests an earlier loss of stem cells. Using the Fancc-deficient mouse model of FA, we demonstrate a developmental deficit in the hematopoietic stem and progenitor cells (HSPC) of the liver, the main hematopoietic organ in the fetus, and a significant reduction in their serial repopulating capacity. While we did not detect increased apoptosis or elevation of inflammatory cytokines in the fetal liver, which are two culprits of the postnatal FA pathology, an increased proportion of *Fancc*$^{-/-}$ fetal HSPCs were in G_1 phase of cell cycle, suggesting cell cycle arrest. We further corroborated the FA fetal liver defect in two other mouse models. As the FA pathway functions in coordination of DNA repair, we assayed HSPC-enriched populations for DNA damage response and found increased γH2AX nuclear

foci and upregulation of several genes involved in nonhomologous end-joining and homologous recombination. Taken together, this suggests that FA fetal liver HSPCs are susceptible to DNA damage in the absence of chemical or radiological induction and that the hematopoietic compartment is a target for congenital defects.

As an alternative approach to the study of developmental vulnerability, we then chose a non-genetic model to further test self-renewal of the HSPC pool. As in FA, DNA damage plays a role in the pathology arising from a high-fat diet (HFD), which drives oxidative stress. The spread of the Western diet, which is composed of an excess of fat, has been accompanied by an epidemic of obesity. Interestingly, epidemiologists have documented a concurrent rise in pediatric diseases involving the immune system, which is derived from HSPCs, and this, along with studies from animal models and patients, suggests a role for excess lipid intake and maternal obesity in the nutritional programming of the hematopoietic system. We investigated the previously unexplored roles of these factors in the developing blood system, using an established mouse model of overnutrition. Maternal obesity and HFD induced losses in fetal liver HSPCs and short-term repopulation defects. Gene expression studies revealed perturbations in several genes involved in DNA repair.

Finally, nutritional and genetic mechanisms of developmental origins of disease were assayed together in a study of FA mice programmed under a HFD.

Fancc$^{-/-}$ fetal mice receiving a HFD appeared to be inhibited from the late gestational growth spurt observed in their wildtype littermates, and both fetal and juvenile mice had modest quantitative defects in the hematopoietic progenitor compartment.

Altogether, these studies reveal that HSPCs are programmable via both genetic and metabolic modification, highlighting an intrauterine vulnerability in hematopoietic cells and revealing a unique opportunity for preventative therapy.

CHAPTER 1: INTRODUCTION

Developmental Origins of Health and Disease

Beginning in the World War II era, England saw a disconcerting rise in adult coronary heart disease of unknown etiology, while in countries such as the United States, the disease rate had risen and then began a downward slide, despite a lack of association with lifestyle changes. It was not until more than a generation later, in the mid-1980s, when a correlation was uncovered that would spawn a new field of study.

Analyzing data from different parts of England and Wales, Barker and colleagues discovered a geographical association between neonatal mortality from 1921-25 and death from cardiovascular disease between 1968-1978. Low birthweight was a common cause of infant death at that time, which suggested a link between poor prenatal growth and adult cardiovascular disease[1]. In a subsequent study, they used data collected by midwives and government health visitors from 1911-1930 in Hertfordshire, England, who had recorded birth weights, weights at one year of age, whether babies were breast- or bottle-fed, and mortality data, and analyzed it against cause of death data for the same individuals between 1951 and 1987. They found a striking correlation between low birth weight and postnatal growth with coronary artery disease. It turned out that war rationing further restricted the diet of the impoverished populations in the industrial and rural areas of Hertfordshire. Those districts had lower birth weights and growth rates in the first year of life, and infant mortality was higher than in more prosperous regions[2,3]. The male infants who were lighter in weight at birth and

failed to thrive grew up to be men who were at increased risk of dying from coronary artery disease. This even impacted those who should have been at lowest risk based on levels of serum cholesterol, smoking, blood pressure, and early symptoms[3]. A follow-up study with living men who had been born in Hertfordshire suggested that fetal liver development may have been impaired in those who were growth-restricted[4].

Hales and Barker later published the “thrifty phenotype” hypothesis with data from individuals in Hertfordshire and Preston, linking impaired glucose tolerance and type 2 diabetes to reduced birth weight and weight at one year[5]. They proposed that poor prenatal and infant nutrition impaired proper metabolism[5]. Smaller body size and altered metabolism were thought to be fetal adaptations for living on minimal nutrients. Later studies linked low birth weight, paired with large placenta weight, to adult hypertension[6]. Along with previous work on coronary artery disease, a compelling body of evidence had emerged for the fetal programming of adult chronic disease.

Since then, the Developmental Origins of Health and Disease (DOHaD) theory has evolved, which states that fetuses respond to environmental cues during critical windows of development to adapt to anticipated life outside the womb[3]. As a result, certain phenotypic characteristics and disease susceptibilities of children can be altered in a way that cannot be accounted for by genetics alone. Other than low birth weight, additional risk factors have been identified, including

adiposity of neonates, umbilical cord leptin concentration, high birth weight, maternal nutrition, and *in utero* exposure to diabetes mellitus[7,8].

Metabolic Programming and High-Fat Diet

Since the seminal studies in DOHaD were performed, an overweight and obesity epidemic has emerged in the United States and other countries that have adopted the contemporary Western-style high-fat diet (HFD). It is typically constituted by about one-third fat (or more), high in refined carbohydrates, low in fiber, and generally consists of an excess of processed foods and meat, with a lack of adequate fruits, vegetables, and whole grains[9,10]. With it have come gains in body mass index, a sharp increase in type 2 diabetes, cardiovascular disease, dysfunction of the immune system, and increased rates of cancer. In the United States, more than half of women and approximately 2/3 of men 20-39 years of age are overweight or obese, and of those, about 1/3 are obese[11]. Obesity is recognized as a state of chronic inflammation, which modulates the immune system, and excess dietary lipids have also been shown to increase inflammation and trigger the production of oxidative radicals.

Recent research in DOHaD has uncovered a concerning role for maternal obesity and prenatal high-fat diet in the programming of cardiovascular disease, type 2 diabetes, and immune defects, including asthma and allergy[12-19].

Genetic Programming

The intricate temporal and spatial regulation of gene expression during development can be modified not only by exogenous, nutritional means, but also by endogenous genetic factors. One such example is in folic acid metabolism. Dietary folic acid deficiency during early embryogenesis results in failed neural tube closure and spina bifida, the incidence of which has been greatly decreased by supplementation of cereals, breads, and other foods. However, spina bifida can also be caused by a loss in function of the folic acid metabolizing enzyme or in a carrier protein, preventing folic acid from being utilized and simulating a nutritional deficiency[20,21]. Interestingly, with extra folic acid supplementation, this genetic defect can be partially overcome, and in the case of folic acid carrier protein deficiency, was demonstrated to rescue mice from embryonic lethality[21]. Thus, a unique therapeutic opportunity during prenatal development may be taken advantage of in order to ameliorate the impact of other genetic conditions with a developmental component to their pathology.

Fanconi Anemia

Defects of the musculoskeletal system representing failure of proper development are among the many symptoms of Fanconi anemia (FA), a rare, recessive, multisystem syndrome caused by mutation in one of at least 16 known genes, for which the carrier rate is estimated to be 1:181 in the United States[22,23]. The most common cause of morbidity and mortality, however, is bone marrow failure. This is characterized by cytopenia due to a deficiency in functional

hematopoietic stem and progenitor cells, ultimately resulting in infection, anemia, and/or bleeding from an insufficient number of neutrophils, erythrocytes, or platelets, respectively. The median age of onset for bone marrow failure is 7 years and it affects 90 percent of FA patients by age 40[24]. Improvements in therapies and supportive care have boosted the life expectancy of patients from 19 years of age in the 1980s to 30 years in 2000[25].

In addition to hematopoietic abnormalities, FA includes a variety of signs and symptoms: skeletal defects, especially in radial and craniofacial formation, small stature, learning disabilities, defects in the cardiac, endocrine, reproductive system, kidney, and gastrointestinal systems, café au lait markings on the skin, infection with human papillomavirus, and an increased susceptibility to cancer, including acute myelogenous leukemia (AML) or myelodysplastic syndrome (MDS)[26,27].

Aplastic anemia and physical anomalies were present in the three brothers who were first diagnosed in Switzerland in 1927, by the pediatrician Guido Fanconi, after whom the condition would come to be named[28,29]. It became characterized as a chromosomal instability disorder in 1964 during clinical cytogenetic tests, and the discovery that the DNA crosslinking chemical, mitomycin-c (MM-C) worsened chromosomal aberrations in FA cells was made a decade later[29]. The use of MM-C for cytogenetic testing of FA cells, which display an increased

incidence of chromosomal radials and breaks, has been a clinical standard for diagnosis[30].

Using MM-C as well as diepoxybutane, which crosslinks DNA with histones[31] and prevents its unwinding for replication or transcription, cell fusion experiments were performed with FA patient cells to test for complementation; in this assay, fusion of cells with a mutation in the same gene does not rescue the DNA repair defect and lowered cell viability in the presence of crosslinkers, whereas fusion of cells with mutations in different genes yields a wild-type phenotype and greater cell survival. Despite some drawbacks, including a misclassification of a patient (which lead to the withdrawal of complementation group H)[32], complementation cloning and retroviral transduction have been important tools for the identification of FA genes[33].

Compound heterozygote mutations in one of at least 16 genes can cause loss of function, leading to FA. They all cooperate in a pathway to direct repair proteins to interstrand crosslinks (ICL), which covalently join the Watson and Crick strands together, and to repair crosslinks between DNA and protein as well as double strand breaks. To accomplish this, the FA gene products direct homologous recombination (HR), translesion synthesis (TLS), and nucleotide excision repair (NER) proteins in a multistep process[34]. Proteins of the Fanconi anemia pathway coordinate DNA repair upon stalling of the replication fork during S-phase[29]. FANCM and an accessory protein, FAAP24, form a heterodimer that

binds to the DNA lesion at the stalled fork, recruiting the core complex, which is composed of FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, and FANCL, along with FANCM. Two other accessory proteins, FAAP100 and FAAP20, have been shown to be associated with the core complex, but like FAAP24, are not classified as FA genes because they have not yet been found to be mutated in any FA patients[35,36]. After the core complex binds to a DNA lesion, the FANCL protein, an E3 ubiquitin ligase, uses ubiquitin-conjugating enzyme UBE2T[37] to add one ubiquitin moiety each to FANCD2 and FANCI, separating them from one another; ubiquitinated FANCD2 ($FANCD2^{Ub}$) then binds to foci (including repair factors such as H2AX and Rad51) that mark the site of damage on the chromatin, followed by FANCI[38]. Next, $FANCD2^{Ub}$ recruits repair proteins to the site. On one side of the ICL, MUS81-EME1 creates a double-strand break, and on the other side, Fanconi anemia associated nuclease 1 (FAN1) creates a single-stranded break to unhook the ICL[39,40]. This creates a gap that is filled in by translesion synthesis[41]. The unhooked crosslink is removed by NER machinery, the conserved strand is resected, and HR machinery forms a D-loop intermediate in the gap, providing a template for extension of the DNA. This is then likely to be disassembled by FANCJ, a helicase that unwinds atypical DNA structures to allow movement of the replication fork in *trans*[35,42,43]. In addition to FANCJ, other downstream FA proteins include FANCD1 (BRCA2), FANCP (SLX4), and FANCN (PALB2). FANCD1 mediates recombination, is required for the formation of Rad51 foci, and colocalizes with FANCD2; it is a well-known tumor suppressor that is often mutated or epigentically inactivated in breast, ovarian, and other

cancers[44-47]. The function of FANCN is still under investigation, though it is needed for the binding of FANCD1 to chromatin and thus for HR[48]. FANCP is a scaffold protein that regulates nucleases participating in ICL repair as well as both topoisomerase I inhibitor-induced and PARP inhibitor-induced DNA damage[49]. FANCQ (ERCC4) is the most recently discovered FA gene and encodes the XPF endonuclease, which participates in NER and ICL[50,51]. Altogether, the FA pathway involves a panel of multifunctional proteins critical to DNA damage control and cellular integrity.

In addition to directing repair proteins to ICL, the FA pathway inhibits NHEJ, an error-prone repair mechanism for double-strand DNA breaks which is a key repair pathway in hematopoietic stem cells (HSC)[52]. In this pathway, double-stranded break ends are ligated without the requirement of a homologous sequence. Though NHEJ uses anywhere from one to a few homologous bases, or microhomologies, to position the ends, illegitimate pairing can lead to mutations and gross cytogenetic abnormalities[53]. These defects were found to be greatly reduced in both yeast and a chicken B cell line lacking *fcd-2* (the yeast homolog of *FANCD2*) and *FANCC*, respectively, when NHEJ was pharmaceutically or genetically inhibited[54,55].

Other than blocking NHEJ and directing the complex process of ICL repair to HR, TLS, and NER proteins, the FA pathway is also activated by other types of DNA damage, including spontaneous damage during replication and lesions from

exogenous causes, including ionizing radiation, ultraviolet light, and the deoxyribonucleotide production inhibitor, hydroxyurea. However, whereas these other types of damage often can be tended by other pathways, ICL repair solely relies on the FA pathway[35].

There are multiple dietary agents and metabolic products that can induce ICL or DNA-protein crosslinks. Nitrates, such as those found in preserved meats, can be converted to nitrous acid in the acidic conditions of the stomach and reacts with guanines to form ICL[56]. Furocoumarins are photoactive organic compounds produced by certain kinds of plants, including foods such as celery and lime oil, as a defense mechanism; these compounds can intercalcate into DNA and then react with UV light to form monoadducts with pyrimidines, and further react to form ICL[57,58]. Interest has recently grown in the role of aldehydes in producing ICL, as they have been demonstrated to exacerbate the FA phenotype, particularly during the sensitive window of fetal development[59,60]. Aldehydes can be produced in the body by many metabolic reactions, including those for carbohydrates, fats, amino acids, vitamins, ethanol, and steroids[61]. In the FA field, aldehyde work has almost solely focused on ethanol metabolism[59,60]. Several steps are involved in the catabolism of ethanol, which involves its conversion to acetaldehyde and then acetate and NADH; the main enzyme that performs the last reaction is aldehyde dehydrogenase 2 (Aldh2). Both this enzyme and *Fancd2* have been inactivated in double knockout mice, which were found to be profoundly sensitive to ethanol, often dying *in utero* from exposure and

developing hematopoietic failure after receiving it postnatally. They eventually developed lymphoblastic leukemia or aplastic anemia even in the absence of alcohol[59,60].

Alcohol metabolism is a factor that is clearly involved in fetal alcohol syndrome and contributes to increased rates of esophageal cancer in East Asian countries such as Japan, Korea, and China, where 36 percent of the population carries a dominant negative mutation in *ALDH2* (8 percent of the world's population carries *ALDH2* mutations). This causes a condition called the Alcohol Flushing Response, in which even a loss of one copy of fully functional ALDH2 causes a more than 100-fold reduction in enzymatic activity, accompanied by skin flushing, nausea, and tachycardia, along with a greatly increased risk of squamous cell carcinoma for even moderate drinkers[62,63]. The buildup of the acetaldehyde metabolite due to Aldh2 deficiency dramatically increases cellular aldehyde exposure. Acetaldehyde is a volatile compound that passes through the placenta; interestingly, at least one functional copy of *Aldh2* is required in *Fancd2*$^{+/-}$ dams to prevent prenatal lethality of *Aldh2*$^{-/-}$ *Fancd2*$^{-/-}$ fetuses, suggesting conversion of endogenous acetaldehyde in the mother protects the fetus[60]. Recent corrobative evidence from Japanese FA patients implicates co-incidence of *ALDH2* mutations in accelerated bone marrow failure, as well as increased rates of congenital defects, such as cardiovascular malformations [64].

Endogenous alcohol production can occur via metabolism of substrates such as pectin, or by gut bacteria, but the levels produced are quite low[65]. It is unclear what the main source of aldehydes is in people for whom alcohol exposure only occurs via endogenous production, such as in pregnant women and children. Dietary fats are another source of aldehydes. The metabolism of fats involves lipid peroxidation, which can yield acrolein, 4-hydroxynonenal (4-HNE), crotonaldehyde, and malondialdehyde which can react with both DNA and protein[66-68].

Lipid peroxidation also produces reactive oxygen species (ROS), which oxidize proteins, lipids, and nucleic acids. ROS can cause a variety of damage to DNA, including crosslinks to protein, single-strand breaks that can be converted to double-strand breaks during replication, and oxidation to bases, such as in the oxidation of guanine to 8-oxoguanine (8-oxoG). Damage to proteins may also exacerbate the situation by crippling enzymes that remove ROS from the cell, such as catalase and glutathione oxidase. Reactive organic radicals can be created upon ROS damage to lipid membranes[67].

Oxidative stress is extremely damaging to FA cells, particularly HSPCs[69,70]. Pang *et al.* showed that HSPC from FA mice are generally more susceptible than WT counterparts to reoxygenation injury[71]. Primary FA patient bone marrow HSPCs survive more successfully under hypoxic conditions (5% O_2), that resemble those in the bone marrow microenvironment, than in ambient tissue

culture oxygen (20% O_2); they are also partially protected by antioxidant supplementation *in vitro*, in particular, *N*-acetyl cysteine (NAC)[72].

Preserving genomic integrity is critical to proper prenatal development, including the HSPC compartment, which undergoes rapid expansion in the fetal liver. Along with obvious challenges of studying *in utero* events in human development, modeling of bone marrow failure in FA had long been focused on predominant postnatal losses of HSPC. Though congenital anomalies, including certain skeletal, heart, eye and ear, kidney, and skin defects, are signs of the disease, potential developmental defects in the hematopoietic compartment have been largely unstudied. The initial suggestion of a possible prenatal defect in hematopoiesis in FA came from the first FA patient cord blood transplant in France in 1990. Compared to progenitor colony formation from cord blood from 3 unaffected infants, cord blood from an FA newborn was found to have a very low frequency of hematopoietic progenitor cells[73]. Corroborating a potentially important role for FA proteins during hematopoietic development, *in situ* hybridization on 13.5 dpc (days post coitum) mice revealed robust *Fanca* gene expression in the fetal liver[74]. Knockdown of *FANCA* and *FANCD2* in human embryonic stem cells (hESC) and subsequent induction of differentiation to the hematopoietic lineage demonstrated a defect in hematopoietic cell formation and progenitor production[75]. Though this suggested that the loss of FA genes hindered hematopoietic development, no studies in a more physiologically relevant model existed to clarify this, although examination of p21 expression in

human FA fetal liver specimens revealed significant overexpression, suggesting p53 induction via replicative stress[76].

Hematopoietic Stem Cells and Development

Hematopoietic stem cells are the source of all differentiated blood cells, which must be continually replenished throughout life. Their development, maintenance, and survival requires DNA repair to preserve faithful function and avoid malignant evolution. This is particularly critical during prenatal development, when the foundations of the blood system are being laid.

The development of the hematopoietic system occurs in waves. During the first wave, primitive HSCs emerge on embryonic day 7.5 (E7.5) in the mouse, in blood islands formed by hemangioblasts, which are progenitor cells that give rise to both endothelial and hematopoietic cells[77]. Primitive HSCs can only give rise to a subset of cells: primitive erythrocytes, megakaryocytes, and macrophages[78]. At E8.25, the second wave yields definitive multipotent HPSCs, produced by hemogenic endothelium in the aorta-gonado-mesonephros (AGM) or para-aortic splanchnopleure (PAS) (the dorsal aorta's ventral wall)[77]. Unlike transient and limited primitive HSCs, definitive HSCs are able to self-renew and thus replicate themselves, as well as produce progenitor cells that will supply the full spectrum of mature, functional hematopoietic cells[78]. Last, the third wave entails further production of definitive HSCs by AGM[79] and in the umbilical and vitelline arteries[80], starting on E10.5[77]. They are detectable in the placenta on E10.5[81]

and yolk sac[79,82] and fetal liver starting on E11.5[83]. The fetal liver, which is downstream of the placenta, is the main hematopoietic niche of the fetus, and HSCs will undergo rapid expansion there, doubling each day until 14.5 dpc. HSC are also detectable in the fetal spleen and thymus at this time[84,85]. At day 15.5, the fetal liver HSC population begins to decrease as the cells migrate to the bone marrow[86] and the hepatoblasts begin to differentiate into hepatocytes[87]. (Mice retain some residual hematopoiesis in the fetal liver for about a week after birth and in the spleen for several weeks[88].)

The bone marrow serves as the main postnatal hematopoietic niche, though mice carry out some hematopoietic functions in the spleen as well[89] and can use the liver for stress hematopoiesis; humans can only carry out extramedullary stress hematopoiesis in the spleen[90]. In humans, the hematopoietically active red marrow regresses from the long bones between age 5 to 7 and blood production is concentrated in their proximal ends, along with the ilium and axial bones (vertebrae, ribs, sternum, and cranium), whereas in mice, hematopoiesis occurs in all of the bones[91].

To isolate HSPCs from a mouse, no known single cell surface marker exists; rather, a combination of markers is used for both positive and negative selection, and though different investigators have different preferences, transplantation studies have verified the ability of certain cell populations to repopulate a mouse. Fetal liver HSCs can also be isolated using signaling lymphocytic activation

molecule (SLAM) markers ($CD150^{+}CD48^{-}CD244^{-}$)[92]. The c-Kit receptor and Sca-1 are commonly used markers for HSPC positive selection, along with negative selection with several hematopoietic lineage (Lin) markers that are expressed by various types of differentiated blood cells; this combination is commonly known as c-Kit^{+} Sca-1^{+} Lin^{-}, or KSL (fetal liver HSPCs are $Lin^{lo/-}$, while adult bone marrow HSPCs are Lin^{-})[93,94]. The bone marrow KSL population is composed of stem and progenitor cells, but HSCs can be more stringently sorted with the addition of CD34 and FLT3 for negative selection, or with two SLAM markers ($CD150^{+}CD48^{-}$)[95,96]. Mac-1, CD144, and AA4.1 (CD93) are epitopes expressed by fetal liver HSPCs and their expression is eventually turned off as they switch to an adult phenotype[86,97-100]. $AA4.1^{+}Sca\text{-}1^{+}Lin^{lo/-}$ (ASL) is a combination of markers used to isolate fetal liver HSPCs which incorporates both the Sca-1 and lineage markers commonly used in sorting adult HSPCs, as well as the fetal liver AA4.1 cell surface marker[100].

Purifying HSCs requires a substantial number of cells from hematopoietic tissue, as they are relatively rare. Murine fetal liver has been reported to contain 0.1-0.2 percent HSCs, whereas adult bone marrow contains less than 0.01 percent HSCs[99]. Whereas 40 percent of fetal liver HSCs ($Thy1.1^{lo}Lin^{-/lo}Sca\text{-}1^{+}$) are actively cycling, only approximately 20 to 30 percent of adult HSCs ($Thy1.1^{lo}Lin^{-/lo}Sca\text{-}1^{+}$ or $CD34^{-}CD48^{-}CD150^{hi}$ KSL cells, respectively) are in cycle under homeostatic conditions[101,102]. Similarly, other transplantation studies have shown that cycling adult HSCs can confer short-term engraftment, whereas those that

are slow-cycling or quiescent are capable of long-term engraftment[101,103,104]. Most adult HSCs are quiescent, and while this protects them from replicative stress, it also comes at the cost of allowing genomic damage to accumulate, as the cell is not undergoing the DNA damage checkpoints that occur during mitosis; this damage greatly inhibits their capacity to respond to stressors such as infection or injury when they are forced back into cycle[105]. Differentiation of HSCs can also be induced by DNA damage[106].

The cell cycle status of HSC is reflected in their tightly regulated metabolic state. Long-term quiescent stem cells occupy a hypoxic microenvironment and fittingly, rely much more on glycolysis than for oxidative phosphorylation to meet their energy requirements and, even carrying fewer mitochondria that their more differentiated progeny[107]. This pathway protects them from DNA-damaging ROS, produced during the oxygen-intensive metabolic reactions in the electron transport chain. However, when HSCs divide to produce one HSC and one more differentiated progenitor cell (termed an asymmetric division due to the dissimilarity in the two resulting daughter cells), they switch to fatty acid oxidation[108]. Overall, HSC metabolism is a field that is relatively new and the metabolic characteristics of fetal liver HSCs are yet to be explored.

Mechanisms of Programming

Metabolism plays an important role in DOHaD, and though various processes are just starting to be elucidated, several studies implicate epigenetics as a major

mechanism[109-114]. Epigenetics refers to modifications made to the transcriptional state of DNA that do not change its sequence. These changes are mediated by chemical modifications on proteins that package DNA, called histones, and direct methylation of DNA[115]. Histones can be chemically tagged by acetylation, methylation, ubiquitination, phosphorylation, and sumoylation, which can recruit factors and enzymes or alter histone charge and its interaction with the negatively charged DNA backbone[116]. Methylation of DNA, which causes gene silencing, typically occurs at cytosine-guanine bases (CpG). The function of non-CpG methylation has been elucidated in plants, though there is more to be learned of its role in mammalian cells; it been detected in embryonic stem cells and in early embryonic development in mice[116-118]. In utero DNA methylation[110,111] and histone modification[119] have both been identified as mechanisms involved in DOHaD.

Both epigenetics and changes to the DNA sequence may be factors in DNA damage as a possible factor in developmental programming. Schuler *et al.* demonstrated that chromatin modifications can accumulate after DNA damage goes unrepaired or is misrepaired in mouse hair follicle stem cells[120]. Chromatin modification after DNA damage is hypothesized to induce epigenetic changes[121].

Damage to mitochondria, which have their own DNA, has also been implicated in both HFD and obesity-driven developmental programming. HFD murine oocytes have proportionally more morphologically abnormal mitochondria, more mtDNA

copy numbers and significantly lower levels of citrate, but normal ATP levels; altogether, this suggests that the mitochondria are stressed and compensating by increasing their numbers[122]. Likewise, diet-induced obesity (DIO) increased mitochondrial potential, mtDNA content, and biogenesis of mitochondria in oocytes and zygotes, and raised reactive oxygen species while depleting glutathione, a key endogenous antioxidant. An example of mitochondrial phenotype compromising HSPC function was recently provided by Mantel *et al*., who demonstrated that deletion of *Stat3*, which, among other things, increases oxidative phosphorylation in mitochondria, leading to an accelerated aging phenotype[123].

Much work on developmental programming has been on the maternal metabolic status and *in utero* effects, but embryonic defects also result from paternal DIO. Binder *et al.* took epididymal sperm from DIO or control diet-fed male mice and, using *in vitro* fertilization, generated embryos for study with time-lapse microscopy and transplantation into pseudopregnant female mice, each of which received embryos from both cohorts into separate uterine horns. They found that embryos from obese fathers had reduced mitochondrial membrane potential, reductions in the allocation of cells to the inner cell mass and trophoectoderm (outer cell layer) of the blastocyst, and reduced outgrowth on fibronectin. For the *in vivo* studies, fetuses from DIO fathers had delayed development, and reduced fetal and placental length and weight[124].

Altogether, mechanisms behind DOHaD are likely to center on sustained modification of gene expression, via epigenetic or mutational mechanisms.

General Hypothesis and Specific Aims

Existing studies on the developmental origins of disease tend to be epidemiologically driven and focus on overt disease outcomes, whereas the mechanisms by which organs are susceptible remain to be clarified. Developmental hematopoiesis in mice closely resembles that in humans and offers a unique opportunity to track and experimentally test both mechanisms and outcomes longitudinally. HSPC govern homeostatic function and organ specific injury response. Intriguingly, the main HSPC niche in the midgestational fetus, the liver, is a predominantly hematopoietic organ previously proposed as a target organ of developmental malprogramming[4]. In this dissertation, I will investigate the effect of developmental programming on the fetal liver in two mouse models of disease: Fanconi anemia and overnutrition by HFD.

Specific Aims:

1. **Determine whether hematopoietic failure is developmentally programmed in Fanconi anemia.**

   The state of HSPCs in FA prenatal development has been largely unexplored, even as the loss of Fanconi anemia genes has been demonstrated to result in congenital defects in limbs and in several organ systems. The conventional model of FA-associated bone marrow failure,

based mainly on work from patient samples, has focused on HSPC loss in the bone marrow and has attributed the pathology to postnatal attrition. However, evidence from cord blood samples and recent differentiation assays with ESCs suggest a potential role for prenatal development in FA-associated bone marrow failure.

2. Identify the effect of metabolic programming by HFD on fetal liver HSPCs.

Recent evidence implicates HFD in malprogramming of the fetal liver. Maternal HFD increased apoptosis, oxidative stress, and triglycerides, and altered expression of genes involved in metabolism and induced tissue resembling nonalcoholic fatty liver disease[14,119,125]. Since the liver has a key role in expansion and maturation of the hematopoietic system in fetal development, HFD may impact this process. I will determine whether HFD alters the quantity and function of fetal liver HSPCs in mouse models of prenatal HFD and maternal obesity.

3. Determine whether HFD exacerbates the prenatal hematopoietic development in FA. Through a double-knockout mouse model, aldehydes from ethanol metabolism have been implicated in fetal defects and lethality, bone marrow failure, and leukemia, but the role of lipid metabolism-derived aldehydes and ROS have not been investigated in fetal hematopoietic development. The dramatic increase in fat

consumption with the Western diet has greatly increased dietary lipids in a large proportion of the population. I will use the *Fancc* mouse model to assay the effect of HFD on DNA damage and the quantity and function of the HSPC compartment in the *Fancc*$^{-/-}$ mouse model.

CHAPTER 2: FETAL ORIGINS OF HEMATOPOIETIC FAILURE IN A MURINE MODEL OF FANCONI ANEMIA

Published in *Blood*. Kamimae-Lanning AN, Goloviznina NA, Kurre P. Fetal origins of hematopoietic failure in a mouse model of Fanconi anemia. *Blood*. 2013;121(11):2008-2013.

Abstract

Hematopoietic failure is the predominant clinical manifestation of Fanconi anemia (FA), a rare, recessively inherited disorder. Mutations in one of 15 genes that coordinately function in a complex pathway to maintain DNA integrity also predispose patients to constitutional defects in growth and development. The hematologic manifestations have long been considered to reflect the progressive loss of stem cells from the postnatal bone marrow microenvironment. Ethical concerns preclude the study of human hematopoiesis *in utero*. Here we report significant late gestational lethality and profound quantitative and qualitative deficiencies in the murine *Fancc*$^{-/-}$ fetal liver hematopoietic stem and progenitor cell (HSPC) pool. *Fancc*$^{-/-}$ fetal liver HSPC revealed a significant loss of quiescence and decline in serial repopulating capacity, but no substantial difference in apoptosis or levels of reactive oxygen species. Taken together, our studies suggest that compromised hematopoiesis in *Fancc*$^{-/-}$ animals is developmentally programmed and does not arise *de novo* in the bone marrow compartment.

Introduction

Bone marrow (BM) failure is the most common cause of morbidity and mortality from Fanconi anemia (FA), a recessively inherited disorder resulting from biallelic mutations in one of 15 known genes that cooperate in a DNA repair pathway.[29,35] The presumptive postnatal loss of hematopoietic stem and progenitor cells (HSPC) from the marrow is thought to account for patient

symptom onset during early school age and is generally consistent with experimental evidence.[24] However, cytopenias often precede clinical symptoms in FA patients or affected siblings.[126] While systematic studies of *in utero* development of FA patients have not been conducted, reports of reduced cord blood progenitor frequency, evidence in embryonic stem cell lines, and studies of rare fetal tissues all support the notion of early compromise in HSPC function.[73,75,76,127-129] Here, we studied fetal liver (FL) hematopoiesis in a murine model of FA complementation group C (*Fancc*$^{-/-}$) to determine if hematopoietic deficits were limited to HSPC losses from the BM compartment, or whether they may have origins earlier in development.[130] Our study for the first time reveals broad defects of hematopoietic development in *Fancc*$^{-/-}$ animals, which include a compromised fetal liver HSPC pool as well as late gestational lethality.

Results and Discussion

A wide range of FA-associated constitutional defects arising *in utero* are diagnosed at birth. Hematopoietic manifestations of FA in infants, by comparison, are rare.[126] *Fancc*$^{-/-}$ mice provide a validated, if imperfect, model of FA hematopoiesis. Here, we focused on FL hematopoiesis (14.5 dpc) as a key stage during hematopoietic development.[83] Using heterozygous breeder animals, the genotype distribution across 28 litters yielded 26% *Fancc*$^{-/-}$ fetuses (n=59$^{-/-}$, 81$^{+/+}$, 87$^{+/-}$), at near-Mendelian frequency. This exceeds the frequency at weaning of 18.9% (n=659 total; *P*=0.004), also reported by others, and for the first time reveals late gestational lethality in FA mice (Fig. 2.1A).[131,132]

Beyond occasional gross morphological abnormalities (microphthalmia, anophthalmia, anencephaly), *Fancc*$^{-/-}$ fetuses weighed on average 8% less than WT littermates, with a concomitant 34% reduction in whole FL cellularity and a 15% decrease in placental mass (Fig. 2.1B-H). *Fancc*$^{-/-}$ FL cells exhibited characteristic mitomycin-c (MM-C) induced radial formation (Fig. 2.1I) and chromosomal breaks (not shown).[130,133] Clonogenic hematopoietic progenitor growth in methylcellulose (CFU-C) was reduced by 20% in *Fancc*$^{-/-}$ FL compared with WT (Fig. 2.1J), implying an aggregate 47% decline in progenitor frequency per fetus. While interleukin-1, tumor necrosis factor-α (TNF-α) and interferon-γ can suppress postnatal hematopoiesis in *Fancc*$^{-/-}$ animals, we found no differences in cytokine secretion by unfractionated FL cells to account for the reduction in fetal *Fancc*$^{-/-}$ progenitor cells (Fig 2.1K).[134] Our data reveal other phenotypic differences between fetal FA HSPC and those present in the postnatal bone marrow compartment. Unlike adult FA HSPC, characterized by exaggerated apoptosis and elevated levels of reactive oxygen species (ROS), we found no difference in the frequency of apoptotic events (Annexin V and expression of pro-apoptotic genes *Puma* and *Noxa*) or ROS levels (by carboxy-H_2DCDFA stain) between *Fancc*$^{-/-}$ and WT Sca-1$^+$ AA4.1$^+$ (fetal HSPC marker[100]) FL cells (Fig. 2.2A-C).[135,136] Cell cycle analysis revealed fewer *Fancc*$^{-/-}$ than WT c-Kit$^+$ Sca-1$^+$ (KS) Ki-67^{neg} (G_0) HSPC (Fig. 2.2D), echoing the reported loss in quiescence in murine FA BM KSL cells, and suggested potential G_1 arrest and senescence.[133,137,138] This is further consistent with qRT-PCR studies of sorted FL HSPCs demonstrating *Fancc*$^{-/-}$ upregulation of p21

A

Age	*Fancc*$^{+/+}$	*Fancc*$^{+/-}$	*Fancc*$^{-/-}$	% *Fancc*$^{-/-}$	Total	*p*-value
14.5 dpc	81	87	59	26.0%	227	0.004
P21	202	333	124	18.8%	659	

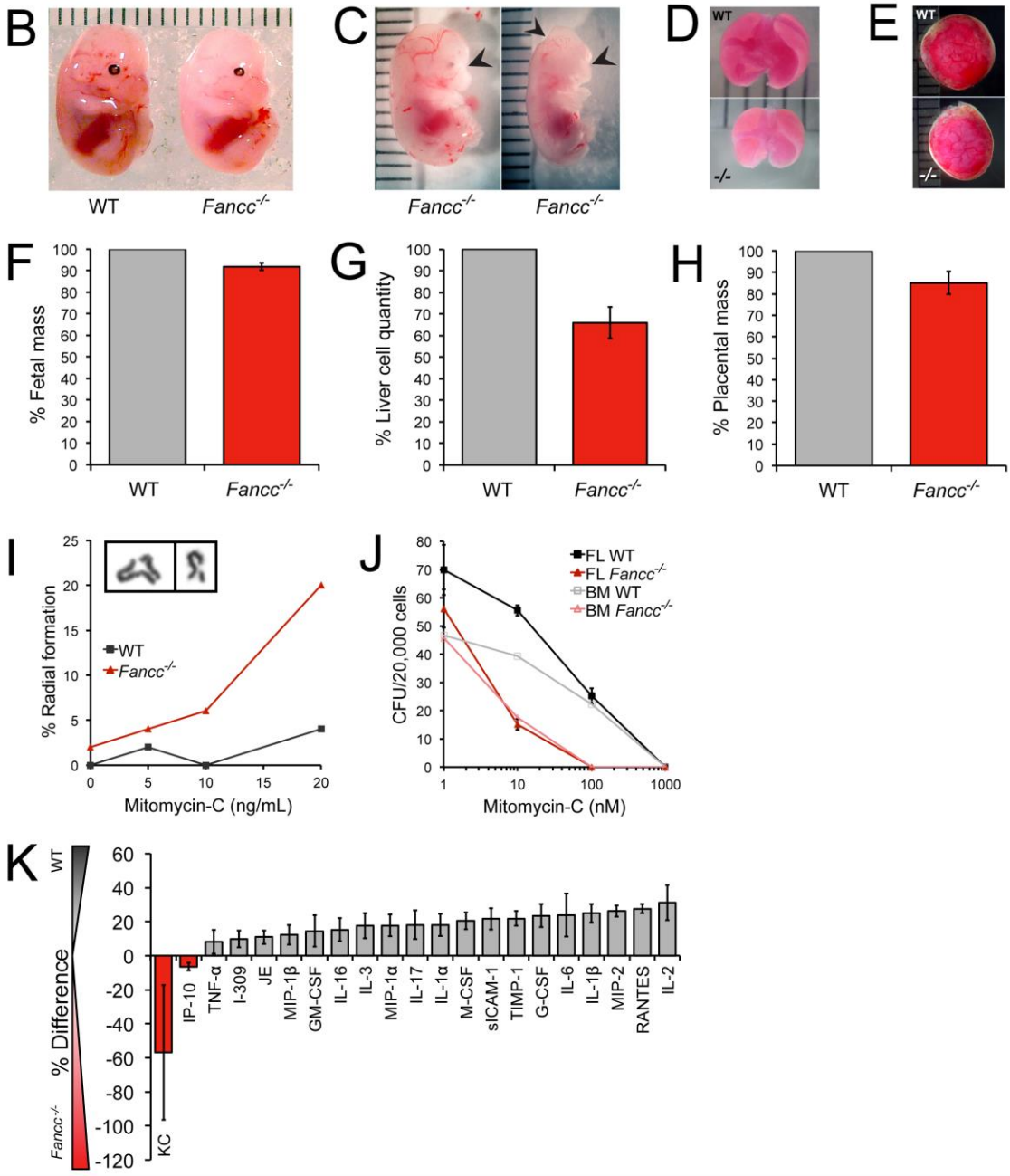


Figure 2.1. Prenatal manifestation of several FA-associated defects. (A) *Fancc*$^{+/-}$ crosses yielded an approximately Mendelian frequency of *Fancc*$^{-/-}$ fetal mice, whereas frequencies of *Fancc*$^{-/-}$ weanlings were sub-Mendelian. χ^2 test comparing fetuses to weanlings yielded a p-value of 0.004. (B) Representative photo of WT and *Fancc*$^{-/-}$ day 14.5 fetal mice. Units are mm. (C) Malformations observed in some *Fancc*$^{-/-}$ fetuses: microphthalmia (left), anophthalmia and anencephaly (right). (D) Representative photos of 14.5 dpc WT and *Fancc*$^{-/-}$ fetal livers, and (E) placentas. (F) Fetal mass

normalized to WT mean within each litter ($n_{litters}$= 5, n_{WT}= 19, $n_{Fancc-/-}$= 14). (G) Cell counts from single cell suspensions of fetal livers normalized to WT mean ($n_{litters}$= 6, n_{WT}= 16, $n_{Fancc-/-}$= 13). (H) Placenta weights (n_{WT}= 25, $n_{Fancc-/-}$= 14), *P*= 0.009. (I) Chromosome radial formation (%) in WT and *Fancc*$^{-/-}$ fetal liver cells cultured in MM-C (n= 50/condition). Inset image of representative radial (left) and break (right). (J) FL and adult BM hematopoietic progenitor colony formation in methylcellulose (CFU-C) (n_{FL}= 3/genotype, n_{BM}= 1/genotype). Unfractionated cells were plated in the presence or absence of MM-C at 2x10^4 per 35 mm dish and colonies of >50 cells were counted on day 12. (K) Mean percent difference in the secretion of select cytokines from *Fancc*$^{-/-}$ versus WT fetal liver-conditioned media assayed by cytokine arrays in 3 independent experiments; the cytokines shown are those that yielded consistent results across all 3, whereas those that did not show differential expression are not shown. Error bars= SEM; BM, bone marrow; FL, fetal liver; MM-C, mitomycin-c; WT, wild type.

and p18, cell cycle regulators involved in hematopoietic failure in FA (Fig. 2.2E).[76,128]

We previously showed that the frequency of immunophenotypically defined HSPC (c-Kit$^+$ Sca-1$^+$ Lin$^-$; KSL) in *Fancc*$^{-/-}$ BM is not substantially different from WT animals.[139] Similarly, we found a minimal decrease in *Fancc*$^{-/-}$ FL AA4.1$^+$ Sca-1$^+$ Lin$^{low/-}$ (ASL) HSPCs (Fig. 2.2G). Deficient repopulation is widely considered a hallmark of compromised FA HSPC function.[127,138] We therefore tested the serial repopulating ability by transplanting 2x10^6 CD45.2 *Fancc*$^{-/-}$ or WT unfractionated FL cells into sublethally irradiated, congenic CD45.1 mice. Peripheral blood donor chimerism five months after transplantation showed an average genotype difference of 24% (*P*= 0.14) (Fig. 2.2F, H). However, during serial repopulation, the average donor chimerism between 1^o and 2^o recipients decreased from 87% to 66% in WT, compared with 66% to 20% for *Fancc*$^{-/-}$ donor cells (21% versus 46% decline, respectively) (Fig. 2.2I). Thus, the average difference in chimerism between genotypes grew from 24% in 1^o recipients to

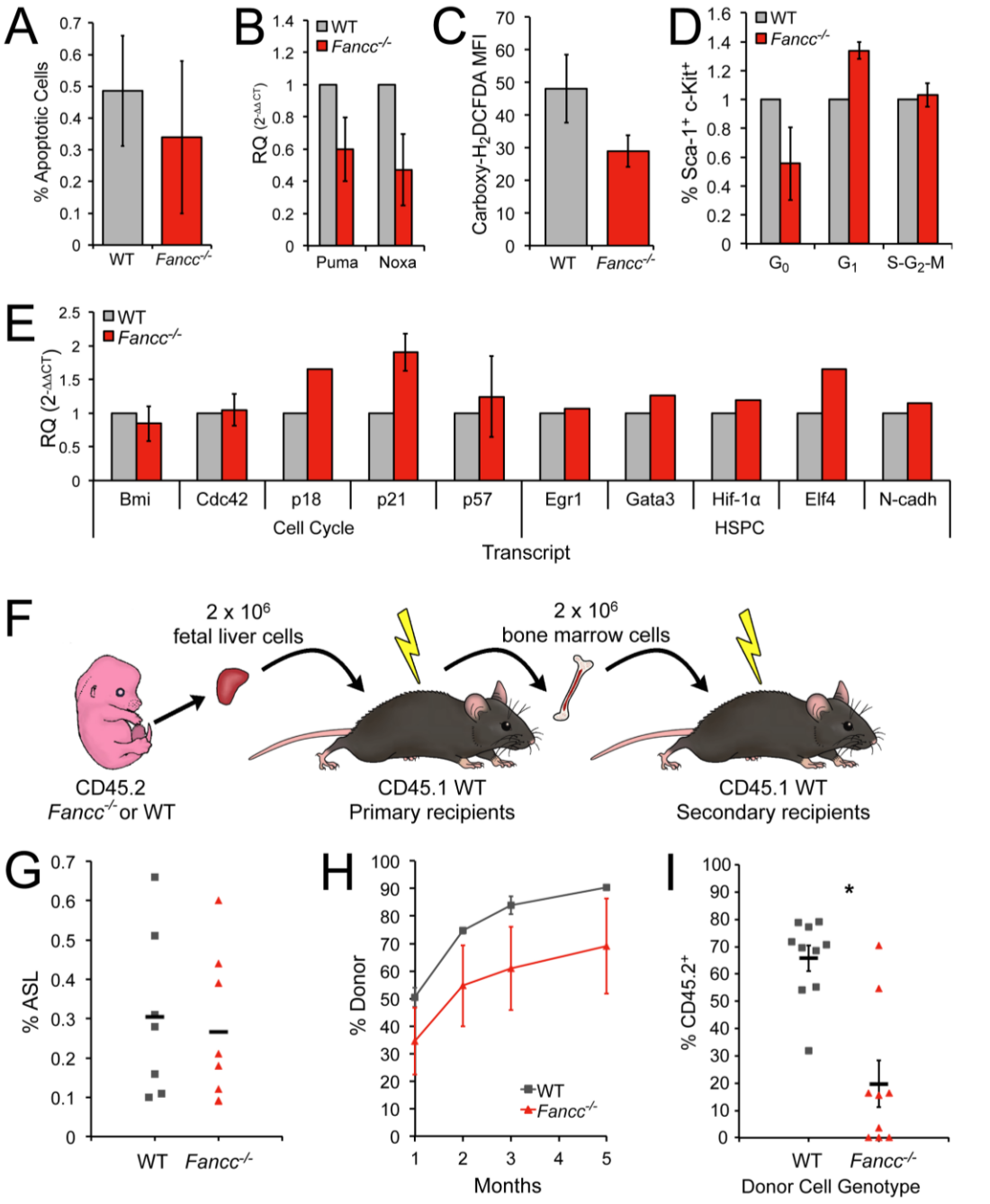


Figure 2.2. Serial repopulating defects and cell cycle abnormalities in *Fancc*$^{-/-}$ fetal liver HSPCs. (A) Annexin V flow cytometry analysis for AA4.1^{+} Sca-1^{+} cells, P= 0.65. (B) QRT-PCR gene expression analysis of *Puma (p53-upregulated modulator of apoptosis)* and *Noxa (Phorbol-12-myristate-13-acetate-induced protein-1),* pro-apoptotic, p53 target genes in *Fancc*$^{-/-}$ and WT FL sorted ASL cells (n = 3/genotype), P_{Puma}= 0.17, P_{Noxa}= 0.13. (C) Reactive oxygen species flow cytometry analysis for AA4.1^{+} Sca-1^{+} cells, shown as median fluorescence intensity, P= 0.17. (D) Cell cycle analysis for c-Kit^{+} Sca-1^{+} cells. *Fancc*$^{-/-}$ average 45% decrease in G_0 cells, 34% increase in G_1, and 3% increase in S-G_2-M cells. Results normalized to WT littermates. (E) QRT-PCR gene expression analysis of transcripts from sorted ASL or LSK cells

from *Fancc*$^{-/-}$ and WT FL. *Bmi1* (BMI1 polycomb ring finger oncogene), Cdc42 (small GTPase cell division control protein42), *p57* (cyclin-dependent kinase inhibitor 1C), EGR1 (early growth response 1), Gata3 (GATA binding protein 3), Hif-1α (hypoxia inducible factor-1α), Elf4 (E74-like factor 4), N-cadh (N-cadherin). (F) Serial transplantation scheme: 2x10^6 unfractionated CD45.2 FL cells were transplanted into conditioned CD45.1 recipients. Five months later, 2x10^6 whole BM cells from 1^o recipients were transplanted into CD45.1 hosts. (G) Percent ASL cells in WT and *Fancc*$^{-/-}$ FL, *P*= 0.36. (H) Donor chimerism (% CD45.2$^+$) of peripheral blood in 1^o transplant ($n_{WT\ FL\ donors}$= 4, $n_{Fancc\text{-}/\text{-}\ FL\ donors}$= 3; $n_{WT\ recipients}$= 7, $n_{Fancc\text{-}/\text{-}\ recipients}$= 5), *P*= 0.13 for 1 month, 0.12 for 2 months, 0.10 for 3 months and 0.14 for 5 months. (I) CD45.2$^+$ chimerism (% FL donor-derived) for 2^o transplantation. Two 1^o recipients served as donors from each cohort ($n_{WT\ recipients}$= 10, $n_{Fancc\text{-}/\text{-}\ recipients}$= 10), *P*= 0.0002. Error bars= SEM; BM, bone marrow; FL, fetal liver; WT, wild type.

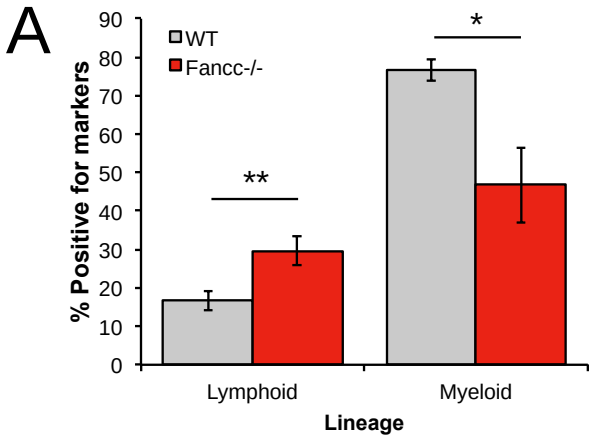


Figure 2.3. Lineage skewing in secondary transplantation of *Fancc*$^{-/-}$ fetal liver-derived bone marrow cells. (A) Bone marrow was harvested from 2° recipients and analyzed for lymphoid (CD3$^+$/B220$^+$) and myeloid (Mac-1$^+$/Gr-1$^+$) lineage marking. T-tests were 2-tailed and asterisks indicate P ≤ 0.05 for *, P ≤ 0.01 for **.

70% in 2^o recipients (*P*= 0.0002 for 2^o transplant). Furthermore, the *Fancc*$^{-/-}$ donor-derived (CD45.2) bone marrow lymphoid (CD3$^+$/B220$^+$) cells were significantly increased, compared to WT controls, and the myeloid (Mac-1$^+$/Gr-1$^+$) population was significantly decreased, indicating that a lineage bias accompanied secondary engraftment (Fig. 2.3A). Altogether, these results demonstrate a substantial loss of serial repopulating potency in *Fancc*$^{-/-}$ fetal liver-derived cells.

Fancc$^{-/-}$ mice do not demonstrate the slowly progressive cytopenias seen in patients, but they have provided valuable insight into FA HSPC biology. Our data reveal that the hematopoietic defect in *Fancc*$^{-/-}$ mice has origins in development without compartmental or temporal restriction to the bone marrow. These observations provide the cohesive developmental context for prior reports that suggested prenatal HSPC loss and have potential implications for ongoing efforts to harness induced pluripotency for FA.[73,75,76] It will be important to delineate how the FA phenotype impacts HSPC pool expansion and seeding of the fetal BM niche during development. Finally, compromised prenatal hematopoiesis may determine the timing of diagnostic procedures, a reassessment of its mechanistic origin, and prompt strategies to preempt hematopoietic failure in FA patients.

Materials and Methods

Mice

Mice were handled in accordance with the OHSU IACUC. FL from 14.5 days post coitum (dpc) timed pregnancies (vaginal plug method) from C57BL/6 *Fancc*$^{+/-}$ (CD45.2) dams were dissected to prepare single-cell suspensions. Fetal tails were genotyped using the Phire© PCR Kit (Thermo Scientific). BM cells from adult animals were harvested as previously described.[139]

Cellular Assays

Cytogenetic analyses were conducted on FL cells by the OHSU Cytogenetics Core Laboratory. Methylcellulose progenitor assays (M3434, StemCell

Technologies) were performed according to manufacturer's instructions.

Cytokine arrays

Unfractionated FL cells were cultured in StemSpan SFEM (StemCell Technologies) at $1.6x10^7$ cells/mL. Conditioned media was applied to Mouse Cytokine Antibody Array, Panel A (R&D Systems) following manufacturer's instructions and imaged on a FujiFilm LAS2000. Pixel density was analyzed using Multi Gauge software (Fujifilm).

Transplantation

Unfractionated FL cells ($2x10^6$) were injected via tail vein into irradiated (530 cGy) congenic CD45.1 recipients. Serial chimerism was analyzed as described.[139] For 2^o transplant, $2x10^6$ whole BM cells from 1^o recipients (n=2/genotype) were transplanted (each) into 5 irradiated (775 cGy) CD45.1 recipients.

Flow cytometry

For c-Kit^+ Sca-1^+ Lin^- (KSL) and AA4.1^+ Sca-1^+ $Lin^{low/-}$ (ASL) HSPC analyses, cells were stained using antibodies; lineage antibodies were against B220, Gr-1, CD3, CD4, CD5, and Ter119 (excluding Mac-1[86]). For cell cycle analysis, we used anti-Ki-67 antibody and Hoechst 33342.[137] Apoptosis assays used Annexin V (BD) and ROS levels were assayed by carboxy-H_2DCFDA staining (Invitrogen). Data were collected on FACSCalibur and BD Influx (BD

Biosciences) and analyzed using FlowJo software (TreeStar).

Quantitative real-time PCR

KSL or ASL FL cells were sorted by FACS on a BD Influx. RNA was purified using RNeasy Mini Kit (Qiagen), and then converted to cDNA using SuperScript III First Strand (Invitrogen). The cDNA was analyzed by real-time PCR (StepOne Plus) using Power SYBR Green Master Mix (both Applied Biosystems). *Fancc*$^{-/-}$ and WT samples were normalized to *Gapdh*, and *Fancc*$^{-/-}$ gene expression was normalized to WT. Relevant transcripts were previously described.[128,140]

Statistical analysis

Numerical results are expressed as mean (+/- standard error of the mean as indicated) and compared using an independent Student's t-test. Genotype distributions were compared by chi-square test.

CHAPTER 3: VALIDATION OF FETAL HEMATOPOIETIC DEFECTS IN FANCONI ANEMIA AND INVOLVEMENT OF PRENATAL DNA DAMAGE

Abstract

Fanconi anemia is a disease of heterogeneous symptoms and severity, in part depending on the specific mutation and the FA gene affected. Our previous studies in the C57BL/6 *Fancc*$^{-/-}$ mouse model revealed a prenatal onset of defects in the hematopoietic system, involving an increased proportion of hematopoietic stem and progenitor (HSPC)-enriched cells in G_1 phase of cell cycle and compromised serial repopulating ability, likely predisposing hematopoietic stem cells to failure later in life. Here, we investigated a different Fanconi anemia complementation group, Fancd2, and the 129Sv *Fancc*$^{-/-}$ mouse, demonstrating that although the fetal hematopoietic phenotypes vary in severity, both models validate our previous hypothesis that the FA HSPC compartment is developmentally constrained. We find evidence of endogenous DNA-damage in *Fancc*$^{-/-}$ and *Fancd2*$^{-/-}$ fetal liver cells enriched for HSPCs and transcriptional activation of the non-homologous end-joining and homologous recombination pathways. Altogether, these results corroborate our hypothesis that hematopoietic defects are present prenatally in FA, and implicate endogenous DNA damage in FA hematopoietic development.

Introduction

The complex array and severity of symptoms seen in Fanconi anemia patients are at least partially manifestations of the different types of genetic lesions and the FA genes involved[141]. While the majority of patients harbor mutations in FA core complex genes, others lose function of the FANCD2-FANCI complex or

downstream factors. Severity of the FA phenotype can also be affected by whether the mutation is hypomorphic or null, the status and expression level of interacting factors. The genomic instability that results from the disease may cause chromosomal rearrangements and additional mutations. In murine models as in patients, complementation group and strain-specific differences have been reported[142,143]. Indeed, most murine models do not replicate the spontaneous postnatal bone marrow failure phenotype seen in patients. Considering these factors, and given that our previous work was done in an FA core complex mutant model (*Fancc*) we were prompted to validate our previous findings in a different FA mutant mouse to determine whether there is a core complex-independent phenotype, as well as in another mouse strain.

Although FA knockout mice do not develop spontaneous bone marrow failure and must be induced with agents such as lipopolysaccharide, they do exhibit several hematological symptoms similar to patients. *Fancc*$^{-/-}$ fetal liver and adult bone marrow have defects in short- and long-term hematopoietic repopulation[71,133,134,138,144-147]. The *Fancd2*$^{-/-}$ mouse is also deficient in repopulating ability and has fewer bone marrow HSPCs[129]. Using C57BL/6 *Fancd2*$^{-/-}$ 14.5 dpc mice, we found that the hematopoietic defects observed in C57BL/6 *Fancc*$^{-/-}$ mice (carrying a disruption in exon 8) are recapitulated, and that this model exhibits an even more severe developmental defect in hematopoiesis. Likewise, the 129Sv (C57BL mixed background) *Fancc*$^{-/-}$ mouse (in which exon 9 is replaced by a transgene) showed prenatal hematopoietic

defects, though somewhat subtler than those seen in the C57BL/6, exon 8 *Fancc*$^{-/-}$ mouse.

FANCC and FANCD2 work in the FA pathway to help coordinate three DNA repair pathways—homologous recombination, mutation translesion synthesis, and nucleotide excision repair[35]—to repair interstrand crosslinks and double-strand breaks, and to guard replication forks from degraded when they stall at a lesion during S-phase[148]. Although FA gene deficiency can also increase apoptosis, both of which can contribute to stem cell attrition, we have not found evidence of this in the 14.5 dpc fetus. Thus, we tested for evidence of increased DNA damage and activation of DNA repair pathways. Our results suggest a role for *in utero* DNA damage in the absence of induction by chemicals or radiation.

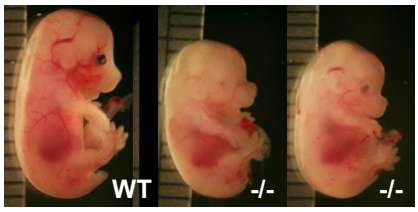
Results

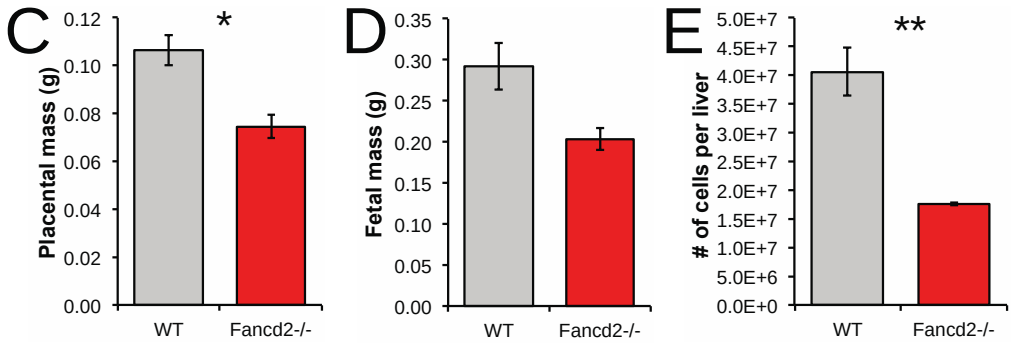
C57BL/6 Fancd2$^{-/-}$ *14.5 dpc fetal mice exhibit a more severe hematopoietic phenotype than C57BL/6 Fancc*$^{-/-}$

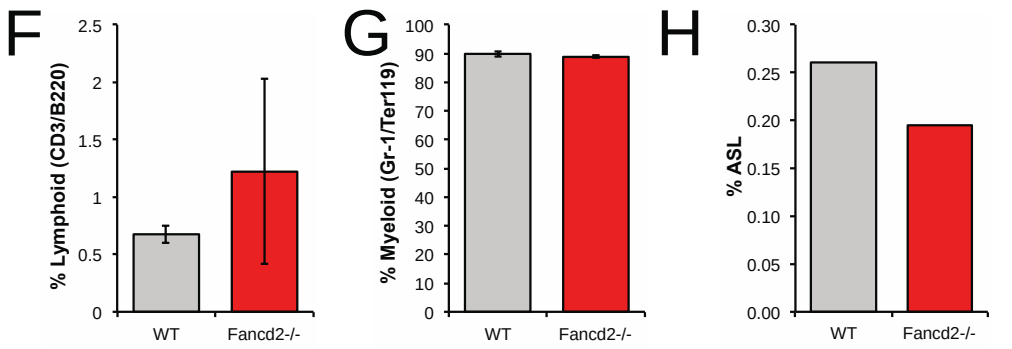
To validate our findings in a different Fanconi anemia complementation group, we examined C57BL/6 Fancd2 14.5 dpc fetal mice[131]. *Fancd2*$^{-/-}$ fetuses occurred at about 13 percent at day 14.5, which is the same as at postnatal day 1, however by day 7, this number dropped to 2.5 percent (Qingshuo Zhang, personal communication), indicating an earlier and more severe gestational lethality than we observed in *Fancc*$^{-/-}$ [147], as well as early postnatal demise (Fig. 3.1A). Occasional *Fancd2*$^{-/-}$ fetuses exhibited microthalmia and anophthalmia

A

	Fancd2 WT	*Fancd2*$^{+/-}$	*Fancd2*$^{-/-}$	Total
n	19	41	9	69
%	28	59	13	100







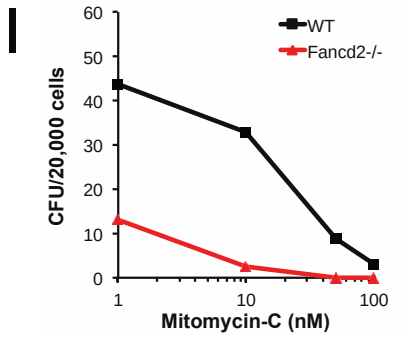


Figure 3.1. *Fancd2*$^{-/-}$ 14.5 dpc fetal mice exhibit a more severe hematopoietic phenotype than *Fancc*$^{-/-}$. (A) Genotype yields of *Fancd2*$^{+/-}$ x *Fancd2*$^{+/-}$ timed pregnancies, harvested at 14.5 dpc. (B) Photos of WT fetuses and two *Fancd2*$^{-/-}$ littermates exhibiting anophthalmia (center) and microphthalmia (right) (marks = mm). (C) Average masses of *Fancd2*$^{-/-}$ and WT placentas and (D) fetuses (n= 5/genotype). (E) Mean cells per fetal liver (n= 3/genotype). (F) Percent of lymphoid and (G) myeloid (n_{WT}= 5, $n_{Fancd2-/-}$= 3), as well as (H) ASL cells in WT vs *Fancd2*$^{-/-}$ fetal livers (n= 2/genotype). (I) Frequency of fetal liver hematopoietic progenitor cells and response to mitomycin-c. Error bars reflect SEM. T-tests were 2-tailed; P ≤ 0.05 for *, P ≤ 0.01 for **.

(Fig. 3.1B), as others have reported in this mouse model postnatally[131]. The average mass of *Fancd2*$^{-/-}$ placentas was 30 percent lower than in WT (Fig. 3.1C). Fetal masses trended downward for *Fancd2*$^{-/-}$ mice, compared to WT, although the difference was nonsignificant (Fig. 3.1D), while *Fancd2*$^{-/-}$ fetal liver cellularity was 57 percent lower than that of WT (Fig. 3.1E). Lymphoid and myeloid populations in the fetal liver were not significantly different between *Fancd2*$^{-/-}$ and WT (Fig. 3.1F, G). The HSPC-enriched, ASL population was reduced by 25 percent in *Fancd2*$^{-/-}$ fetal livers, compared to WT (Fig. 3.1H); integrating this data with total liver cell counts indicates that *Fancd2*$^{-/-}$ fetuses have 68 percent fewer ASL cells than WT animals. Hematopoietic progenitor frequency of *Fancd2*$^{-/-}$ livers, tested by clonogenic methylcellulose assay, was lower as well and showed a characteristic sensitivity to a DNA crosslinking agent (Fig. 3.1I). Altogether, *Fancd2*$^{-/-}$ mice recapitulate our previous findings in *Fancc*$^{-/-}$ and exhibit severe prenatal defects in the fetal liver HSPC compartment.

Hematopoietic defects in Fancd2$^{-/-}$ *adult blood and bone marrow*

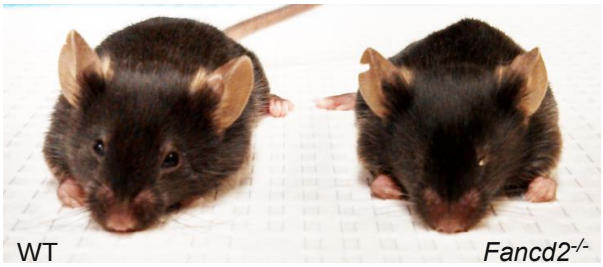
Next, we assessed whether the hematopoietic state of *Fancd2*$^{-/-}$ mice changes with age. Like 14.5 dpc mice, *Fancd2*$^{-/-}$ adults had an increased incidence of microthalmia and anopthalmia (Fig. 3.2A) Complete blood counts (CBC) revealed lymphocytosis and thrombocytopenia in an adult *Fancd2*$^{-/-}$ animal (Fig. 3.2B). While bone marrow lymphoid (CD3^{+}/B220^{+}) populations showed a trend toward being increased in *Fancd2*$^{-/-}$ mice, myeloid (Gr-1^{+}/Mac-1^{+}) populations were the same as WT (Fig. 3.2C, D). Both *Fancd2*$^{-/-}$ hematopoietic progenitor and KSL

frequency were somewhat lower than in WT, and progenitor cells showed a typical sensitivity to mitomycin-c (MM-C) (Fig. 3.2E, F). Transplantation of bone marrow into irradiated WT recipients revealed significantly reduced repopulation capacity for *Fancd2*$^{-/-}$ donor cells at both short- (4 weeks) and long-term (16 weeks) time points (Fig. 3.3A-C, two separate transplants shown). Donor peripheral blood lymphoid cells were significantly reduced in *Fancd2*$^{-/-}$-transplanted animals, while myeloid cells were significantly increased at 4 weeks; by 16 weeks, these populations had normalized (Fig. 3.3D). In sum, the results show substantial repopulation defects in the bone marrow of *Fancd2*$^{-/-}$ mice, and while the deficits in hematopoietic progenitor and immunophenotypic HSPCs were not as severe as in the fetuses, this may be due to a selection process associated with survival to adulthood.

Defects in FA model mice are not strain-specific

We also wanted to ensure that the results in fetal livers of *Fancc* and *Fancd2* mouse models were not C57BL/6 strain-specific. Using 14.5 dpc 129Sv Fancc mice, we found evidence of perinatal lethality (Fig. 3.4A), as seen in C57BL/6 *Fancc*$^{-/-}$ and *Fancd2*$^{-/-}$ mice[147]. The average placental mass of 129Sv *Fancc*$^{-/-}$ fetal mice was approximately equal to that of WT, while fetal masses trended downward (Fig. 3.4B-C) at the current sample size ($n_{Fancc\text{-}/\text{-}}$= 5, n_{WT}= 7). *Fancc*$^{-/-}$ liver cellularity and hematopoietic progenitor frequency also showed a downward trend compared to WT (Fig. 3.4D-E). The lymphoid population was somewhat

A



B

	WBC	*LY*	RBC	PLT
WT	8.7	*7.0*	8.8	789
Fancd2$^{-/-}$	14.2	*11.6*	8.7	517

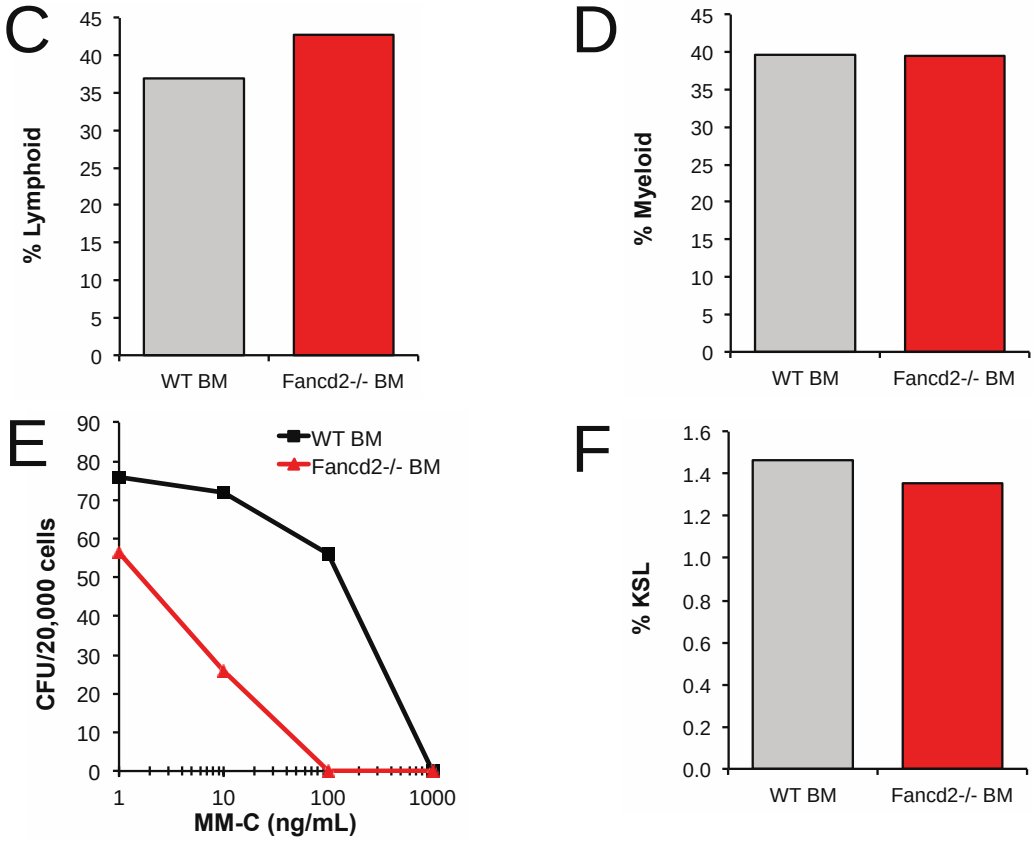


Figure 3.2. Hematopoietic defects in *Fancd2*$^{-/-}$ adult mice. (A) Photo of adult WT mouse and a *Fancd2*$^{-/-}$ animal with anophthalmia. (B) Peripheral blood CBC from adult mice (n= 1/genotype). (C) Lymphoid (CD3^{+}/B220^{+}) and (D) myeloid (Gr-1^{+}/Mac-1^{+}) proportions in bone marrow. (E) Bone marrow hematopoietic progenitor frequency and characteristic *Fancd2*$^{-/-}$ response to MM-C. (F) KSL frequency in bone marrow. For C-F, graphs reflect mean of 2 per genotype.

increased in 129Sv *Fancc*$^{-/-}$ livers, but myeloid cells were present at the same proportion as in WT (Fig. 3.5A-C). Neither the ASL or KSL HSPC population of 129Sv *Fancc*$^{-/-}$ fetal livers were very different from WT for the current sample size (Fig. 3.5D-F). Together, these data further validate our findings in C57BL/6 *Fancc*$^{-/-}$ and *Fancd2*$^{-/-}$ fetal mice.

DNA damage response in Fancc$^{-/-}$ *and Fancd2*$^{-/-}$ *HSPC-enriched fetal liver cells*

Because we have found fewer liver HSPCs in *Fancc*$^{-/-}$ and *Fancd2*$^{-/-}$ fetal mice in the absence of increased apoptosis, we hypothesized that endogenous DNA damage in the fetus would hinder stem cell self-renewal and thus constrain the quantity of HSPCs able to populate the fetal liver. FA cells are characterized by an increased susceptibility to DNA damage, which can be indirectly assayed via detection of proteins such as γH2AX that localize to DNA lesions and form foci. Immunostaining fetal liver HSPC-enriched (Sca-1^{+}) cells, we found that a slightly higher proportion of *Fancc*$^{-/-}$ cells were positive for γH2AX foci (Fig. 3.6A-B). Adult bone marrow *Fancc*$^{-/-}$ Sca-1^{+} cells appeared to have a lower incidence of γH2AX foci (Fig. 3.6C). Next, to test for transcriptional activation of DNA repair pathways, we enriched fetal liver HSPCs by Sca-1 magnetic enrichment or used FACS to sort a more highly enriched population, AA4.1^{+} Sca-1^{+} Lin$^{low/-}$ (ASL), and assayed the expression of several DNA repair genes. *Prkdc*, *Xrcc5*, and *Xrcc6*, which encode DNA-PK, Ku80, and Ku70, respectively, and function together in NHEJ, were all upregulated in Sca-1^{+} *Fancc*$^{-/-}$ and *Fancd2*$^{-/-}$ fetal liver cells, compared to WT (Fig. 3.7A,B). Expression of the homologous recombination

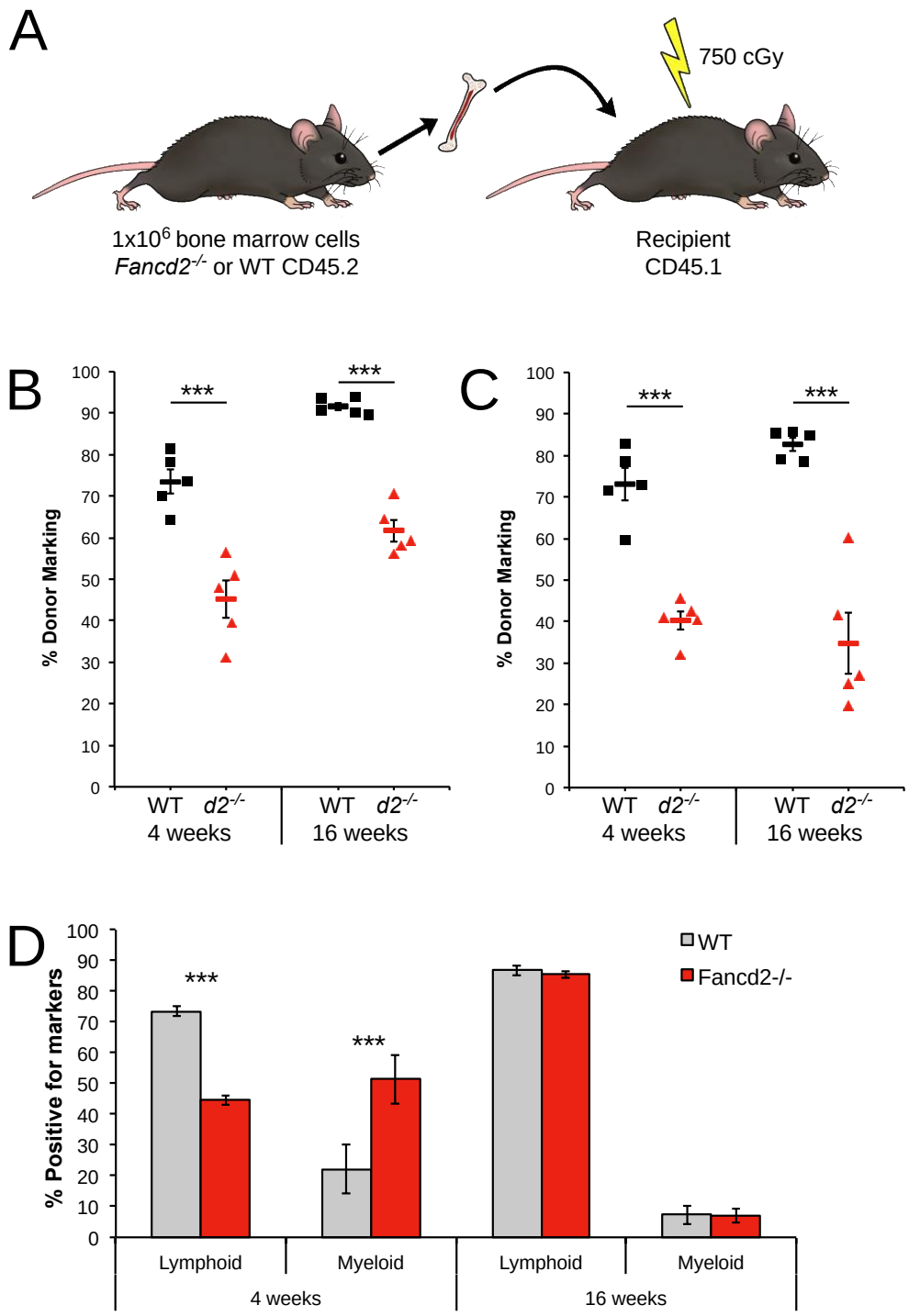


Figure 3.3. Short- and long-term repopulation defects in *Fancd2*$^{-/-}$ adult bone marrow. (A) Schematic for CD45.2 *Fancd2*$^{-/-}$ and WT bone marrow transplantation into irradiated CD45.1 mice. (B) Percent donor chimerism from first transplant and (C) second transplant (each transplant had 1 donor per genotype and 5 recipients per donor). (D) Lymphoid (CD3^{+}/B220^{+}) and myeloid (Gr-1^{+}/Mac-1^{+}) marking in donor cells from peripheral blood of recipients at 4 and 16 weeks post-transplantation (1 transplant experiment, n= 5 per donor genotype). Error bars reflect standard error of the mean. T-tests were 2-tailed and asterisks indicate P ≤ 0.001 for ***.

A

	129Sv WT	129Sv *Fancc*$^{+/-}$	129Sv *Fancc*$^{-/-}$	Total
n	7	22	5	34
%	21	65	15	100

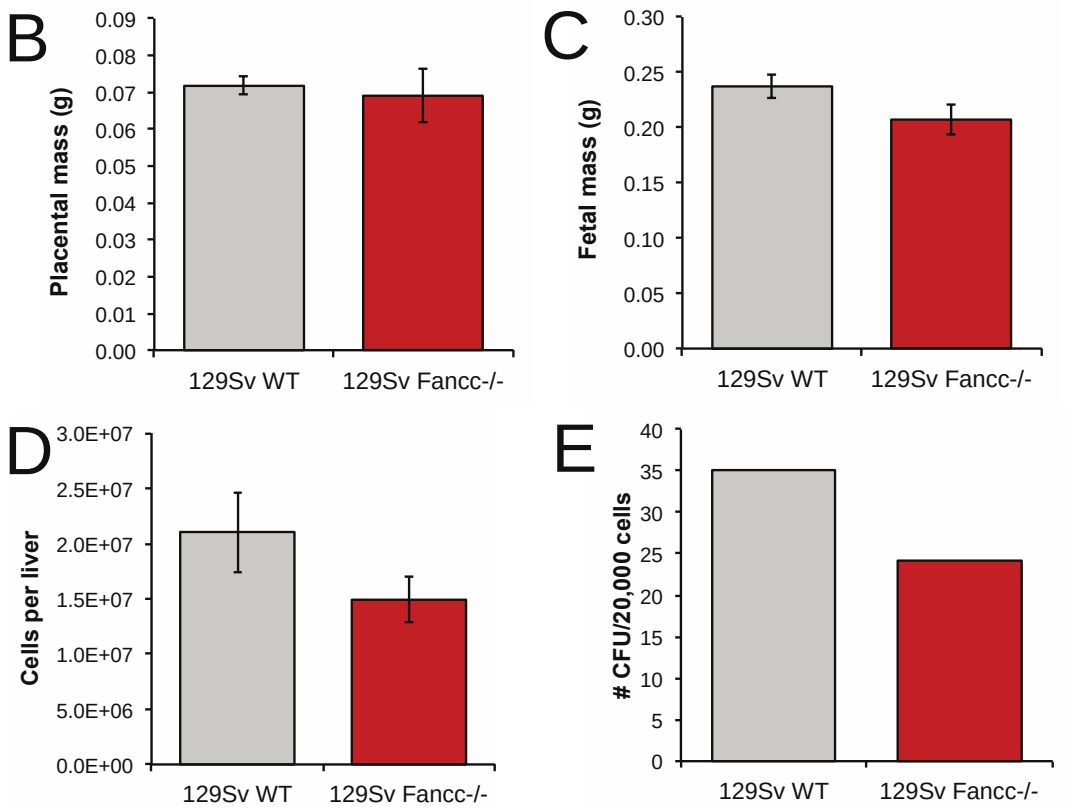


Figure 3.4. Defects in FA model mice are not strain-specific: defects in 129Sv *Fancc*$^{-/-}$ fetal mice. (A) Genotype yields of 129Sv *Fancc*$^{+/-}$ x 129Sv *Fancc*$^{+/-}$ timed pregnancies, harvested at 14.5 dpc. (B) Average masses of 129Sv *Fancc*$^{-/-}$ and WT placentas and (C) fetuses (n_{WT}= 7, $n_{Fancc\text{-}/\text{-}}$= 5). (D) Mean cells per fetal liver, as counted by hemacytometer (n_{WT}= 5, $n_{Fancc\text{-}/\text{-}}$= 5). All error bars reflect SEM. (E) Frequency of fetal liver hematopoietic progenitor cells (n_{WT}= 2, $n_{Fancc\text{-}/\text{-}}$= 2).

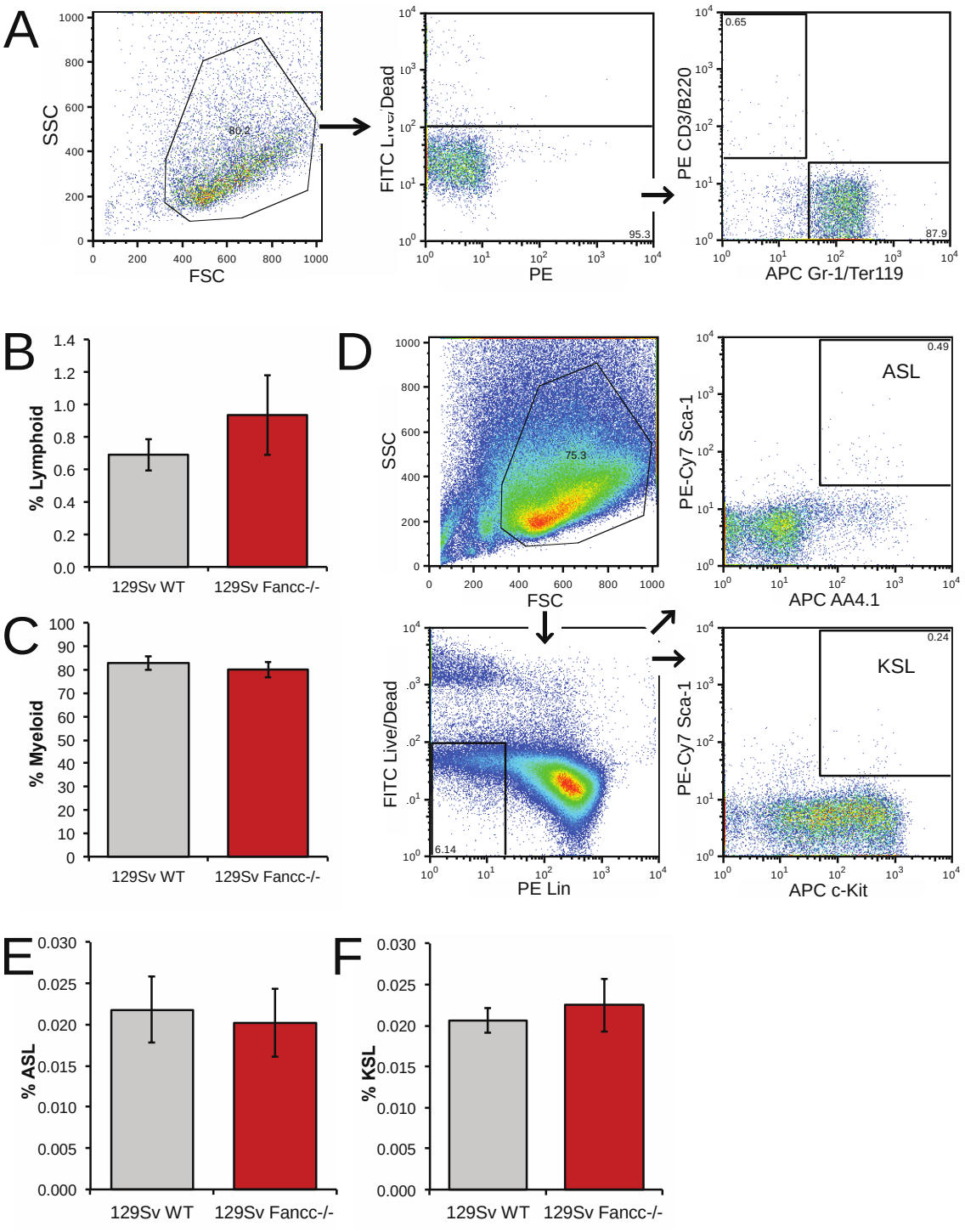


Figure 3.5: Immunophenotyping of 129Sv *Fancc*$^{-/-}$ and WT fetal liver hematopoietic cells. (A) Representative flow cytometry plots in analysis of fetal lymphoid versus myeloid fetal liver cells. (B) Frequency of lymphoid ($CD3^+/B220^+$) and (C) myeloid ($Gr\text{-}1^+/Ter119^+$) cells (n_{WT}= 5, $n_{Fancc\text{-}/\text{-}}$= 3). (D) Representative flow cytometry gating in analysis of ASL and KSL HSPC-enriched cell populations. (E) Proportion of ASL (n_{WT}= 5, $n_{Fancc\text{-}/\text{-}}$= 5) and (F) KSL cells in 129Sv WT vs *Fancc*$^{-/-}$ fetal livers (n_{WT}= 5, $n_{Fancc\text{-}/\text{-}}$= 4). Graphs reflect means and error bars are SEM.

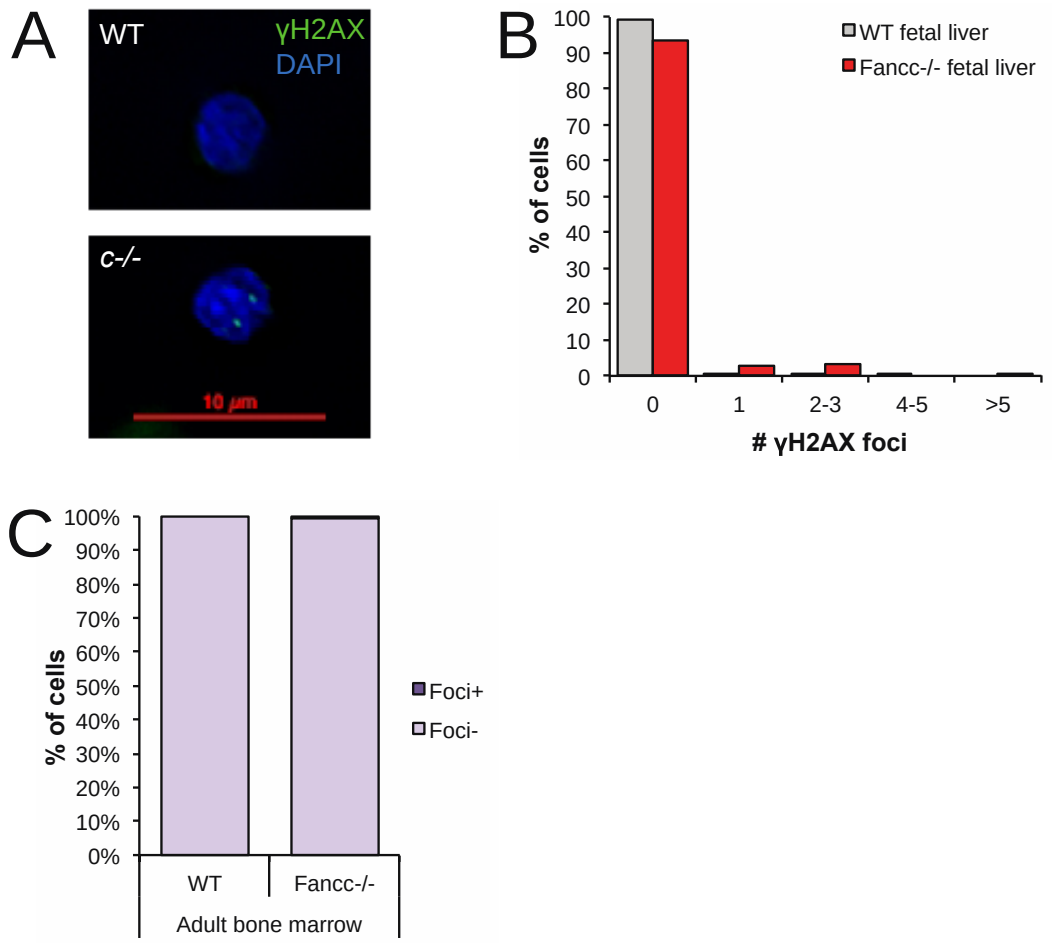


Figure 3.6. Increased γH2AX foci in Sca-1^{+} immunomagnetically enriched *Fancc*$^{-/-}$ fetal liver and adult bone marrow cells. (A) Immunofluorescence microscopy of Sca-1 enriched C57BL/6 WT and *Fancc*$^{-/-}$ fetal liver cells cytospun onto slides and stained for γ H2AX (AF488) and DAPI nuclear stain. (B) Quantification of γH2AX foci in *Fancc*$^{-/-}$ (n= 6 mice) and WT (n= 2 mice) fetal liver cells. (C) Proportions of γH2AX foci in adult bone marrow Sca-1 enriched cells (n_{WT}= 1 mouse, 68 cells; $n_{Fancc-/-}$= 1 mouse, 48 cells).

(HR) gene *Rad51*, but not *Xrcc2*, was also increased in *Fancc*$^{-/-}$ Sca-1^{+} fetal liver cells (Fig. 3.7A). Using more stringent selection, ASL-sorted cells were assayed for expression of *Xrcc2* and *Hmga2*, the latter of which is a non-histone chromatin remodeling factor that regulates self-renewal and inhibits NHEJ; both were upregulated in *Fancc*$^{-/-}$ ASL cells (Fig. 3.7C). Overall, these results suggest DNA repair induction in fetal liver HSPCs in the absence of an intact FA pathway.

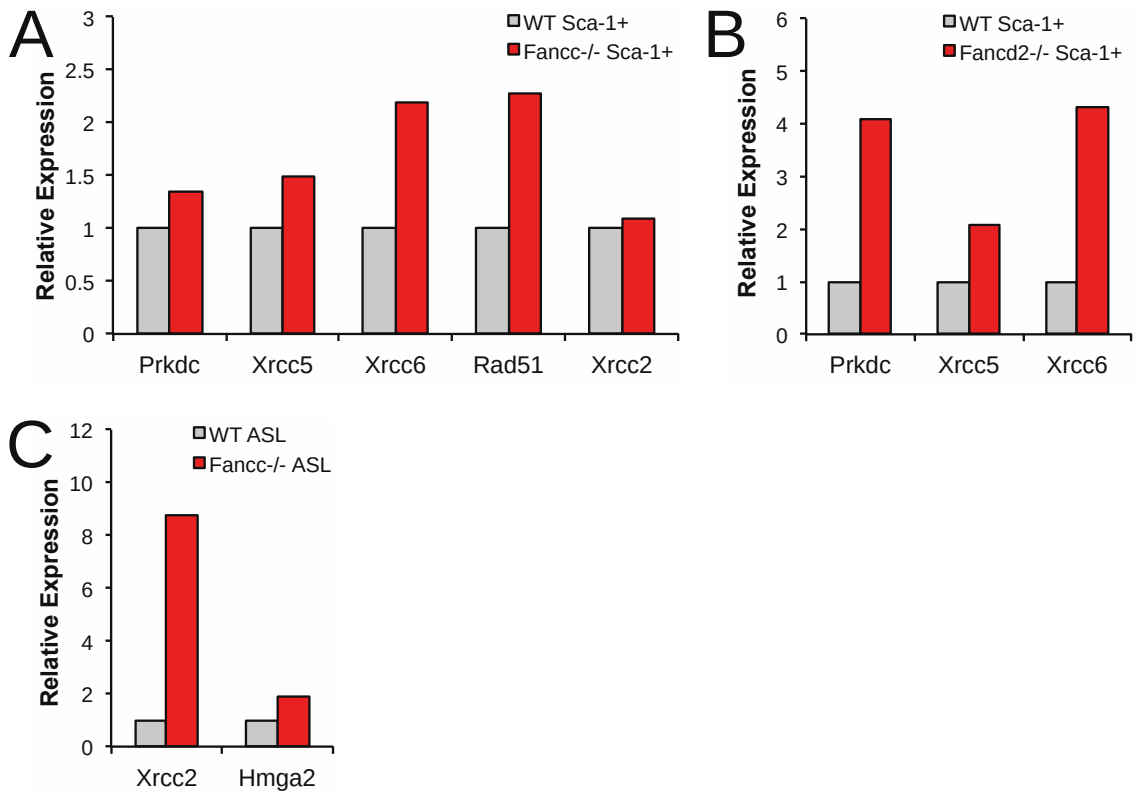


Figure 3.7. Induction of DNA damage response in HSPC-enriched *Fancc*$^{-/-}$ and *Fancd2*$^{-/-}$ fetal liver cells. (A) qRT-PCR analysis of Sca-1 enriched cells from *Fancc*$^{-/-}$ and WT fetal livers (n= 1/genotype) and (B) from *Fancd2*$^{-/-}$ and WT fetal livers (n= 2/genotype). (C) qRT-PCR analysis of *Xrcc2* (n= 1/genotype) and *Hmga2* (n= 2/genotype) in *Fancc*$^{-/-}$ and WT ASL sorted fetal liver cells.

Discussion

Studies in FA patients and mouse models have demonstrated differences in symptoms and severity between different complementation groups and mouse strains[143]. For this reason, we wanted to evaluate a non-core complex FA complementation group and another mouse strain for hematopoietic defects during development. Here, we demonstrate defects in the HSPC compartment in C57BL/6 *Fancd2*$^{-/-}$ and 129Sv (C57BL mixed background) *Fancc*$^{-/-}$ mice, recapitulating the defects we observed in the C57BL/6 *Fancc*$^{-/-}$ model[147].

Our findings in *Fancd2*$^{-/-}$ complement previous reports in postnatal animals[129,131]. *Fancd2*$^{-/-}$ 14.5 dpc fetal mice exhibited the eye defects and perinatal lethality first observed by Houghtaling *et al.*[131] Interestingly, the sub-Mendelian ratios of *Fancd2*$^{-/-}$ fetuses at 14.5 dpc closely matched those of newborn mice, but the reduced frequency at 1 week of age suggests that more than half succumb to postnatal demise. Furthermore, those that reach adulthood have a significantly diminished repopulation capacity, consistent with published reports by Parmar *et al*., using the C57BL/6 model, and Zhang *et al.* in the 129Sv *Fancd2*$^{-/-}$ mouse[129,137]. In the mutant adult animals, though the proportion of HSPC is reduced in the bone marrow and repopulating capacity is significantly impaired, the quantitative defects are less profound than those tested in the fetus, which may be due to selection, in part mediated by survival to adulthood. We investigated *Fancd2*$^{-/-}$ hematopoietic function at an earlier timepoint than has previously been examined, and found evidence of severely stunted growth of the fetal liver and fewer HSPCs in this niche.

Transplantation of the *Fancd2*$^{-/-}$ adult bone marrow revealed a substantial decrease in the proportion donor-derived peripheral blood lymphoid cells and an increase in myeloid cells in primary recipients at 4 weeks post-transplantation, compared to WT-transplanted animals, and these differences normalized by 16 weeks. Many lymphocytes at the 4-week timepoint were passively transferred, and since there was no significant difference by 16 weeks, this suggests that

there may be a microenvironmental defect in the *Fancd2*$^{-/-}$ mouse, which has been largely unexplored in the FA field. In future studies, we will compare the adult data to the repopulating capacity of *Fancd2*$^{-/-}$ fetal liver HSPCs to ascertain whether the defect becomes more or less severe postnatally.

Both the *Fancc*$^{-/-}$ and *Fancd2*$^{-/-}$ mice that we have assayed have been in the C57BL/6 strain, but some heterogeneity across inbred mouse strains has been observed in FA mouse models[132,143]. In the C57BL/6 strain, the number of hematopoietic stem cells increases with age, though repopulation potency is limited compared to younger animals[142,149]. To ensure that our observations were not unique to C57BL/6 mice, we examined the fetal livers of 129Sv (C57BL mixed background) *Fancc*$^{-/-}$ animals. Our findings indicate that FA developmental defects in hematopoiesis are not C57BL/6 strain-specific.

For lifelong hematopoiesis, the survival and maintenance of HSCs is dependent upon DNA repair. Zhang *et al.* reported congenital bone marrow failure in a DNA-dependent protein kinase catalytic subunit (DNA-PKcs) mutant mouse; this enzyme is critical for repair of double strand breaks (DSB) by NHEJ, as well as V(D)J recombination in lymphocytes[150]. Both the FA and HR pathways were affected, resulting in a severe impairment in DSB repair. Mutations in other genes involved in DNA repair, including NHEJ, nucleotide excision repair, interstrand crosslink repair, and DNA damage response have been demonstrated to negatively impact the HSPC compartment as well[151-153]. Although the increase

in γH2AX foci in *Fancc*$^{-/-}$ HSPC-enriched fetal liver cells was modest, evidence of activation in NHEJ and HR from gene expression analysis supports our hypothesis that endogenous DNA damage is increased in *Fancc*$^{-/-}$ and *Fancd2*$^{-/-}$ HSPC-enriched fetal liver cells (limitations in sample size will be addressed in future work). Further studies will ascertain to what extent other DNA repair pathways, including translesion synthesis and nucleotide excision repair, both of which are involved in FA pathway-directed repair, are activated in FA gene-deficient fetal HSPCs. Furthermore, changes in expression must be assayed at the protein level for several genes to rule out posttranscriptional regulation.

Lastly, our gene expression analysis in *Fancc*$^{-/-}$ ASL cells detected increased expression of *Hmga2*, a chromatin-associated protein with DNA-binding domains that participates in transcriptional regulation. *Hmga2* is involved in the high self-renewal activity of fetal HSPCs and is expressed at relatively high levels in these cells, but is translationally downregulated during the transition to a more quiescent, adult phenotype[154]. Its increased expression in *Fancc*$^{-/-}$ ASL cells may be reflective of increased self-renewal activity[154,155], which could compensate for a reduced quantity of HSPCs in the liver, however, further investigation into their replicative activity is warranted. Additionally, studies in cell lines have demonstrated that HMGA2 pauses clearance of γH2AX after induction of double-strand breaks and interferes with NHEJ by delaying the release of the DNA-PKcs complex from double-strand breaks, facilitating an accumulation of DNA damage[156,157]. The absence of an intact FA pathway to coordinate proper double-

strand break repair via HR, with concomitant inhibition of NHEJ by *Hmga2,* may compound the DNA repair defect in these cells. Thus, further research is needed to elucidate whether the increase in *Hmga2* expression in *Fancc*$^{-/-}$ fetal liver HSPCs contributes to genomic instability.

In summary, we have demonstrated that fetal liver hematopoietic defects are not mouse strain- or complementation group-specific, and provide evidence for endogenous DNA damage and increased stimulation of NHEJ and HR pathways in fetal HSPCs. Although much evidence points to DNA damage as the main cause of hematopoietic failure in FA, more research is warranted to definitively demonstrate this and to pinpoint the mechanisms that interfere with HSPC self-renewal in the absence of a functional FA pathway. Further studies to define the mechanisms used to respond to endogenous DNA damage will provide insight into strategies for therapeutic intervention in self-renewing stem cells, which are scarcer in postnatal hematopoietic tissue.

Materials and Methods

Mice

Mice were handled in accordance with the OHSU IACUC. C57BL/6 *Fancd2*$^{+/-}$ and 129Sv (C57BL mixed background) *Fancc*$^{+/-}$ mice were kindly provided by M. Grompe[131,133]. Heterozygotes were mated for timed pregnancy and 14.5 dpc fetuses were harvested and processed as described previously[147]. For Fancd2 adult bone marrow assays, 8-9 week-old mice were anesthetized with isoflurane

and peripheral blood was drawn from the retroorbital sinus. Mice were sacrificed and bone marrow was harvested for use in subsequent assays.

Cellular Assays

Methylcellulose progenitor assays were performed as described previously[147].

Flow cytometry

Fetal liver cells were stained as described previously[147]. Peripheral blood from transplant recipients was stained with CD45.1-FITC, CD45.2-PE-Cy7, CD3-PE, B220-PE, Gr-1-APC, and Mac-1-APC (BD Biosciences) for chimerism and lymphoid versus myeloid analysis. Events were acquired on a FACSCalibur and LSR II (BD Biosciences) and analyzed using FlowJo software (TreeStar).

Transplantation

Unfractionated *Fancd2*$^{-/-}$ and WT bone marrow cells ($1x10^6$) were injected via tail vein into irradiated (750 cGy) congenic CD45.1/.2 recipients. Blood draws were performed at 4 and 16 weeks.

Complete blood counts

Complete blood counts were performed on a HemaVet 950FS (Drew Scientific, Inc.).

Immunofluorescence

Fetal liver or adult bone marrow cells were immunomagnetically enriched with Sca-1-PE antibody (BD Biosciences) and the PE Positive Selection Kit (StemCell Technologies). Cells were fixed in 2% PFA and then cytospun (Shandon Cytospin, Thermo Scientific). Cytospins were stained with anti-γH2AX antibody (Upstate/Millipore), followed by AF488 donkey anti-mouse secondary antibody (Molecular Probes) and DAPI and imaged on an ApoTome with ZenBlue software (Zeiss).

Quantitative real-time PCR

Sca-1^{+} cells were enriched (as above). Fetal liver cells were stained with lineage antibodies (CD3, CD4, CD5, B220, Gr-1, Ter119), Sca-1, and AA4.1 (BD) for ASL sort, and propidium iodide, and sorted into Qiazol (Qiagen) by FACS on a BD Influx. RNA was purified using the RNeasy Micro Kit (Qiagen), and then converted to cDNA using SuperScript III First Strand (Invitrogen). The cDNA was analyzed by quantitative real-time PCR (StepOne Plus, Applied Biosystems) using Power SYBR Green Master Mix (Applied Biosystems). *Fancc*$^{-/-}$ and WT samples were normalized to *ß*-actin, and *Fancc*$^{-/-}$ gene expression was normalized to WT. Relevant transcripts were previously described[52].

Statistical analysis

Numerical results are expressed as mean (+/- standard error of the mean as indicated) and compared using an independent Student's t-test.

CHAPTER 4: NUTRITIONAL PROGRAMMING OF THE HEMATOPOIETIC STEM AND PROGENITOR CELL POOL BY HIGH-FAT DIET AND MATERNAL OBESITY

Abstract

The developing fetus is highly susceptible to metabolic cues, and evidence for *in utero* programming of adult disease has been found in several organ systems. Here, we show that maternal obesity, especially due to gestational high-fat diet, impairs development of the murine fetal hematopoietic stem and progenitor (HSPC) cell pool, leading to a decrease in immunophenotypic HSPCs, a reduction in short-term repopulation after transplantation, and an expansion of the lymphoid compartment. A high-fat diet in the absence of obesity significantly elevated fetal lymphoid and myeloid cells. These outcomes were only partly ameliorated by diet adjustments during breeding and gestation for obese dams. Mechanistically, our studies of fetal hematopoietic tissue revealed perturbations in expression of several critical genes involved in DNA damage response, including *Ogg1*, which functions in base excision repair, along with *Hmga2*, a non-histone architectural factor involved in double-strand break signaling, hematopoietic stem cell self-renewal, obesity, and type 2 diabetes. Our data identify the fetal hematopoietic stem cell pool as a previously unrecognized target of *in utero* metabolic injury that elicits a DNA repair response. Genomic integrity is critical to HSPC expansion and the data suggest that pregnancy constitutes a period of previously unrecognized susceptibility to metabolic injury in the fetal HSPC pool.

Introduction

The rise in obesity rates over the past several decades coincides with an increase in the prevalence of a range of diseases, not only in obese individuals, but progressively more in their offspring.[158-162] Little is known about the mechanisms underlying the fetal programming of postnatal disease, but an association with maternal obesity as well as overnutrition has been noted.[163] Even though unhealthful maternal dietary manipulation generally results in insidious disease onset without gross organ compromise during infancy, compelling evidence indicates the long-term (*in utero*) metabolic programming of postnatal neurological, cardiovascular and endocrine complications[12,13,164,165]. These and other studies reveal prenatal development as a period of global vulnerability for metabolic injury and organ dysfunction, pointing to a crucial role for fetal programming by maternal high-fat diet (HFD)[119,166]. Recent evidence also demonstrates fetal programming of the immune system, with increased disease susceptibility later in life (as reviewed in [17,167,168]). Obesity and excess dietary fat induce alterations in immune cell numbers, impair immune responses, and promote inflammation[109,169,170]. Mice receiving HFD have altered leukocyte subpopulations, thymic enlargement, and show changes in chemokine signaling[171,172]. This is consistent with maturation of the immune system during development, when adaptive immunity and central and peripheral tolerance first arise *in utero*, and may point to the origin of these permanent adaptations.[173]

Hematopoietic stem and progenitor cells (HSPC) give rise to the complete spectrum of blood and immune cells. Their self-renewal and differentiation are responsive to metabolic signaling cues, as they heavily rely on fatty acid oxidation and glycolysis[107,108,174,175]. HSPC maintenance and injury response are critically dependent on intact DNA repair[52,105]. Indeed human cells are known to lose competitive repopulation capacity as a result of accruing DNA damage[176]. Much less is known about the HSPC injury response during development, when the fetal liver serves almost exclusively as a hematopoietic organ for the more than 40-fold net expansion of the HSPC pool[83,177].

We recently showed that maternal overnutrition severely impacts fetal body composition and disproportionately stunts neonatal development[177]. Here, we hypothesized that fetal metabolic programming via HFD would compromise HSPC pool development. Using a validated model of maternal dietary manipulation, we report that maternal obesity, with and without *in utero* exposure to HFD, compromises fetal HSPC number and function, revealing lymphoid skewing of fetal hematopoiesis and deficits in short-term repopulation after competitive transplantation. Additionally, we demonstrate alterations in transcriptional activity among candidate genes involved in DNA repair, which is critical to preserving HSPC function. Our observations of diet- and obesity-induced compromise in the fetal HSPC pool provide compelling evidence for metabolic programming of the hematopoietic system.

Results

We used three dietary strategies to test the effect of a HFD, a HFD with maternal obesity, and obesity without HFD on fetal hematopoietic development at 14.5 dpc (days post coitum). We monitored weight gain from 0.5 to 14.5 days of gestation in dams for all three sets. A representative image of nonpregnant control and chronic HFD female mice is shown in Fig. 4.1A. Weights were significantly elevated in chronic HFD dams, compared to controls, for both 0.5 and 14.5 dpc (Fig. 41B). The percent weight gain of dams during gestation was also calculated in order to compare different treatments; acute HFD dams gained significantly more than obese dams, as they began gestation with a lower body mass (Fig. 4.1C).

Acute HFD leads to expansion of the lymphoid compartment

To investigate the effect of HFD without preexisting obesity on fetal hematopoietic programming, we fed HFD or control diet to a cohort of female mice for 2 weeks and during subsequent mating and gestation, harvesting fetuses at 14.5 dpc. Two weeks was chosen in order to allow the animals to adjust to the new diet, and is a short enough timepoint in order to prevent them from becoming obese. Acute HFD litter sizes were larger than controls (Fig. 4.2A, $P = 0.02$). While HFD fetuses had approximately equivalent placenta size, body mass and liver cellularity were significantly increased, compared to controls (Fig. 4.2B-D). Despite having a higher liver cell content, the hematopoietic progenitor cell (HPC) frequency in acute HFD livers was somewhat lower than in controls

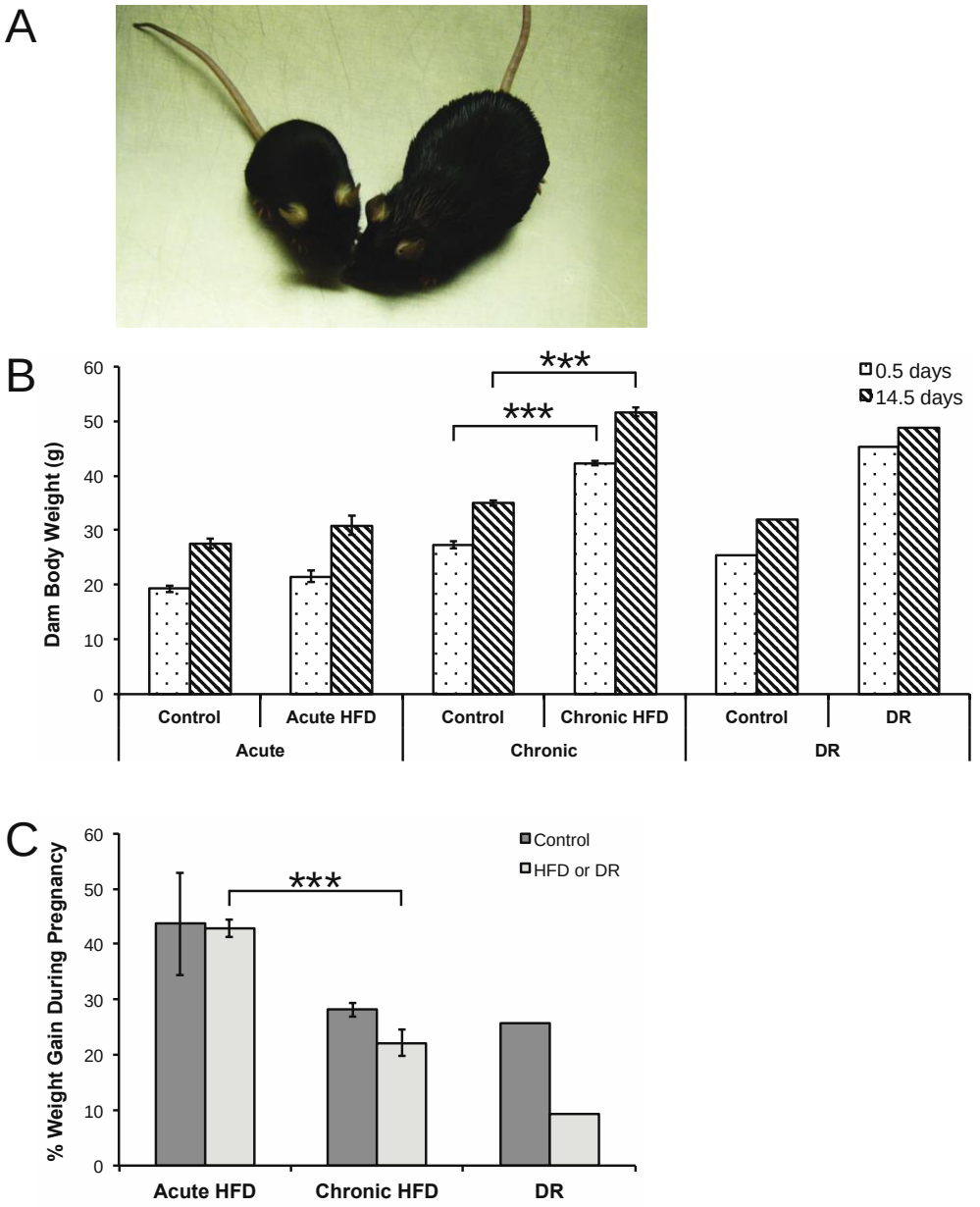


Figure 4.1: Maternal weight gain during pregnancy. (A) Representative photo of a non-pregnant control diet-fed (left) versus a chronic HFD-fed (right) female mouse. (B) Weights for dams on day 0.5 and 14.5 of gestation (acute HFD $n_{Control}$ = 3, n_{HFD} = 5; chronic HFD $n_{Control}$ = 4, n_{HFD} = 5; diet reversal $n_{Control}$ = 2, n_{DR} = 2). 2-tailed Student's T-test: 0.5 dpc acute HFD to control P = 0.17, for 14.5 dpc P = 0.22; 0.5 dpc chronic HFD to control P < 0.001, for 14.5 dpc P < 0.001. (C) Percent weight gain during pregnancy (expressed as percent of weight by 14.5 dpc to starting weight on gestational day 0.5) was calculated in order to compare dam weight gain across experiments, as the three experimental setups each contained dams at different ages (acute HFD $n_{Control}$ = 3, n_{HFD} = 5, P = 0.92; chronic HFD $n_{Control}$ = 4, n_{HFD} = 5, P = 0.08; DR $n_{Control}$ = 2, n_{DR} = 2). Comparing across experiments, for acute control vs. chronic control, P = 0.11, for acute HFD vs chronic HFD, P < 0.001. Error bars are standard error of the mean. T-tests were 2-tailed and asterisks indicate P ≤ 0.05 for *, P ≤ 0.01 for **, and P ≤ 0.001 for *** (T-test was not used for DR group because n < 3).

A

Cohort	# Litters	Mean Litter Size	# Fetuses
Control	6	6.7	40
Acute HFD	5	8.6	43

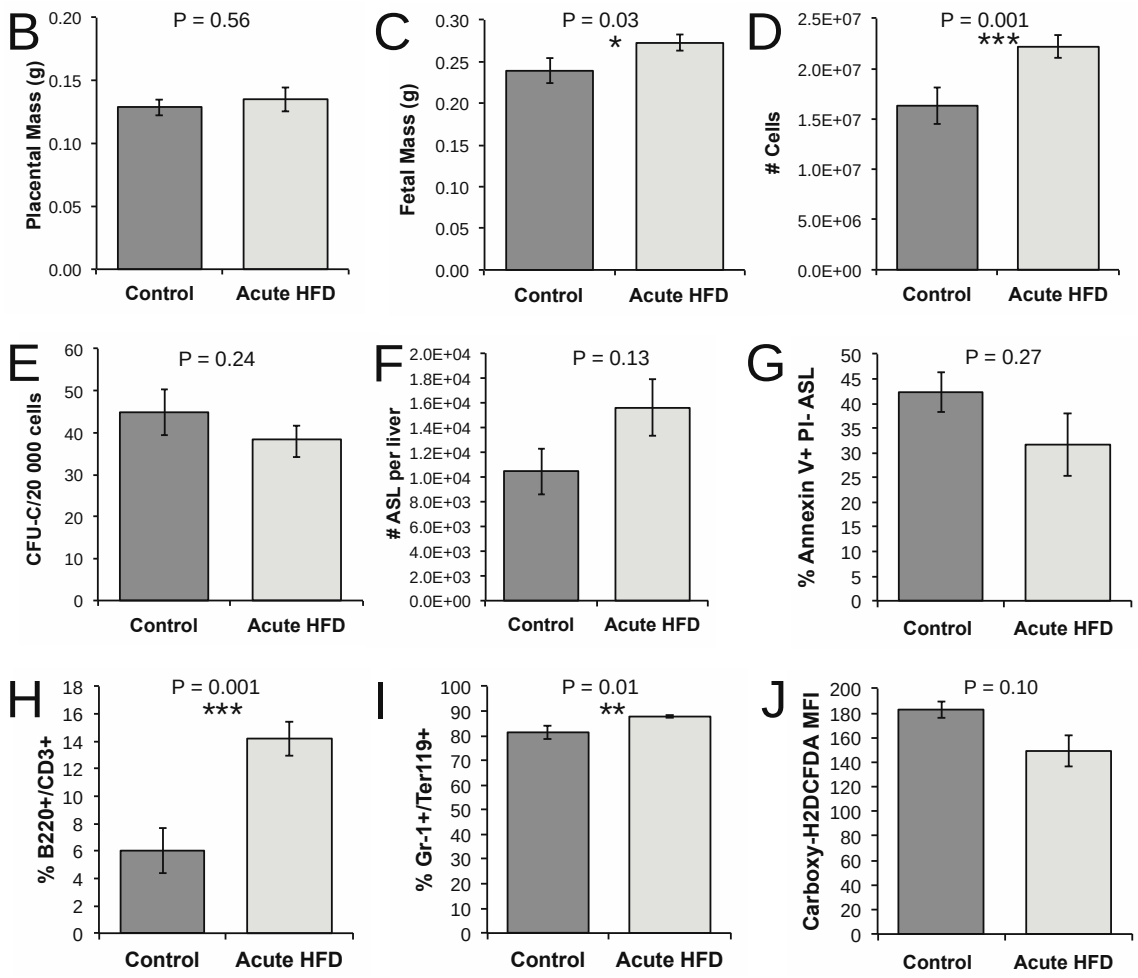


Figure 4.2: Acute HFD induces a significant increase in fetal liver cellularity, including lymphoid and myeloid cells. (A) Litter data. (B) Placental weights. (C) Fetal weights. (D) Cells per liver. For B-D, $n_{Control}$ = 40, n_{HFD} =43. (E) Hematopoietic progenitor cell frequency as colony forming units per 20,000 cells, $n_{Control}$ = 10, n_{HFD} = 8. (F) The number of ASL HSPCs per liver was calculated from flow cytometry and liver cell counts (ASL per liver: $n_{Control}$ = 6, n_{HFD} = 9). (G) Apoptotic ASL fetal liver cells. (H) Percent lymphoid ($B220^+$ or $CD3^+$) cells in fetal liver. (I) Percent myeloid ($Gr\text{-}1^+$ or $Ter119^+$) cells in fetal liver. (J) Median fluorescence intensity of the reactive oxygen species detector carboxy-H_2DCFDA in ASL fetal liver cells. Error bars reflect standard error of the mean T-tests were 2-tailed and asterisks indicate P ≤ 0.05 for *, P ≤ 0.01 for **, and P ≤ 0.001 for ***.

A

Cohort	# Litters	Mean Litter Size	# Fetuses
Control	4	8	32
Chronic HFD	5	6.4	27

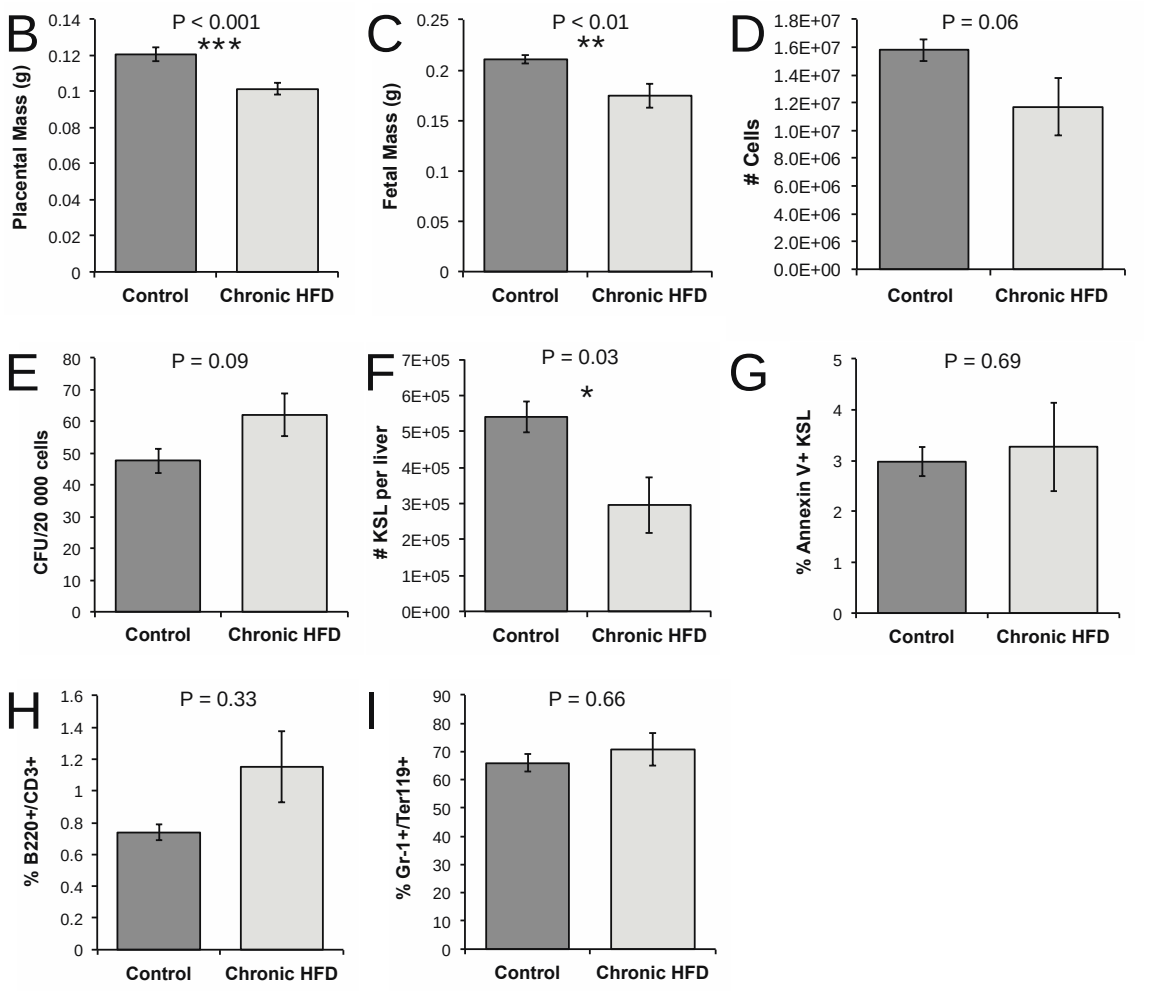


Figure 4.3: Maternal obesity with HFD causes a significant decrease in fetal liver KSL cells. (A) Litter data. (B) Placental weights ($n_{Control}$ = 32, n_{HFD} =27). (C) Fetal weights ($n_{Control}$= 32, n_{HFD}=27). (D) Cells per liver ($n_{Control}$ = 30, n_{HFD} =27). (E) Hematopoietic progenitor cell frequency as colony forming units per 20,000 cells. (F) Number of c-Kit^{+} Sca-1^{+} Lin$^{low/-}$ cells in HFD vs control-diet fetal livers ($n_{Control}$ = 9, n_{HFD} = 15). (G) Annexin V^{+} KSL fetal liver cells (n = 3 per cohort). (H) Percent lymphoid (B220^{+} or CD3^{+}) cells in fetal liver. (I) Percent myeloid (Gr-1^{+} or Ter119^{+}) cells in fetal liver. Error bars reflect standard error of the mean and T-tests were 2-tailed and asterisks indicate P ≤ 0.05 for *, P ≤ 0.01 for **, and P ≤ 0.001 for ***.

A

Cohort	# Litters	Mean Litter Size	# Fetuses
Control	2	5.3	16
Diet Reversal	3	6.5	13

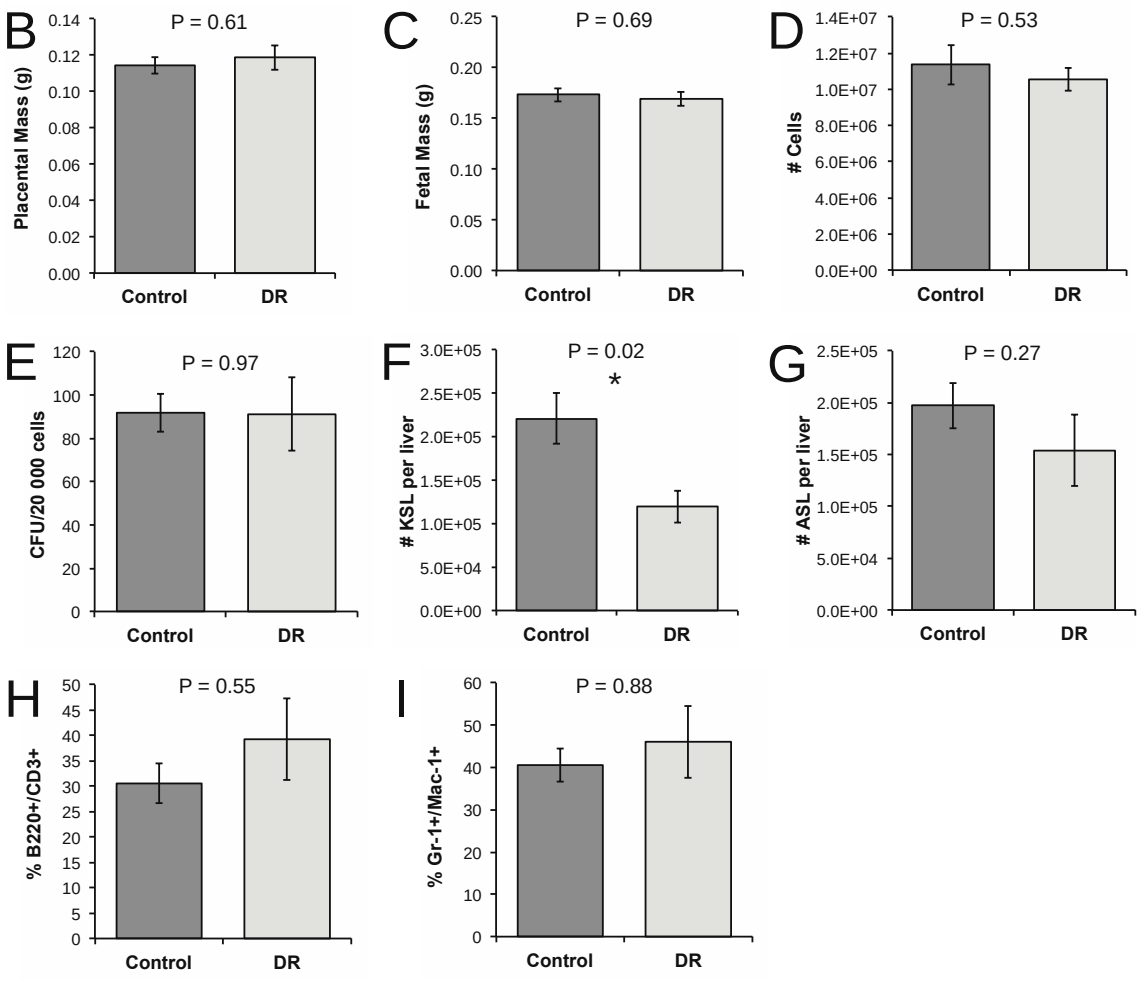


Figure 4.4: Maternal diet reversal ameliorates some fetal defects, but not the diminished KSL content. (A) Litter data. (B) Placental weights. (C) Fetal weights. (D) Cells per liver. For B-D, $n_{Control}$ = 16, n_{DR} =13. (E) Hematopoietic progenitor frequency, $n_{Control}$ = 3, n_{DR} =3. (F) Percent KSL and (G) ASL cells in fetal liver ($n_{Control}$ = 13, n_{DR} = 9). (H) Percent lymphoid cells and (I) myeloid cells in fetal liver and asterisk indicates P ≤ 0.05. DR = diet reversal.

(Fig. 4.2E), though the number of AA4.1^{+} Sca-1^{+} Lin$^{low/-}$ (ASL) fetal HSPCs was increased (Fig. 4.2F). However, when the total liver cell counts were not considered, the proportion of ASL cells in acute HFD livers was lowered by 71 percent (P = 0.001) (data not shown). Annexin V staining suggested that apoptosis did not account for the relative decrease of acute HFD fetal liver ASL cells at 14.5 dpc (Fig. 4.2G). In comparison to the control cohort, acute HFD fetal livers had a 58% increase in lymphoid cells (P = 0.002) but no signficant increase in myeloid cells (P = 0.01) (Fig. 4.2H-I). Lastly, since increased fat peroxidation has been shown to increase levels of reactive oxygen species (ROS), we used flow cytometry to measure relative ROS in fetal liver cells with the fluorescent detection dye, carboxy-H_2DCFDA, but did not detect a significant difference between acute HFD and control livers (Fig. 4.2J). In sum, acute HFD fetuses exhibit increased body mass and liver cellularity, a stunted HSPC pool, and a larger proportion of lymphoid and myeloid cells in the fetal liver.

Chronic HFD reduces overall fetal liver cellularity and constrains the HSPC pool

Next, we wanted to examine the long-term effect of a HFD on hematopoietic development. In dams fed a HFD for ≥6 months, the average litter size at day 14.5 of gestation was not significantly different from litters of dams fed a control diet (P = 0.1, Fig. 4.3A). Chronic HFD 14.5 dpc placentas were an average of 16% smaller than those of controls (Fig. 4.3B), while fetuses were 17% smaller (Fig. 4.3C). The cellularity of chronic HFD livers trended downward, compared to controls (Fig. 4.3D), and the frequency of liver HPCs was not significantly

increased (Fig. 4.3E). Chronic HFD livers contained significantly fewer c-Kit^{+} Sca-1^{+} Lin$^{low/-}$ (KSL) HSPCs than controls, totaling a 45% decrease (Fig. 4.3F). To investigate whether some of the KSL cell loss is attributed to apoptosis at this particular time of development, we stained them with Annexin V and analyzed them by flow cytometry, but did not find a difference between the two cohorts (Fig. 4.3G). The B220^{+}/CD3^{+} lymphoid populations were moderately increased in chronic HFD fetal livers, but the Gr-1^{+}/Ter119^{+} myeloid populations were similar to controls (Fig. 4.3H-I). Overall, offspring of chronic HFD-fed, obese dams were significantly smaller in size, with a disproportionate decrease in liver cellularity and KSL cells.

Diet reversal during pregnancy ameliorates liver cellularity but not HSPC population

We next tested the effect of obesity without HFD by feeding former chronic HFD-fed, obese dams a control diet through mating and gestation, and then assaying fetal mice at 14.5 dpc. We harvested 3 diet reversal (DR) litters and 2 control litters (Fig. 4.4A). In contrast to the chronic and acute HFD cohorts, diet reversal (DR) offspring showed no significant difference in their placental or fetal masses, compared to controls (Fig. 4.4B-C), nor did they exhibit significant differences in fetal liver cellularity and HPC frequency (Fig. 4.4D-E). Calculation of HSPCs per liver revealed a 46% loss in KSL cells and a downward trend in ASL cell content in DR offspring (Fig. 4.4F-G). There were no significant increases in lymphoid and myeloid populations in DR livers (Fig. 4.4H-I). Overall, DR ameliorated the

effects of chronic maternal HFD on placental and fetal mass as well as whole fetal liver cellularity, despite persistence of maternal obesity, but did not improve KSL numbers.

Reduction in repopulating capacity of fetal liver HSPC after chronic maternal HFD exposure

Due to HSPC deficits in chronic HFD fetal livers, we wanted to definitively test the function of this population by transplantation. Unfractionated chronic HFD and control fetal liver cells were mixed with an equivalent number of competing, wild-type adult bone marrow cells and injected into CD45.1 adult mice conditioned with 750 cGy (Fig. 4.5A). At 4 weeks post-transplant, peripheral blood was drawn; donor chimerism from chronic HFD fetal liver-derived cells showed a downward trend compared to that of control fetal liver-derived cells. By 28 weeks post-transplant, donor chimerism in HFD-transplanted animals was not significantly higher than that of the control group (Fig. 4.5B).

To determine whether a similar trend is present in offspring from obese dams switched to control diet, we transplanted DR and control fetal liver cells using the same method as above, and measured peripheral blood chimerism at 4 and 18 weeks. Mice transplanted with DR fetal liver cells showed improved short-term donor chimerism relative to the chronic HFD transplant, but were still somewhat lower than controls, whereas repopulation at 18 weeks trended lower for DR donor cells than controls (Fig. 4.5C). Together, the data illustrate the broad

impact of HFD on immunophenotypically defined stem and progenitor cell population with compromised repopulating capacity that is followed by rebound gains in chimerism that coincide with expansion of the lymphoid compartment.

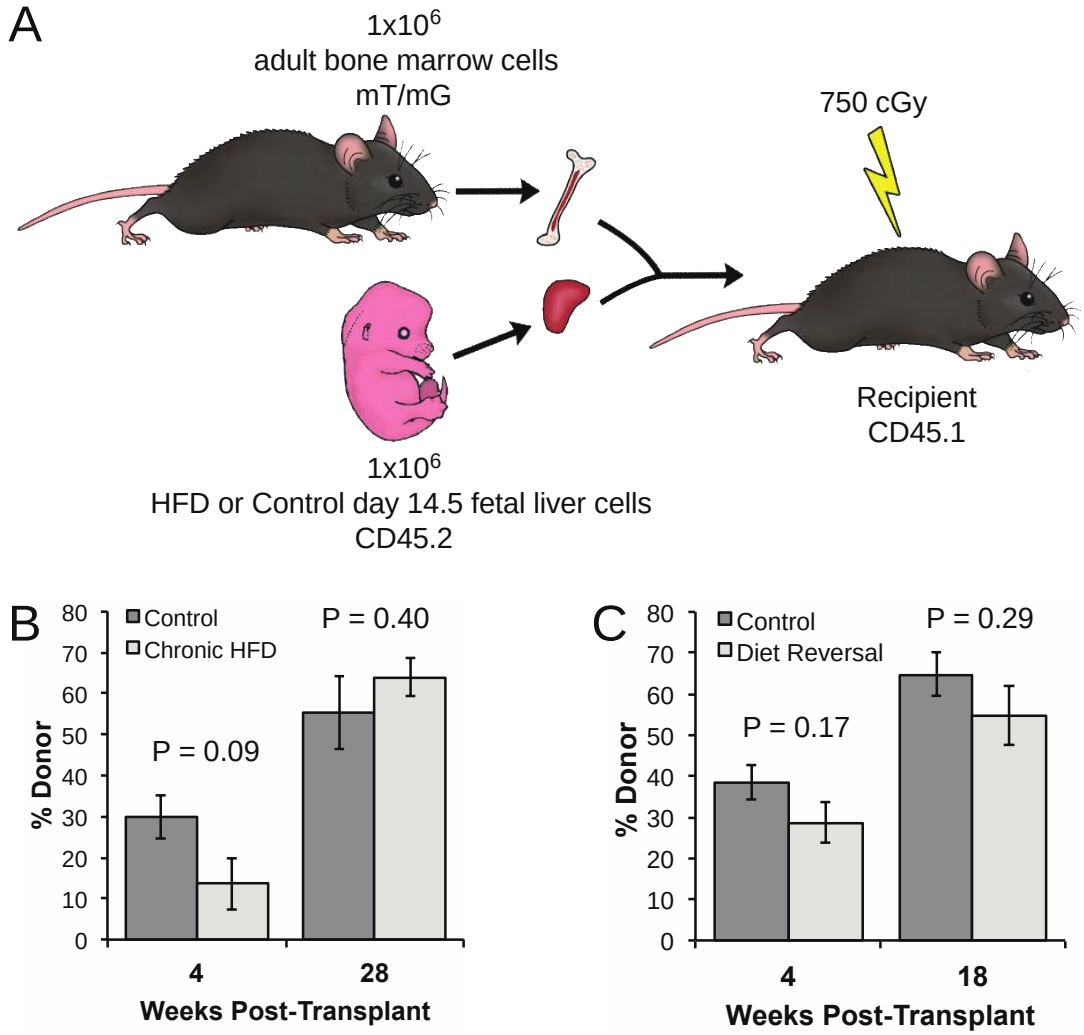


Figure 4.5: Defects in short-term engraftment of HFD and DR HSPCs. (A) Schematic for competitive transplantation. B. Chronic HFD at 4 and 28 weeks ($n_{Control}$ = 5, n_{HFD} =6). (B) Diet-reversal at 4 and 18 weeks ($n_{Control}$ = 5, n_{DR} = 5).

In utero exposure to maternal HFD elicits a broad DNA damage response in fetal liver

Results from the hematopoietic assays led us to hypothesize that HFD programming and maternal obesity stressed highly replicative fetal liver HSPCs. Others have shown that HFD increases oxidative stress and oxidative DNA damage, to which HSCs are especially vulnerable[176,178-180]. Thus, we assayed fetal livers for expression of candidate genes critically involved in DNA damage response and repair. *Trp53bp1* encodes 53BP1, a DNA damage binding protein. *Xrcc5* (Ku80) is required for non-homologous end-joining (NHEJ) DNA repair and V(D)J recombination. Both genes were significantly upregulated in acute HFD livers, along with the base excision repair (BER) glycosylase, Ogg1 (Fig. 4.6A). *Atm* is involved in initiation of the cell cycle checkpoint for DNA damage, and *Ercc1* mostly functions in nucleotide excision repair (NER); neither of these were differentially expressed between acute HFD and control livers. Similar to the acute group, *Trp53bp1* was also upregulated in chronic HFD livers, though *Ogg1* was slightly downregulated (Fig. 4.6B). Interestingly, *Hmga2*, a gene that functions in DNA repair[156,157,181,182], regulation of adipocyte proliferation[183], and neural and hematopoietic stem cell self-renewal[154,155], was upregulated more than two-fold in chronic HFD livers. Diet reversal was the only group in which *Atm* expression was increased, though *Ogg1* was downregulated as in the chronic HFD livers (Fig. 4.6C). In postnatal day 1 mice from dams fed HFD for approximately 25 weeks, expression of *Trp53bp1* was significantly upregulated, by almost two-fold in the liver (Fig. 4.6D). Interestingly, there was a larger

difference in HFD versus control female pup livers than in their male counterparts. Altogether, HFD induced upregulation of *Trp53bp1*, while obesity without HFD did not. For BER-involved genes, *Ogg1* expression increased with non-obese HFD programming and decreased with maternal obesity, but only maternal obesity accompanied by HFD increased fetal liver *Hmga2* expression. The data in aggregate demonstrate a broad response to metabolic injury that is only partially ameliorated by diet reversal during pregnancy.

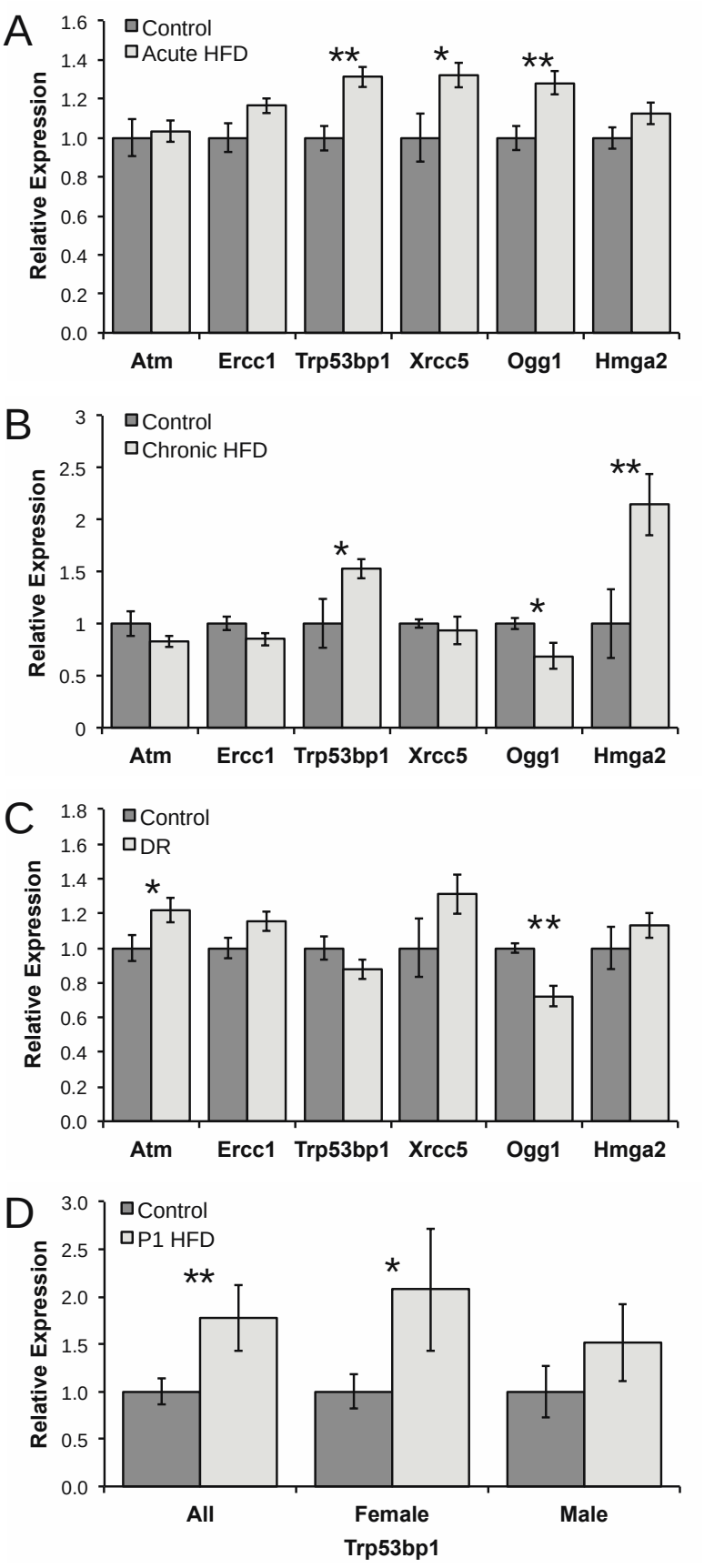


Figure 4.6: Fetal liver induction of DNA repair genes by HFD and obese intrauterine environ- ment

(A) Acute HFD fetal livers, P_{ATM} = 0.74, P_{Ercc1} = 0.08, $P_{Trp53bp}$ < 0.01, P_{Xrcc5} = 0.02, P_{Ogg1} = 0.01, P_{Hmga2} = 0.16 ($n_{Control}$ = 5, n_{HFD} = 5). (B) Chronic HFD fetal livers, P_{ATM} = 0.22, P_{Ercc1} = 0.18, $P_{Trp53bp}$ = 0.02, P_{Xrcc5} = 0.73, P_{Ogg1} = 0.04, P_{Hmga2} = 0.01 ($n_{Control}$ = 3, n_{HFD} = 5, n_{HFD} =4 for *Ogg1* only). (C) Diet reversal fetal livers, P_{ATM} = 0.03, P_{Ercc1} = 0.09, $P_{Trp53bp}$ = 0.19, P_{Xrcc5} = 0.13, P_{Ogg1} < 0.01, P_{Hmga2} = 0.28 ($n_{Control}$ = 6, n_{DR} = 9). (D) Expression of *Trp53bp1* in postnatal day 1 livers from the 3rd litters of dams fed HFD for 25 weeks. n=8 all together per condition, n=4 per condition and sex, P_{All} < 0.01, P_{Female} = 0.03, P_{Male} = 0.14. T-test was performed on ΔC_T values and asterisks indicate P ≤ 0.05 for *, and P ≤ 0.01 for **.

Discussion

Several lines of evidence implicate metabolic injury by maternal overnutrition in fetal programming of chronic diseases and long-term consequences have been documented in multiple organ systems. Murine models of maternal HFD and obesity demonstrate a pervasive effect on offspring, including reproductive defects, fetal growth restriction, defects in brain development, cardiac abnormalities, and endocrine dysfunction[12,165,184-186]. In humans, disease onset is frequently delayed, but the late effects of fetal programming are apparent in epidemiological studies of maternal obesity and offspring. The hematopoietic stem cell pool has not been studied as a target of developmental programming. In a series of dietary manipulations, we systematically evaluated the impact of acute and chronic maternal HFD on fetal hematopoietic development, as well as the potential rescue by gestational diet reversal in the context of established maternal obesity. We show that metabolic injury from maternal obesity, with or without a gestational HFD, significantly constrains HSPC pool size and elicits a transcriptional DNA repair response at a critical time for hematopoietic development *in utero*[83].

During development, the fetal liver provides little metabolic function relative to its postnatal activity, but is a critical site for expansion of the HSPC pool. Accordingly, the major long-term impact of metabolic damage to this organ during gestation is anticipated to be hematopoietic. This is borne out by the gross differences in liver cellularity we observed during midgestation (14.5 dpc) in

chronic HFD-programmed fetal mice, when 90% of the fetal liver mass is composed of hematopoietic cells. These initial observations are consistent with previous reports in neonatal mice and fetal macaques that were developmentally programmed under HFD and showed a disproportionate stunting of liver growth relative to body weight, although hematopoietic function was not tested in those studies[125,177].

Indeed, immunophenotypic analysis of the fetal HSPC compartment showed not only that programming via acute HFD significantly compromised the stem and progenitor cells (KSL/ASL) but was associated with significant increases in lymphoid and myeloid populations, indicating the production of more differentiated blood cells. Considering that acute HFD produced a significant expansion of the lymphoid cell population without an increase in apoptosis, we conclude that maternal HFD exerts pressure on the fetal hematopoietic system to prioritize lymphocyte development. This corresponds to a study in which mice fed HFD starting at 6-7 weeks of age were shown to have an elevated proportion of bone marrow lymphocytes and larger thymuses after 3-6 months of HFD[171]. The data illustrate the profound vulnerability of fetal liver HSPCs that matches prior reports of accumulation of toxic lipid metabolites and changes in hepatic innervation all occurring secondary to the initiation of maternal HFD[14,125]. Our findings suggest that HFD metabolic injury may developmentally program immune dysfunction via a direct influence on HSPC differentiation toward lymphopoiesis. Recent studies demonstrate that (adult) HSPCs are susceptible

to changes in fatty acid oxidization via PPARδ by altering the divisional symmetry of self-renewal[108]. Here, we found that both chronic HFD and DR groups had significantly diminished KSL populations. Along with the gain in differentiated progeny and lack of apoptosis, this strongly suggests a lack of canonical symmetric self-renewing divisions, and the resulting deficits during the critical phase of net expansion in this population.

Moreover, HSPC function, tested by transplantation, was diminished in short-term repopulation capacity for chronic HFD fetal liver, with donor chimerism averaging less than half that of the control, although these results did not reach statistical significance. Intriguingly, at 28 weeks post-transplant, chronic HFD donor cell chimerism was slightly higher than controls, suggesting that the stem cell compartment was able to rebound long-term repopulation capacity after experimental transfer from a suboptimal *in utero* environment at 14.5 dpc. This is supported by our observations that DR fetal liver donor cells had a more modest repopulation defect (26% lower repopulation at 4 weeks) that was not significantly different than controls and twice as high as chronic HFD cell engraftment. At 18 weeks post-transplant, mean DR donor engraftment remained lower than controls, but not at levels that achieved statistical significance. Taken together, this suggests that an obese gestational environment and excessive lipid intake may reduce short-term fetal liver hematopoietic engraftment capacity via HSPC quantity and function, but that switching to a lower fat diet during pregnancy may rescue this defect.

Injury response by adult HSPCs is constrained by DNA damage, and the successive accrual of double-strand breaks during serial repopulation of HSPC correlates with a loss of xenograft repopulating ability[105,176]. HFD increases oxidative DNA lesions[179,180]; thus, we hypothesized that increased lipid metabolism would induce a transcriptional DNA damage response in the fetus[187]. Among genes critical to DNA repair, our investigation revealed that a base excision repair (BER) enzyme, *Ogg1*, was transcriptionally downregulated in obese conditions but upregulated in acute HFD fetal livers. Double-strand lesions are the most deleterious to cellular integrity, and DNA damage response via NHEJ or homology-directed repair is critical to cellular function. The non-histone DNA-binding protein, *Hmga2,* not only interferes with NHEJ, but is also uniquely induced by HFD in adult animals[156,183]. Our studies show for the first time that *Hmga2* is also upregulated in the chronic HFD fetal livers. This suggests that the BER pathway and DNA double-strand DNA repair are perturbed by maternal obesity when paired with HFD. Upregulation of *Trp53bp1* in HFD-fed livers but not DR livers indicates that diet was critical to induction of this DNA double-strand break signal mediator[188]. The data highlight a need for further investigation into the effect of obesity and HFD on the genomic integrity of fetal liver cells.

Besides its role in NHEJ interference, increased *HMGA2* expression in white adipose tissue has recently been linked to obesity and type 2 diabetes, and this

gene functions in the induction of cellular senescence and adipocyte precursor cell proliferation[183,189]. It has also been implicated in the self-renewal of neural stem cells and in fetal HSCs[154,155]. Our data indicates *Hmga2* upregulation may participate in the obesity- and HFD-driven hematopoietic defects in the fetal liver.

Current data indicate that nearly half of women of childbearing age in the U.S. are either overweight or obese[190], demonstrating that there is a rapidly emerging need to evaluate the impact of maternal overnutrition and obesity on health and disease risk in offspring[166]. Mounting experimental evidence now indicates that maternal metabolic status (e.g. obesity, diabetes) and an obesogenic diet and are important determinants of vulnerability for a number of childhood diseases involving components of the hematopoietic system, including atopy[191-193], autoimmune diabetes[18,194], and the inflammatory complications of early-onset obesity[17]. This broad spectrum of childhood disease linked to maternal health supports the concept that fetal HSPCs are a target for developmental programming of disease. In light of recent evidence that maternal obesity aggravates late cardiovascular risk for premature death in offspring, we would predict that the constraints placed on developing HSPCs by HFD and maternal obesity may not manifest until later life[164]. Our data show for the first time that maternal diet and metabolic status adversely program hematopoietic stem cells during development, suggesting that long-term disease susceptibility in postnatal life has its origins during fetal HSPC expansion.

Materials and Methods

Mice

Animals were handled in accordance with OHSU IACUC. For HFD studies, C57BL/6 (CD45.2) female mice were fed a 60% kcal% fat diet (D12492, Research Diets, New Brunswick, NJ) or a 13.5% kcal% fat diet (Laboratory Rodent Diet 5001, Lab Diet, St. Louis, MO) *ad libitum*. Chronic HFD female mice were fed the diet starting at 5 weeks of age, and were sacrificed for fetal harvests at 33-37 weeks of age. Acute mice were fed HFD for 2 weeks starting at 9-11 weeks of age; both groups were kept on diet through breeder pairing and pregnancy. For diet reversal experiments, mice from the chronic HFD cohort were switched to the control diet at 42 weeks of age, bred, kept on control diet through gestation, and sacrificed at 45-49 weeks of age for fetal harvests. Postnatal day 1 mice were harvested from dams fed HFD for approximately 25 weeks and which had 2 prior litters. Male breeders were only fed HFD when paired with females for breeding. Pregnancies were timed using the vaginal plug method; fetal mice were harvested at day 14.5 of gestation and livers were dissected and prepared in single cell suspensions by pipetting. Placental and fetal masses were collected as wet weights.

Cell Culture

Unfractionated fetal liver cells were plated in mouse methylcellulose complete media (R&D Systems, Minneapolis, MN) at 20,000 cells per mL and performed according to manufacturer's instructions.

Flow Cytometry

Cells were prepared from fetal liver and adult bone marrow. The following antibodies were used for analysis: CD3, CD4, CD5, B220, Gr-1, Ter119, c-Kit/CD117 CD45.2 (BD, Franklin Lakes, NJ), CD45.1, Sca-1, AA4.1/CD93 (eBioscience, San Diego, CA). Staining reagents were also used for analysis: LIVE/DEAD Fixable Dead Cell Stain (Invitrogen, Carlsbad, CA), propidium iodide (Sigma-Aldrich, St. Louis, MO), Annexin V, and carboxy-H_2DCFDA (Molecular Probes, Eugene, OR). Cells were analyzed on a BD LSR II and a BD FACS Calibur.

Molecular

For qRT-PCR, RNA was extracted with a Qiagen RNeasy Mini Kit (Valencia, CA), cDNA synthesis was performed with TaqMan Reverse Transcription reagents (Invitrogen), and reactions were run on ABI 7300 (software v1.4) (Invitrogen). The primer sequences employed in qRT-PCR were: ATM (Mm01177457_m1), Ercc1 (Mm00468337_m1), Trp53bp1 (Mm01271860_m1), Xrcc5 (Mm00550142_m1), Ogg1[187], and Hmga2[140]. Two-sided Student's t-test was performed on the ΔCt of each gene.

Competitive Transplantation

Recipient and rescue cell donor mice were fed a standard chow diet (Laboratory Rodent Diet 5001, Lab Diet, St. Louis, MO), *ad libitum*. Recipient mice (CD45.1

or CD45.1 GFP) were irradiated with 750 cGy in a J.L. Shepherd Cesium Irradiator approximately 24 hours prior to transplantation. $1x10^6$ unfractionated CD45.2 HFD or control fetal liver cells were mixed with an equal number of unfractionated, hemolyzed mT/mG (Tomato) adult bone marrow cells, and injected via tail vein. Retroorbital blood draws were performed at serial timepoints and blood was hemolyzed and analyzed for chimerism by CD45.1 and CD45.2 immunophenotype as described[139], as well as GFP and Tomato expression.

Statistical Analysis

Data is presented as mean +/- standard error of the mean and compared using two-tailed, unpaired Student's t-test; a *P* value of 0.05 or less is considered significant.

CHAPTER 5: EFFECT OF HIGH-FAT DIET ON HEMATOPOIESIS IN *FANCC*$^{-/-}$ MICE

Abstract

A high-fat diet has been demonstrated to induce DNA damage, to which Fanconi anemia (FA) hematopoietic stem cells are particularly sensitive, and the accumulation of which has been implicated as a factor leading to HSC loss. DNA damage in FA hematopoietic stem cells eventually leads to hematopoietic failure. We previously demonstrated a fetal origin of hematopoietic failure in Fancc-deficient mice. In this mouse model, postnatal hematopoiesis is not grossly abnormal without induction with agents such as mitocmycin-c or lipopolysaccharide (LPS)[71,144,146]. The high-fat, Western-style diet is thought to increase DNA damage via oxidative stress. We wanted to test the hypothesis that excessive lipid intake worsens the hematopoietic status of the Fancc-deficient murine fetal liver, which we previously demonstrated to be stunted. Here, we observed that a high-fat diet during pregnancy can exacerbate existing hematopoietic deficiencies in the fetal liver and increase DNA damage in cells that form the foundation of the hematopoietic and immune system. Our evidence suggests that a lipid-rich diet increases DNA damage in FA hematopoietic stem and progenitor cells (HSPC) and that the prenatal period presents an opportunity for nutritional therapy in preventing disease development.

Introduction

Fanconi anemia is a multisystem syndrome involving defects in the repair of interstrand crosslinks and double-stranded DNA breaks. Evidence has implicated both aldehydes and reactive oxygen species (ROS) as contributing to DNA

damage accumulation in FA, both of which are involved in lipid peroxidation[60,67,135].

ROS are free radicals and molecules containing oxygen. While they can be produced endogenously by processes such as normal oxygen metabolism or by immune effector cells to combat microbes, they can also be generated via ionizing radiation and pollutants[67,195,196]. ROS can directly oxidize DNA to form adducts such as 8-oxoguanine (8-oxoG) and single-strand breaks, which may be converted to double-strand breaks[67]. In addition, ROS can react with lipids in a chain reaction to form genotoxic products such as malondialdehyde and 4-hydroxynonenal (4-HNE), both of which contain aldehyde groups[68,197].

Aldehydes are mutagenic and can react with DNA and proteins. Endogenous sources include lipid peroxidation or metabolic reactions such as ethanol catabolism. Impaired ethanol metabolism in FA mice and patients has been demonstrated to increase the incidence of congenital defects, hematopoietic failure, and leukemia[59,60,64]. However, a major source of endogenously produced aldehydes, especially in non-drinkers, is from the peroxidation of lipids by ROS, generating yet more ROS as end products[197,198].

Despite the well-documented sensitivity of FA cells to both ROS and aldehydes, little is known about the impact of excess lipid consumption in FA, despite the widespread adoption of the high-fat, Western-style diet. Studies in non-FA animal

models have shown evidence of increased oxidative stress and DNA damage with both high-fat diet and obesity. Oocytes from diet-induced obese (DIO) female mice have significantly increased rates of aneuploidy, as well as chromosome alignment and spindle defects[165]. Another DIO study found increased mtDNA damage and markers of oxidative stress in liver and muscle tissue[199]. DNA damage, as measured by 5-hydroxymethyl-2'-deoxyuridine, a type of oxidative lesion, correlated with increasing fat intake in nucleated blood and mammary gland cells of rats[200]. Taken together, this suggests that excessive lipids drive genotoxic reactions.

DNA damage and oxidative stress are particularly harmful to HSC, which are the lifelong source of the continually regenerating population of blood cells. Repair of genotoxic damage is essential to their maintenance and survival[52,76,105,106,144,176,201]. These cells reside in a hypoxic niche within the bone marrow[202], which protects them from oxidative stress, to which FA cells are hypersensitive[69,71,146,203]. As a principal hematopoietic niche during development, the fetal liver is a target of damage under prenatal high-fat diet (HFD) [125,204]. This lipid-rich diet provides more substrate for lipid peroxidation near hematopoietic cells.

We hypothesized that a maternal HFD would exacerbate the defective hematopoietic phenotype of 14.5 dpc Fancc-deficient fetal livers by increasing DNA damage via lipid peroxidation. Building on our previous work that delineated

the developmental programming of hematopoietic defects in FA (Chapter 2 herein) and with HFD (Chapter 4 herein), we found that a prenatal HFD increased the difference between *Fancc*$^{-/-}$ and WT in liver cellularity and HSPC content of fetal livers. Reactive oxygen species were mildly elevated in HFD-programmed *Fancc*$^{-/-}$ unfractionated fetal liver cells. In 5-week-old HFD-programmed *Fancc*$^{-/-}$ mice that remained on HFD postnatally, there was a downward trend in hematopoietic progenitor frequency. Overall, these results suggest that fetal liver HSPC are more sensitive to HFD than their adult bone marrow counterparts.

Results

Acute maternal HFD alters the weights and liver cell counts of Fancc$^{-/-}$ *fetal mice*

To test whether a maternal HFD would exacerbate defects in the fetal liver hematopoietic compartment, we fed *Fancc*$^{+/-}$ females a HFD for 4 weeks and then began breeding them for timed pregnancies. This amount of time on the diet allowed the animals to adjust to the diet without developing obsesity. Day 14.5 *Fancc*$^{-/-}$ offspring programmed under a HFD had placentas that were 21 percent lighter than their HFD WT counterparts, but with the current sample size, were not significantly different from control diet-programmed *Fancc*$^{-/-}$ placentas (Fig. 5.1A). HFD *Fancc*$^{-/-}$ fetuses were 18 percent smaller than HFD WT, while those from the control diet cohort were 17 percent smaller than their WT counterparts; however, there were no significant differences between the HFD cohorts and their corresponding controls (Fig. 5.1B). Control diet *Fancc*$^{-/-}$ fetal livers

displayed a downward trend in cellularity compared to WT, but HFD expanded the difference between *Fancc*$^{-/-}$ and WT, producing a significant difference even with a small sample size (Fig. 5.1C). These data suggest that *Fancc*$^{-/-}$ placenta and fetal liver are unable to increase growth under HFD.

Effect of HFD on Fancc$^{-/-}$ *fetal liver hematopoietic stem and progenitor cells*

Next, we wanted to assess the hematopoietic status of the HFD-programmed *Fancc*$^{-/-}$ fetal liver. There was no significant difference between lymphoid ($CD3^+/B220^+$) and myeloid ($Gr\text{-}1^+/Ter119^+$) populations within cohorts, but HFD significantly lowered the lymphoid population in WT livers, compared to their corresponding controls (Fig. 5.2A, B). The ASL population was somewhat reduced in control diet *Fancc*$^{-/-}$ livers, juxtaposed to control diet WT livers, but HFD widened the disparity between *Fancc*$^{-/-}$ and WT, inducing expansion of the WT but not the mutant ASL pool (Fig. 5.2C). A similar trend was observed in the c-Kit^+ Sca-1^+ $Lin^{low/-}$ (KSL) HSPC pool (Fig. 5.2D). Likewise, the hematopoietic progenitor frequency in fetal liver from HFD *Fancc*$^{-/-}$ fetuses was 32 percent lower than HFD WT, whereas there was no significant difference in control diet *Fancc*$^{-/-}$ colony formation compared to control WT, or between HFD and control groups at the current sample size (Fig. 5.2E). Together, these data indicate that the hematopoietic compartment is altered by HFD programming.

Slight elevation of ROS in Fancc$^{-/-}$ *fetal livers*

To test for elevated ROS as a possible consequence of excessive dietary lipids, fetal liver cells were stained with the ROS indicator, carboxy-H_2DCFDA. Results

suggest a slight increase in ROS in *Fancc*$^{-/-}$ cells in both control and HFD cohorts, compared to respective WT cells (Fig. 5.3A, B).

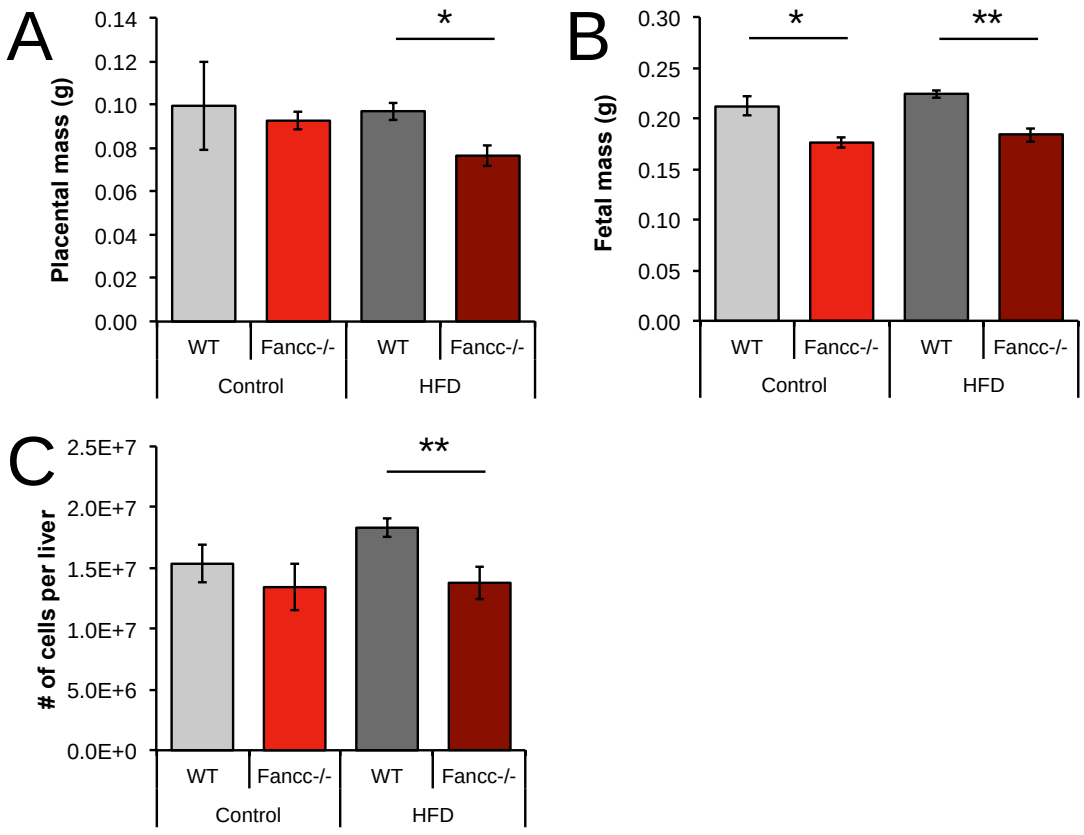


Figure 5.1: Significant decrease in mass and cellularity of fetal tissues for 14.5 dpc HFD *Fancc*$^{-/-}$ mice. (A) Placental mass and (B) fetal mass ($n_{ControlWT}$ = 4, $n_{ControlKO}$ = 4, n_{HFDWT} = 9, n_{HFDKO} = 10). (C) Number of cells per liver ($n_{ControlWT}$ = 9, $n_{ControlKO}$ = 9, n_{HFDWT} = 4, n_{HFDKO} = 3). T-tests were 2-tailed and asterisks indicate P ≤ 0.05 for *, P ≤ 0.01 for **, and P ≤ 0.001 for ***

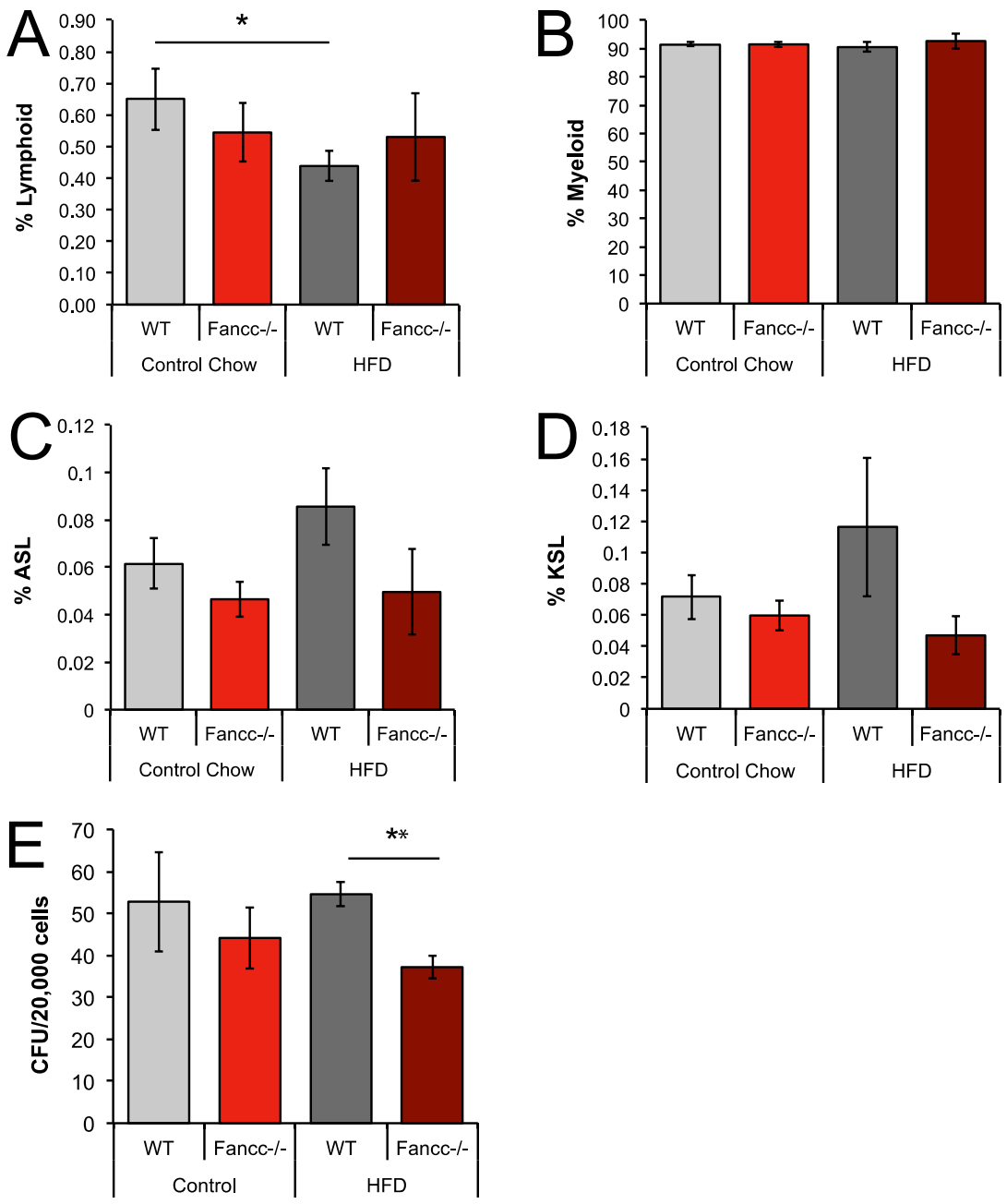


Figure 5.2: Analysis of HSPCs in HFD- and control diet-programmed 14.5 dpc *Fancc*$^{-/-}$ and WT fetal livers. (A) Fetal liver lymphoid, (B) myeloid frequency and (C) ASL, and (D) KSL HSPC-enriched populations ($n_{ControlWT}$ = 6, $n_{ControlKO}$ = 7, n_{HFDWT} = 13, n_{HFDKO} = 5). (E) Hematopoietic progenitor frequency ($n_{Control}$ = 5, n_{HFD} = 6). T-tests were 2-tailed and asterisk indicates P ≤ 0.05 for *.

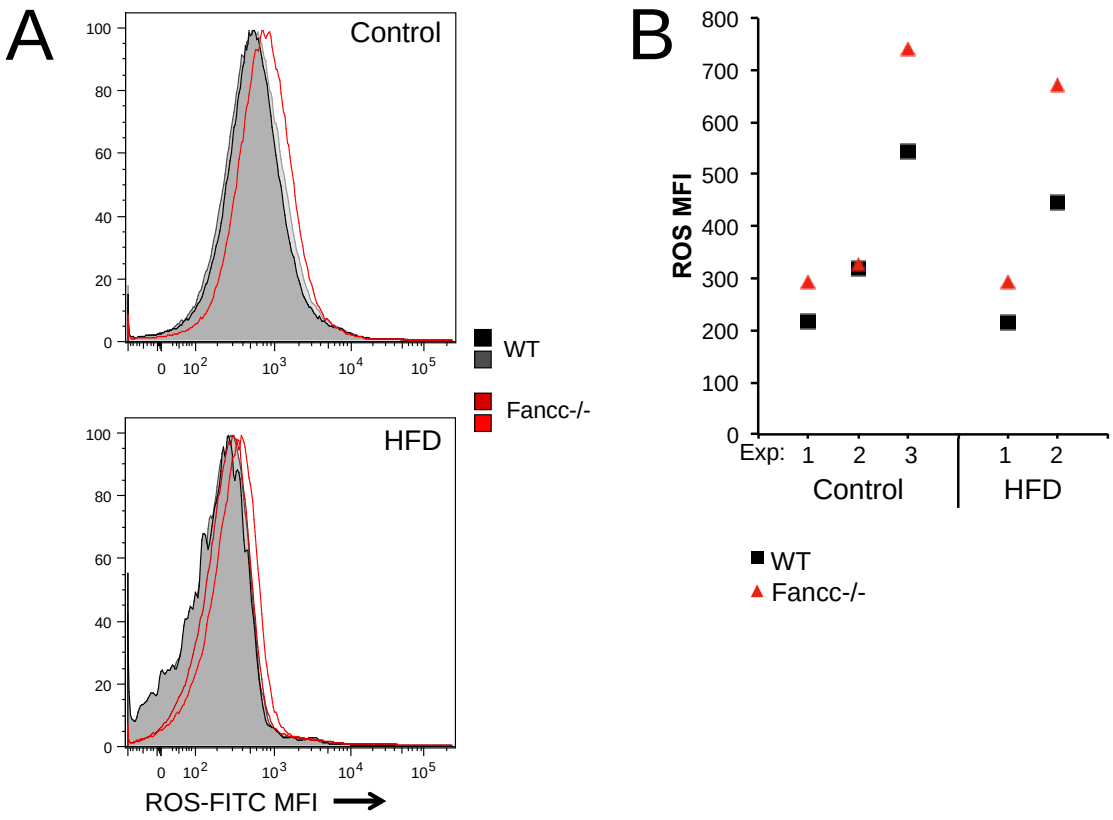


Figure 5.3: ROS in *Fancc*$^{-/-}$ fetal liver cells. (A) ROS median fluorescence intensity (MFI) in unfractionated control diet- and HFD-programmed fetal liver cells. (B) Graph of ROS median fluorescence intensity (MFI) in HFD versus control diet *Fancc*$^{-/-}$ and WT livers. Exp= experiment

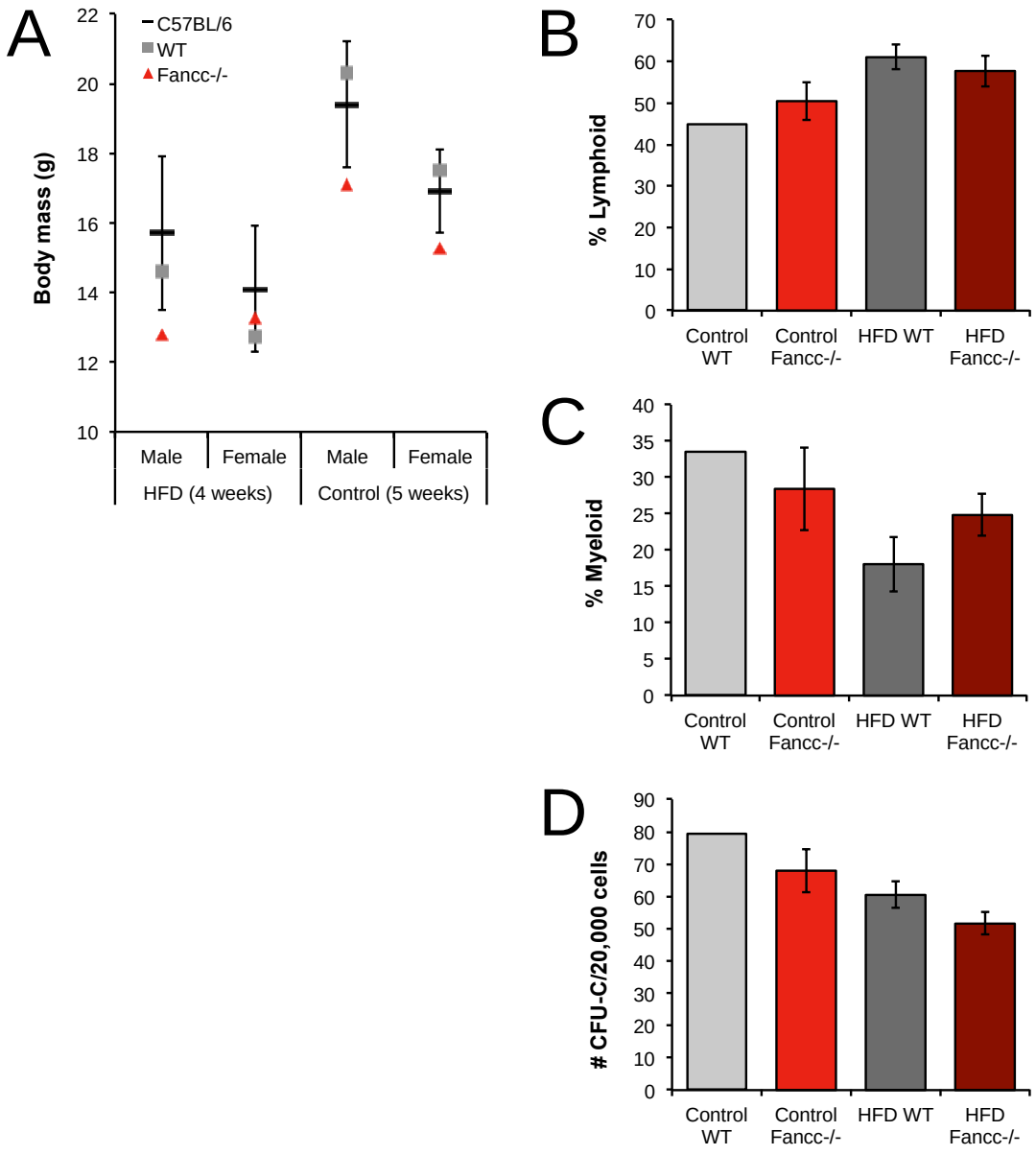


Figure 5.4: Effect of pre- and post-natal HFD on hematopoiesis in *Fancc*$^{-/-}$ 5-week-old mice. *Fancc*$^{-/-}$ and WT mice were prenatally programmed on a HFD (n_{WT}= 3; $n_{Fancc-/-}$= 3) or control diet (n_{WT}= 2; $n_{Fancc-/-}$= 3) and nursed and weaned onto the same diet, then harvested at 5 weeks of age. (A) Mean body masses of HFD and control WT and *Fancc*$^{-/-}$ juvenile mice, compared to standard mean weights of C57BL/6 mice from Jackson Labs[205] (error bars reflect one standard deviation from Jackson Labs). (B) Peripheral blood CBC (WBC, white blood cells; RBC, red blood cells; PLT, platelets). (C) Bone marrow immunophenotyping by flow cytometry for lymphoid ($CD3^+$ or $B220^+$) and (D) myeloid ($Gr\text{-}1^+$ or $Mac\text{-}1^+$) subsets. (E) Bone marrow hematopoietic progenitor frequency by methylcellulose culture assay. (F) Flow cytometry analysis for bone marrow KSL HSPC-enriched cells.

Prenatal and postnatal HFD decreases hematopoietic progenitor cell frequency, but not KSL cells in the bone marrow of juvenile Fancc$^{-/-}$ mice

To determine whether fetal programming under HFD would predispose *Fancc*$^{-/-}$ animals to have a more severe hematopoietic phenotype later in life, we analyzed peripheral blood and bone marrow of 5-week-old *Fancc*$^{-/-}$ and WT mice that were developmentally programmed under HFD or control chow, nursed under, and weaned onto the same diet. Though the sample size (n_{HFD}= 3 per genotype; $n_{ControlWT}$= 2, $n_{ControlFancc}$= 3) to date constrains the calculation of statistical significance, body weights were compared to age-matched standard weights for C57BL/6 mice on a similar diet[205]. The body weights of male HFD *Fancc*$^{-/-}$ juvenile mice at 4 weeks of age fell more than 1 standard deviation below the average weight for age-matched C57BL/6 mice[205], while WT males and females of both genotypes were within one standard deviation of the mean (Fig. 5.4A). Control mice were weighed at 5 weeks and *Fancc*$^{-/-}$ mice of both sexes fell below one standard deviation of the standard for C57BL/6. Both the HFD and control litter were comprised of 8 pups and were the first litters born to the respective dams. Although there were no large differences in the proportions of peripheral blood lymphoid (CD3^{+}/B220^{+}) cells in WT versus *Fancc*$^{-/-}$ mice within each dietary cohort, there appeared to be more lymphoid cells in both HFD groups when compared to control diet, though the sample size is currently too small to detect significance (Fig. 5.4B). There was also a trend toward a decreased myeloid (Gr-1^{+}/Mac-1^{+}) population in HFD cohorts, compared to respective control diet genotypes (Fig. 5.4C). Colony formation of hematopoietic progenitors

trended downward in HFD *Fancc*$^{-/-}$ bone marrow, compared to HFD WT, and both HFD cohorts appeared to have fewer progenitors than their respective controls (Fig. 5.4D). Together with our findings in HFD-programmed *Fancc*$^{-/-}$ fetal liver, these data suggest the hematopoietic tissue is susceptible to HFD programming.

Discussion

Recent research indicates the fetal liver is susceptible to damage by HFD[125,177,204]. Building on prior work that identified deficits in the prenatal FA fetal liver HSPC compartment, we predicted that the FA fetal liver would be more susceptible to HFD-induced damage. HFD is thought to increase ROS and lipid peroxidation, which in turn elevate aldehyde and ROS levels. FA cells are sensitive to both of these by-products, which react with nucleic acids and proteins, potentially causing DNA-DNA and DNA-protein crosslinks and DNA strand breakage. The toxicity of aldehydes and ROS have been well-documented in FA, particularly for HSPCs[59,60,70,71,135]. While further investigation is needed to determine the exact mechanism(s) by which HFD perturbs HSPCs, our data suggests that it negatively impacts the fetal liver and HSPC compartment.

The trends in our data suggest HFD increases growth in WT fetuses, whereas this process is stunted in *Fancc*$^{-/-}$ fetuses. FA patients exhibit short stature, and a study found approximately half to be growth hormone-deficient, which may at least partially account for fetal microsomia[126,206]. HFD widened the gap between *Fancc*$^{-/-}$ fetuses and WT littermates in most assays, compared to the gap

between control diet *Fancc*$^{-/-}$ and WT, which may have been due to growth restriction in *Fancc*$^{-/-}$. Interestingly, placental mass and frequency of KSL cells and hematopoietic progenitor cells in the *Fancc*$^{-/-}$ fetal liver somewhat decreased with HFD, relative to their control diet counterparts. However, HFD did not lower *Fancc*$^{-/-}$ ASL frequency compared to control diet. Based on our HFD studies in WT mice (Chapter 4 herein), it is possible that prolonged maternal HFD exposure would induce a more drastic defect in growth and HSPC numbers in the *Fancc*$^{-/-}$ fetal liver. Studies in HFD programming have not shown an increase in a key ß-oxidation transcription factor in newborn mouse livers which, unlike non-human primates, do not accumulate triglycerides[14,177]. Since this is likely the case for the fetal liver, which is primarily an organ for hematopoiesis rather than metabolism, any potential lipid peroxidation is unlikely to be due to accumulation of substrate (e.g. lipids). However, it could be that small molecules such as aldehydes and ROS may pass through the placenta, through the umbilical vein, and directly into the liver to initiate lipid peroxidation. HFD-induced inflammation and hyperinsulinemia in the *Fancc*$^{+/-}$ dam are unlikely, unless loss of the residual functional *Fancc* allele removes this protection[177,207]. However, mild hyperglycemia is inducible by HFD in female C57BL/6 mice, and glucose transport across the placenta is robust[208].

Culture of human endothelial cells in high glucose media increases the expression of several antioxidant enzymes[209]. In vivo, hyperglycemia, induced by intravenous infusion of glucose, has been reported to induce oxidative stress in

normal, non-diabetic subjects[209,210]. This raises the possibility that dietary triglycerides are utilized for maternal gluconeogenesis, increasing maternal blood glucose and the amount transported through the placenta, and subsequently inducing oxidative stress in the fetal liver from an overabundance of this compound[211].

Though it is unknown whether lipids, their sugar catabolites, or other compounds or signaling mediate the growth restriction and HSPC defects we have observed in HFD-programmed *Fancc*$^{-/-}$ fetuses, other clues from patients may need to be considered. Metabolism itself may play a role: FA patients are prone to metabolic disorders, and have a higher risk of developing type 2 diabetes, which could be aggravated by a diet heavy in lipids[206]. They are also susceptible to liver tumors, which has been hypothesized to be due to a defect in neutralizing ROS resulting from lipid peroxidation[212,213].

Altogether, this study suggests a role for maternal nutrition in the developmental onset and severity of the FA phenotype *in utero,* as well as the establishment of the FA hematopoietic system prior to birth. Future studies will be instructive in elucidating what nutrients or metabolites may be increased or decreased in transport across the placenta, what mechanisms are responsible for the dietary effect on *Fancc*$^{-/-}$ mice, and in determining whether a high-fat or high-glucose diet induces DNA damage in the fetus.

Materials and Methods

Mice

C57BL/6 *Fancc*$^{+/-}$ nulliparous female mice 6 to 17 weeks of age were fed a 60% kcal% fat diet (D12492, Research Diets, New Brunswick, NJ) or standard rodent chow (Laboratory Rodent Diet 5001, Lab Diet, St. Louis, MO) containing 13.5% kcal% fat, *ad libitum*, for 4 weeks, and were then bred to *Fancc*$^{+/-}$ males for timed pregnancies (vaginal plug method); mice were maintained on HFD or control chow throughout breeding and gestation and were weighed weekly. Fetal mice were harvested at day 14.5 of gestation and were processed as previously described[147]. For studies of juvenile mice, dams were kept on the same diet throughout nursing, and pups continued this diet after weaning; peripheral blood and bone marrow were harvested at 5 weeks of age.

Colony forming assays

Whole fetal liver cells were cultured as previously described[147].

Complete blood counts

Peripheral blood cells were analyzed on a HemaVet 950FS (Drew Scientific, Inc.).

Flow cytometry

Cells were stained as previously described[147] and analyzed on a BD FACSCalibur and a BD LSR II.

Statistics

Bar graphs reflect means and error bars are the standard error of the mean. For body mass of juvenile mice, the error bars reflect the standard deviation of the weights for typical C57BL/6 mice (Jackson Labs). Significance was determined by independent, 2-tailed Student's t-test.

CHAPTER 6: FUTURE DIRECTIONS AND CONCLUSIONS

Future Directions

About 25 years of research in DOHaD have uncovered prenatal contributions for some of the most common chronic illnesses, including cardiovascular disease, type 2 diabetes, and obesity. We now demonstrate developmental programming of hematopoietic stem and progenitor cells in multiple mouse models of Fanconi anemia and in wild-type animals under prenatal HFD and maternal obesity, with evidence for the mechanistic involvement of DNA repair.

Genetic Programming of Hematopoietic Development in Fanconi Anemia

The inadvertent discovery of compromised cord blood colony formation from an infant subsequently found to have FA in 1990 first suggested that the HSC pool in this disease could be constrained during development and there may be a prenatal hematopoietic defect[73]. Nearly a decade later, *Fanca* was demonstrated to be richly expressed in the murine fetal liver at 13.5 dpc, with lower expression in newborn mice[74]. Twenty years after the cord blood study was published, knockdown of FANCA and FANCD2 in human embryonic stem cells was demonstrated to reduce hematopoietic differentiation[75]. Another study in human tissue, using 14-18 week fetal livers, found significant upregulation of p21 (*CDKN1A*), suggesting cell cycle arrest and concurring with our results showing increased p21 expression and a greater proportion of HSPC-enriched cells in G_1[76]. Fetal tissues were also evaluated in *Fancd2*$^{-/-}$ *Aldh2*$^{-/-}$ mice; many of these perished in mid-gestation and *in utero* ethanol exposure caused abnormalities in head and eye development[60]. No studies to date have evaluated the role of the

FA pathway during hematopoietic development in a physiological context. Our research in C57BL/6 *Fancc*$^{-/-}$ and *Fancd2*$^{-/-}$, as well as 129Sv *Fancc*$^{-/-}$ (mixed background) mice now provides unambiguous *in vivo* evidence for a prenatal onset of hematopoietic dysfunction in FA (Fig. 6.1A) and we present evidence of genetic programming of postnatal hematopoietic failure.

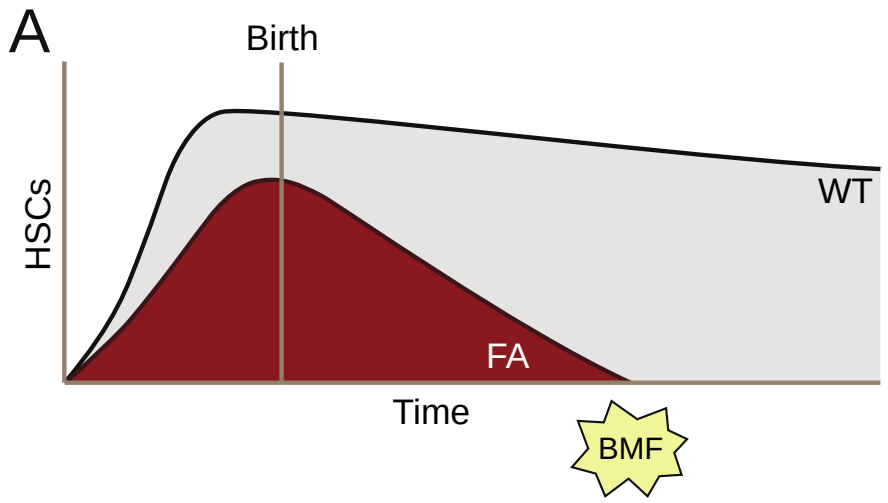


Figure 6.1: Revised model of bone marrow failure in Fanconi anemia. (A) Investigation of the status of FA fetal liver hematopoietic stem cells has revealed prenatal deficits in the blood system, likely predisposing it to undergo bone marrow failure in postnatal life.

The molecular activities of FA genes during embryonic and fetal development have been largely unexamined, although the hematopoietic deficiencies and skeletal, skin, cardiac, renal, reproductive, and gastrointestinal congenital defects that may accompany the disease suggest an important prenatal role for the pathway[35]. It is currently unknown whether FA proteins function in developmental pathways outside of their coordination of DNA repair, or if they are involved in the repair of endogenous damage in specific embryonic or fetal tissues in which

deformities otherwise arise, perhaps from damage at critical windows of organogenesis or skeletal development. One way of addressing this would be with a conditional knockout model, in which FA genes are disrupted either globally or in certain tissues at specific timepoints in prenatal development. For the hematopoietic lineage in particular, knocking out an FA gene such as *Fancd2* during the emergence of primitive (E7.5) versus definitive HSCs (starting at E8.25), or examining FA mice at earlier timepoints than 14.5 dpc would be helpful in ascertaining when the defect arises, and in what prenatal hematopoietic niche[214]. Data from *in vitro* embryonic stem cell studies suggest it may even arise at the earliest stages[75].

It may also be informative to assess FA gene loss in the various phases of hematopoietic differentiation, as this has not yet been systematically dissected. Although it is generally assumed that bone marrow failure is a stem cell-intrinsic consequence of FA gene deficiency, this has not been examined by conditionally knocking out FA genes in the long-term HSC, intermediate-term HSC, short-term HSC, common lymphoid progenitor, and common myeloid progenitor. While patients often present with pancytopenia, which has conventionally been assumed to herald a defect in the LT-HSC population, progenitor cells must divide many times to produce a sufficient number of mature blood cells. Interestingly, we observed a significant increase in *Fancc*$^{-/-}$ fetal liver donor lymphoid cells, accompanied by a significant decrease in donor myeloid cells in the bone marrow of secondary recipients (Chapter 2). We also saw a significant

decrease in *Fancd2*$^{-/-}$ bone marrow donor lymphoid cells and an increase in myeloid cells 4 weeks after primary transplantation, and this difference was completely normalized by 16 weeks. These data suggest potential bias in the HSC compartment[215] or selection of distinct progenitor subsets. Distinct effects on progenitor cells have been demonstrated in a leukemia microenvironment model, in which HSCs retained repopulation capacity while HPCs increased their cycling kinetics and became exhausted, halting the production of functional blood cells[216]. A hierarchal analysis of hematopoietic cells would more specifically identify the defective cell(s) in FA that are the root of hematopoietic failure.

HSCs proliferate relatively rapidly, nearly doubling each day in the fetal liver[86]. The observations underlying this thesis point to the unique capacity of our fetal mouse model for study of hematopoietic stem cell self-renewal and replicative stress under physiological conditions. In contrast, bone marrow transplant models typically involve conditioning, damaging the microenvironment via radiation or chemotherapeutic agents and triggering the bystander effect; this raises levels of ROS and inflammatory cytokines such as tumor necrosis factor-alpha, to which FA cells are hypersensitive, creating a suboptimal environment for engraftment[217-220]. The latter would be especially true for the study of FA stem cells with known susceptibility to inflammatory cytokines, including tumor necrosis factor-α[134,218]. Results from bone marrow transplantation may also be confounded by the existence of potential homing defects in FA HSCs[128,221]. Furthermore, unlike postnatal hematopoietic cells, fetal liver HSCs reside in an

immune privileged, sterile, and generally protected environment, in which they do not have to mount a response to infection (except for situations such as HIV infection) or bleeding[222]. All things considered, the fetal liver may be an ideal system in which to investigate HSC self-renewal, whilst avoiding the introduction of multiple confounding variables to the analysis.

Fetal development is also a time at which the role of FA proteins may be assayed on a molecular level, through protein co-immunoprecipitation (Co-IP), to investigate potential interactions with other proteins, particularly genes that are only expressed prenatally and have thus gone undetected by previous biochemical studies in FA. For example, developmentally timed expression of Lin28b enables it to block Let-7 miRNAs from downregulating *Hmga2* in fetal HSCs (K. Lawson, personal communication) and thus allow robust self-renewal activity; their expression levels are reversed in the transition to an adult phenotype, when cells adopt a primarily quiescent state, illustrating a cell-intrinsic developmental switch[154]. Discovery of novel FA protein interactions may bring exclusive developmental functions of the FA pathway to light or provide novel therapeutic targets. Altogether, much has yet to be investigated in the *in utero* functions of FA genes.

Metabolism and Oxidative Stress

Likewise, fetal HSPCs have yet to be studied for metabolism, and differences in prenatal energy demand and principal energy source utilization remain to be

determined; research on the identification of metabolic pathways used by HSCs has been exclusively conducted in adult cells and is still in its early stages. Unlike adult HSCs, which primarily reside in a hypoxic microenvironment within the bone marrow and usually are in a quiescent state, HSCs in the fetal liver divide relatively rapidly and receive a portion of the freshly oxygenated blood directly traveling back to the fetus from the placenta[86,223-225]. It is unknown whether they rely on anaerobic metabolism via glycolysis, as adult HSC do, or utilize more aerobic oxidative phosphorylation, which generates more ROS; furthermore, whether fetal HSCs must use fatty acid oxidation in asymmetric division, as mature cells do, remains to be discovered[107,108]. However, the high delivery rate of glucose across the placenta to the fetus[208], along with gene expression analysis of fetal liver HSCs showing high transcript levels of the glucose importer Slc2a1, as well as several glycolysis enzymes, including hexokinase 1 and 2 (Hk1, Hk2), and pyruvate dehydrogenase kinase isoenzyme 1 and 3 (Pdk1, Pdk3), as well as hypoxia-inducible factor (HIF)1α[226] suggest that glycolysis may be a preferred method of ATP production in these cells. Future studies will need to define energy utilization pathways in fetal liver HSCs in order to determine how a HFD or maternal hyperglycemia may manipulate their metabolism, and whether this increases ROS or aldehyde levels. This may also provide much-needed mechanistic insight into the nutritional effects that have been at the heart of DOHaD research.

Considering the rapid and efficient transport of glucose across the placenta and limited transport of fat[208], as well as the conspicuous lack of lipid deposition in the newborn murine liver under HFD programming, future studies must also address whether a high-glucose diet exerts similar effects on fetal HSPCs as HFD, as both cause hyperglycemia[177]. It also must be determined whether the expression of glycolysis, lipid metabolism, and antioxidant enzymes are perturbed in fetal HSPCs by maternal diet or obesity, and whether levels of ROS or aldehydes are raised. Unfortunately, markers of oxidative stress from lipid peroxidation, such as 4-hydroxynonenal (4-HNE) and malondialdehyde (MDA), will not clarify whether ROS are being produced as a result of excessive fat or glucose, as surpluses of both nutrients can cause oxidative stress[68]. Glucose-induced oxidative stress may at first seem contradictory, given that anaerobic glycolysis helps to limit ROS formation. However, current evidence suggests hyperglycemia may cause an imbalance between cellular antioxidants and ROS by depleting cellular cofactors used in redox reactions, decreasing ROS-scavenging enzymes, perturbing expression of developmental genes, and reducing expression and activity of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which functions in glycolysis[227,228]. GAPDH activity was also reduced by excessive glucose in rat embryos *in vitro*, and this was accompanied by reduced growth and increased malformation, while addition of the antioxidant *N*-acetylcysteine (NAC) ameloriated these effects[229]. However, it is unknown whether hyperglycemia is toxic to HSCs, in which glycolysis is used preferentially over oxidative phosphorylation. Overall, testing HFD-programmed fetal liver HSPCs

for ROS and aldehyde-induced damage to the DNA or other cellular structures will be a necessary next step to determine whether more DNA damage is taking place, and characterization of fetal hematopoietic metabolism and modulation of nutrient uptake by the maternal diet will clarify basic effects of HFD on the developing HSPCs.

Potential Involvement of Mitochondria

In addition to studying other organs, we may need to examine other organelles; the nucleus has been the primary site of study for DNA damage, yet mitochondria have their own DNA and are a key site of metabolism and cellular signaling. In fact, mitochondrial DNA (mtDNA) is substantially more vulnerable to damage, as it is closer in proximity to ROS (mostly produced in the electron transport chain) and is unshielded by histone proteins[230,231]. Mitochondria share several DNA repair proteins with the nucleus, including FANCD1 (BRCA1) and FANCG, and have some of their own repair genes[232-235]. These organelles are the site of ALDH2 activity, which has been intensively studied in recent years for its role in neutralizing genotoxic endogenous aldehydes in FA, but no studies to date have addressed its potential role in protection of mtDNA, despite reports of mitochondrial dysfunction in FA[234,236-239]. Central to these recent studies is the impairment of ROS scavengers such as superoxide dismutase, which results in a pro-oxidant state in FA cells[237,239]. Mitochondrial dysfunction has also been documented in HFD and with high-fat, high-sucrose diet-induced diabetes[240,241]. Energy generation via oxidative phosphorylation and fatty acid oxidation (FAO)

take place within the mitochondrion, however, glycolysis occurs in the cytoplasm, and as a result, long-term HSCs, which primarily use FAO, contain a relatively small number of mitochondria[223]. Conversely, the more differentiated progenitor cells have increased mitochondrial mass and perform more oxidative phosphorylation. Studies to quantify relative mitochondrial mass in fetal HSCs and hematopoietic progenitors, and testing for mtDNA damage will broaden our understanding of FA and HFD pathology as well as fetal HSC metabolism.

The Potential to Ameliorate Effects of Fetal Programming by Antioxidant Therapy

Due to the damage incurred by oxidative stress in both FA and HFD, there has been renewed interest in antioxidant therapy. *N*-acetyl cysteine (NAC), combined with alpha-lipoic acid, was found to genetically stabilize FA patient lymphocytes[242]. NAC supplementation in *ex vivo* cultures of primary bone marrow from FA patients, combined with hypoxia (which reduces oxidative stress and mimics the oxygen tension of the bone marrow microenvironment), improved their survival, viability, and clonogenicity[72]. In hematopoietic research outside of FA, NAC was demonstrated to ameliorate myeloproliferation and increase lifespan in *Stat3*$^{-/-}$ animals that had a ROS overproduction phenotype[123]. NAC also ameliorated GAPDH deficiency and developmental defects in rat embryos that were cultured under high glucose[229]. A different antioxidant, Tempol, which mimics the effect of superoxide dismutase, lowered DNA damage in FA fibroblasts and delayed cancer onset in *Fancd2*$^{-/-}$ *Trp53*$^{+/-}$ mice[243]. Yet another antioxidant tested in FA mice is resveratrol, which partially normalizes the cell

cycle status and quantity of *Fancd2*$^{-/-}$ KSL cells[137]. Quercetin is an antioxidant that has been shown to improve insulin signaling in HFD-fed, insulin-resistant FA mice and prevent damage to the endothelium in HFD-programmed placentas in C57BL/6 mice[244,245]. Overall, a diverse collection of data support the hypothesis that antioxidants may be used in FA programming to protect HSPCs and their DNA from oxidative damage (Fig. 6.2A).

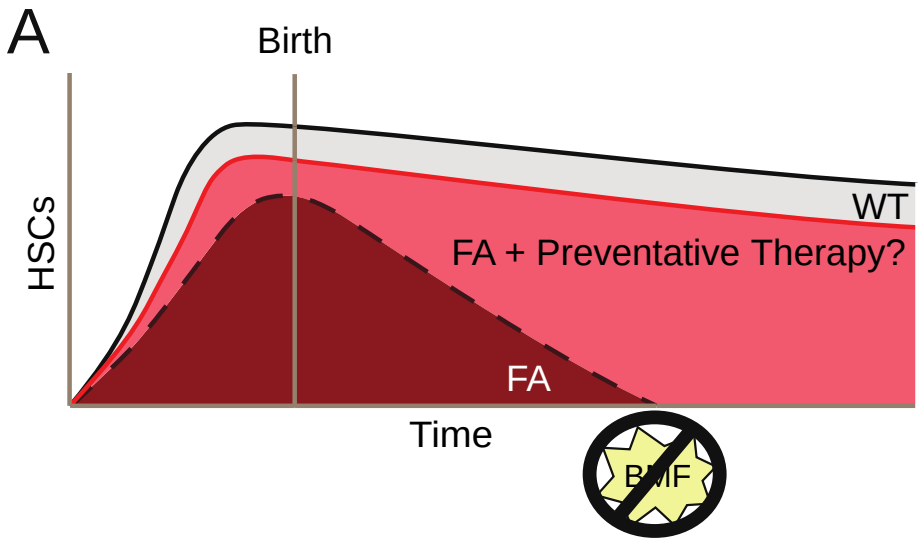


Figure 6.2: Potential for preventative therapy during development. (A) Prenatal and early postnatal life are critical windows of development during which preventative therapy, such as with antioxidant compounds, may be uniquely powerful to boost hematopoietic stem cell numbers and reduce the risk of bone marrow failure.

Epigenetics

DNA damage has been demonstrated to modify the chromatin state, which may epigenetically alter gene expression[121]. Epigenetic changes are one of the main mechanisms by which fetal programming is thought to occur, and though this usually occurs by histone modification and DNA methylation, damage-driven chromatin modification may occur in the cases of FA, HFD-programming, or

maternal obesity. We found that *Hmga2*, a chromatin-associated protein involved in stem cell self-renewal, genomic instability, and obesity, was upregulated in both *Fancc*$^{-/-}$ HSPC-enriched cells and chronic HFD whole fetal liver, and we also found evidence of DNA damage in these models (Chapters 3 and 4 herein). Further studies will need to clarify whether *Hmga2* expression and DNA damage coincide, or if they are independent processes.

Potential Indirect Effects on Fetal Liver HSPC

Another effect of HFD and maternal obesity that would be a fascinating avenue for investigation is whether the effects on fetal liver HSPCs are direct or indirect, as effects on other tissues could impact surrounding cells. Both the placenta and the nervous system appear to be targets, and toxicity to the surrounding stromal cells, such as endothelial cells or hepatoblasts, which are in close contact with HSCs, may need to be considered as well[14,165,245-248]. We and others have documented changes in placental size from HFD (Chapter 4 herein), and inflammation in this tissue has been reported from HFD or maternal obesity[246]. Furthermore, a prenatal diet high in saturated fat was found to cause lipid peroxidation and vasculopathy in the murine placenta[245]. Placental dysfunction could affect nutrient and oxygen transport to the liver, impacting HSPC expansion or survival, or given that the placenta is a hematopoietic niche, could have a negative impact on resident HSPCs there which later seed the liver[249,250]. Additionally, Grant *et al.* reported a reduction in sympathetic innervation of the liver in nonhuman primates that were 1 year old, though it is unknown whether

this defect arose *in utero* or postnatally[125]. Little is known about the crosstalk between the brain and the liver, especially in the fetus, but the brain is involved in metabolic control of the liver and the sympathetic nervous system regulates circadian HSC trafficking[251,252]. Altogether, much remains to be elucidated in the potential indirect effects of HFD and maternal obesity on fetal liver HSPC, and these may need to be considered in future investigations.

In summary, the molecular pathology of FA, HFD programming, and maternal obesity have been documented to share several characteristics, including involvement of ROS and aldehydes, a propensity for oxidative stress and DNA damage, mitochondrial dysfunction, and promising responses to antioxidants. Though aspects have yet to be examined for potential contributions to the pathology of the FA or HFD-programmed 14.5 dpc fetal mouse, our current findings illustrate some common phenotypic characteristics of the liver between these models. We have found reductions in fetal liver size in FA mice and in chronic HFD fetuses, and smaller HSPC pools in these and diet reversal fetal livers. Impaired short-term repopulation was seen in chronic HFD, diet reversal HSPC, and *Fancc*$^{-/-}$ fetal livers, with significantly diminished serial repopulation in the latter. Transcript analysis has identified upregulated DNA repair genes in HFD and obese-programmed fetal livers, as well as *Fancc*$^{-/-}$ and *Fancd2*$^{-/-}$ HSPC. Finally, transcriptional upregulation of chromatin-organizing protein *Hmga2* occurred in both chronic HFD livers and *Fancc*$^{-/-}$ ASL cells; it may indicate a push for self-renewal in these models or further potentiate genomic instability by

inhibition of NHEJ repair. Future investigations in both FA and HFD-programmed fetal livers will focus on delineating mechanisms that impair HSC self renewal, particularly the nature of DNA damage and the cellular response.

Conclusions

Until now, fetal programming of the hematopoietic stem and progenitor cell compartment has been largely unexplored. The onset of Fanconi anemia-associated bone marrow failure at a median of 7 years of age has long been thought to imply the predominant, or even isolated, postnatal loss of HSCs, but in this dissertation, I have demonstrated in three different FA mouse models that there is a prenatal deficiency in HSPCs, with a repopulating defect at least in *Fancc*$^{-/-}$, and that this occurs spontaneously and in the absence of an additional gene knockout or chemical induction to reveal hematopoietic deficiencies[59,60,253]. Evidence of DNA damage response via γH2AX foci, along with the induction of genes involved in DNA repair in HSPC-enriched populations of the fetal liver, suggest that the early accumulation of genomic damage during HSPC pool expansion marks the onset of hematopoietic failure in FA. Here, the relative deficiency of HSPCs in the fetus may exert additional replicative stress on existing stem cells, as fewer of them are present to form the pool of definitive HSC that form the blood system. This is expected to be exacerbated postnatally in times of stress, such as infection or blood loss[134,254]. Increased proportions of FA fetal liver HSPCs are in G_1 phase of cell cycle, suggesting cell cycle arrest. Further analysis of the FA HSPC-enriched population revealed a modest

increase in foci formed by γH2AX, implicating the DNA damage response, and this was supported by gene expression analysis in FA fetal liver HSPC-enriched cells, revealing upregulation of NHEJ and HR genes. My work is the first to assess developmental hematopoiesis both quantitatively and functionally in whole animal models of FA.

In an alternative model, I have examined fetal programming and demonstrated that a prenatal high-fat diet and maternal obesity impact the fetal liver HSPC compartment. While HFD in the absence of obesity, elevates fetal weight and liver cellularity, HFD paired with maternal obesity reduces fetal weight, liver cell counts, and KSL cell content, and induces a deficiency in short-term hematopoietic repopulation capacity. Though diet reversal (obesity without HFD) ameliorates some of these effects and improves short-term engraftment, a reduction in HSPC content in the fetal liver persists. All three conditions increased fetal liver lymphoid cell content, suggesting an early skewing of differentiation. Gene expression analysis of HFD fetal livers and postnatal day 1 HFD livers revealed upregulation of *Trp53bp1*, which encodes a DNA damage binding protein, and a candidate panel of DNA damage checkpoint and repair genes was upregulated in fetal livers as well. Notably, the chromatin regulatory gene *Hmga2* was transcriptionally upregulated by more than two-fold in animals on chronic HFD supplementation, suggesting a potential role for chromatin modification, perturbed self-renewal, or suppression of NHEJ[154,156,157,255].

High-fat diet aggravates the existing hematopoietic defects in the *Fancc*$^{-/-}$ fetus. In addition to decreasing *Fancc*$^{-/-}$ placental and fetal weights, and liver cellularity, it also worsens the deficiency of hematopoietic progenitor cells and modestly increases ROS in the *Fancc*$^{-/-}$ fetal liver.

I have investigated the contribution of the developmental origins of disease in the fetal hematopoietic stem and progenitor cell compartment in Fanconi anemia, prenatal high-fat diet, and maternal obesity. I have demonstrated that fetal HSPCs are both quantitatively and functionally deficient in FA, which likely means the system is built with a hematopoietic handicap from the earliest point in life. Understanding the basis of HSPC pool constraints in FA presents a compelling opportunity to explore preventative therapies that mitigate the risk of hematopoietic failure as a chief cause of morbidity and mortality in patients. The effect of HFD on FA HSPCs should also encourage nutritional guidance and intervention for both FA parents and patients, especially since patients are at increased risk for type 2 diabetes. Likewise, the effects of HFD on hematopoietic development, even in the absence of a genetic defect such as FA, opens the DOHaD field to hematopoietic stem cell research, providing the first evidence of nutritional programming in the hematopoietic stem cell compartment. Overall, this thesis demonstrates the vulnerability of fetal liver HSPCs to prenatal nutritional and genomic insults and provides the basis for the studies that will fully elucidate pathways that can be targeted for therapeutic intervention.

REFERENCES

1. Barker DJ, Osmond C. Infant mortality, childhood nutrition, and ischaemic heart disease in England and Wales. *Lancet*. 1986;1(8489):1077-1081.
2. Barker DJ, Winter PD, Osmond C, Margetts B, Simmonds SJ. Weight in infancy and death from ischaemic heart disease. *Lancet*. 1989;2(8663):577-580.
3. Barker DJP. The origins of the developmental origins theory. *J Intern Med*. 2007;261(5):412-417.
4. Barker DJ, Meade TW, Fall CH, et al. Relation of fetal and infant growth to plasma fibrinogen and factor VII concentrations in adult life. *BMJ*. 1992;304(6820):148-152.
5. Hales CN, Barker DJ. Type 2 (non-insulin-dependent) diabetes mellitus: the thrifty phenotype hypothesis. *Diabetologia*. 1992;35(7):595-601.
6. Barker DJ, Bull AR, Osmond C, Simmonds SJ. Fetal and placental size and risk of hypertension in adult life. *BMJ*. 1990;301(6746):259-262.
7. Yajnik CS. Early life origins of insulin resistance and type 2 diabetes in India and other Asian countries. *J Nutr*. 2004;134(1):205-210.
8. Kuzawa CW, Adair LS. Lipid profiles in adolescent Filipinos: relation to birth weight and maternal energy status during pregnancy. *Am J Clin Nutr*. 2003;77(4):960-966.
9. Popkin BM. The nutrition transition and obesity in the developing world. *J Nutr*. 2001;131(3):871S-873S.
10. Cordain L, Eaton SB, Sebastian A, et al. Origins and evolution of the Western diet: health implications for the 21st century. *Am J Clin Nutr*. 2005;81(2):341-354.
11. Flegal KM, Carroll MD, Kit BK, Ogden CL. Prevalence of obesity and trends in the distribution of body mass index among US adults, 1999-2010. *JAMA*. 2012;307(5):491-497.
12. Drake AJ, Reynolds RM. Impact of maternal obesity on offspring obesity and cardiometabolic disease risk. *Reproduction*. 2010;140(3):387-398.
13. Li M, Sloboda DM, Vickers MH. Maternal obesity and developmental programming of metabolic disorders in offspring: evidence from animal models. *Experimental Diabetes Research*. 2011;2011:592408.
14. McCurdy CE, Bishop JM, Williams SM, et al. Maternal high-fat diet triggers lipotoxicity in the fetal livers of nonhuman primates. *J Clin Invest*. 2009;119(2):323-335.
15. Catalano PM, Presley L, Minium J, Hauguel-De Mouzon S. Fetuses of Obese Mothers Develop Insulin Resistance in Utero. *Diabetes Care*. 2009;32(6):1076-1080.
16. Leddy MA, Power ML, Schulkin J. The impact of maternal obesity on maternal and fetal health. *Rev Obstet Gynecol*. 2009;1(4):170-178.
17. Catalano PM, Farrell K, Thomas A, et al. Perinatal risk factors for childhood obesity and metabolic dysregulation. *Am J Clin Nutr*. 2009;90(5):1303-1313.

18. Lamb MM, Myers MA, Barriga K, Zimmet PZ, Rewers M, Norris JM. Maternal diet during pregnancy and islet autoimmunity in offspring. *Pediatr Diabetes*. 2008;9(2):135-141.
19. Tam WH, Ma RCW, Yang X, et al. Glucose intolerance and cardiometabolic risk in children exposed to maternal gestational diabetes mellitus in utero. *PEDIATRICS*. 2008;122(6):1229-1234.
20. Momb J, Lewandowski JP, Bryant JD, et al. Deletion of Mthfd1l causes embryonic lethality and neural tube and craniofacial defects in mice. *Proc Natl Acad Sci USA*. 2013;110(2):549-554.
21. Zhao R, Russell RG, Wang Y, et al. Rescue of embryonic lethality in reduced folate carrier-deficient mice by maternal folic acid supplementation reveals early neonatal failure of hematopoietic organs. *J Biol Chem*. 2001;276(13):10224-10228.
22. Fanconi G. Familial constitutional panmyelocytopathy, Fanconi's anemia (F.A.). I. Clinical aspects. *Semin Hematol*. 1967;4(3):233-240.
23. Rosenberg PS, Tamary H, Alter BP. How high are carrier frequencies of rare recessive syndromes? Contemporary estimates for Fanconi Anemia in the United States and Israel. *Am J Med Genet*. 2011;155A(8):1877-1883.
24. Kutler DI. A 20-year perspective on the International Fanconi Anemia Registry (IFAR). *Blood*. 2003;101(4):1249-1256.
25. D'Andrea AD. Susceptibility pathways in Fanconi's anemia and breast cancer. *N Engl J Med*. 2010;362(20):1909-1919.
26. Tischkowitz MD, Hodgson SV. Fanconi anaemia. *Journal of Medical Genetics*. 2003;40(1):1-10.
27. Alter BP. Fanconi's anemia and malignancies. *Am J Hematol*. 1996;53(2):99-110.
28. Lobitz S, Velleuer E. Guido Fanconi (1892-1979): a jack of all trades. *Nat Rev Cancer*. 2006;6(11):893-898.
29. De Winter JP, Joenje H. The genetic and molecular basis of Fanconi anemia. *Mutat Res*. 2009;668(1-2):11-19.
30. Auerbach AD, Wolman SR. Susceptibility of Fanconi's anaemia fibroblasts to chromosome damage by carcinogens. *Nature*. 1976;261(5560):494-496.
31. Loecken EM, Dasari S, Hill S, Tabb DL, Guengerich FP. The bis-electrophile diepoxybutane cross-links DNA to human histones but does not result in enhanced mutagenesis in recombinant systems. *Chem Res Toxicol*. 2009;22(6):1069-1076.
32. Joenje H, Levitus M, Waisfisz Q, et al. Complementation analysis in Fanconi anemia: assignment of the reference FA-H patient to group A. *Am J Hum Genet*. 2000;67(3):759-762.
33. Chandra S, Levran O, Jurickova I, et al. A Rapid Method for Retrovirus-Mediated Identification of Complementation Groups in Fanconi Anemia Patients. *Molecular Therapy*. 2005;12(5):976-984.
34. Niedzwiedz W, Mosedale G, Johnson M, Ong CY, Pace P, Patel KJ. The Fanconi anaemia gene FANCC promotes homologous recombination and error-prone DNA repair. *Mol Cell*. 2004;15(4):607-620.

35. Moldovan G-L, D'Andrea AD. How the fanconi anemia pathway guards the genome. *Annu Rev Genet*. 2009;43:223-249.
36. Leung JWC, Wang Y, Fong KW, Huen MSY, Li L, Chen J. Fanconi anemia (FA) binding protein FAAP20 stabilizes FA complementation group A (FANCA) and participates in interstrand cross-link repair. *Proc Natl Acad Sci USA*. 2012;109(12):4491-4496.
37. Machida YJ, Machida Y, Chen Y, et al. UBE2T is the E2 in the Fanconi anemia pathway and undergoes negative autoregulation. *Mol Cell*. 2006;23(4):589-596.
38. Sareen A, Chaudhury I, Adams N, Sobeck A. Fanconi anemia proteins FANCD2 and FANCI exhibit different DNA damage responses during S-phase. *Nucleic Acids Res*. 2012;40(17):8425-8439.
39. MacKay C, Déclais A-C, Lundin C, et al. Identification of KIAA1018/FAN1, a DNA repair nuclease recruited to DNA damage by monoubiquitinated FANCD2. *Cell*. 2010;142(1):65-76.
40. Hanada K, Budzowska M, Davies SL, et al. The structure-specific endonuclease Mus81 contributes to replication restart by generating double-strand DNA breaks. *Nat Struct Mol Biol*. 2007;14(11):1096-1104.
41. Crossan GP, Patel KJ. The Fanconi anaemia pathway orchestrates incisions at sites of crosslinked DNA. *The Journal of pathology*. 2011.
42. Gupta R, Sharma S, Sommers JA, Jin Z, Cantor SB, Brosh RM. Analysis of the DNA substrate specificity of the human BACH1 helicase associated with breast cancer. *J Biol Chem*. 2005;280(27):25450-25460.
43. Schwab RA, Nieminuszczy J, Shin-ya K, Niedzwiedz W. FANCJ couples replication past natural fork barriers with maintenance of chromatin structure. *J Cell Biol*. 2013;201(1):33-48.
44. Howlett NG, Taniguchi T, Olson S, et al. Biallelic inactivation of BRCA2 in Fanconi anemia. *Science*. 2002;297(5581):606-609.
45. Godthelp BC, Wiegant WW, Waisfisz Q, et al. Inducibility of nuclear Rad51 foci after DNA damage distinguishes all Fanconi anemia complementation groups from D1/BRCA2. *Mutat Res*. 2006;594(1-2):39-48.
46. Hussain S, Wilson JB, Medhurst AL, et al. Direct interaction of FANCD2 with BRCA2 in DNA damage response pathways. *Hum Mol Genet*. 2004;13(12):1241-1248.
47. Wang X, Andreassen PR, D'Andrea AD. Functional interaction of monoubiquitinated FANCD2 and BRCA2/FANCD1 in chromatin. *Molecular and Cellular Biology*. 2004;24(13):5850-5862.
48. Xia B, Sheng Q, Nakanishi K, et al. Control of BRCA2 cellular and clinical functions by a nuclear partner, PALB2. *Mol Cell*. 2006;22(6):719-729.
49. Kim Y, Spitz GS, Veturi U, Lach FP, Auerbach AD, Smogorzewska A. Regulation of multiple DNA repair pathways by the Fanconi anemia protein SLX4. *Blood*. 2013;121(1):54-63.
50. Bogliolo M, Schuster B, Stoepker C, et al. Mutations in ERCC4, encoding the DNA-repair endonuclease XPF, cause Fanconi anemia. *Am J Hum Genet*. 2013;92(5):800-806.

51. Gregg SQ, Robinson AR, Niedernhofer LJ. Physiological consequences of defects in ERCC1-XPF DNA repair endonuclease. *DNA Repair*. 2011;10(7):781-791.
52. Mohrin M, Bourke E, Alexander D, et al. Hematopoietic Stem Cell Quiescence Promotes Error-Prone DNA Repair and Mutagenesis. *Cell Stem Cell*. 2010;7(2):174-185.
53. Van Gent DC, Van Der Burg M. Non-homologous end-joining, a sticky affair. *Oncogene*. 2007;26(56):7731-7740.
54. Adamo A, Collis SJ, Adelman CA, et al. Preventing Nonhomologous End Joining Suppresses DNA Repair Defects of Fanconi Anemia. *Mol Cell*. 2010;39(1):25-35.
55. Pace P, Mosedale G, Hodskinson MR, Rosado IV, Sivasubramaniam M, Patel KJ. Ku70 corrupts DNA repair in the absence of the Fanconi anemia pathway. *Science*. 2010;329(5988):219-223.
56. Edfeldt NBF. Solution structure of a nitrous acid induced DNA interstrand cross-link. *Nucleic Acids Research*. 2004;32(9):2785-2794.
57. Cole RS. Light-induced cross-linking of DNA in the presence of a furocoumarin (psoralen). Studies with phage lambda, Escherichia coli, and mouse leukemia cells. *Biochim Biophys Acta*. 1970;217(1):30-39.
58. Cole RS. Repair of DNA containing interstrand crosslinks in Escherichia coli: sequential excision and recombination. *Proc Natl Acad Sci USA*. 1973;70(4):1064-1068.
59. Garaycoechea JI, Crossan GP, Langevin F, Daly M, Arends MJ, Patel KJ. Genotoxic consequences of endogenous aldehydes on mouse haematopoietic stem cell function. *Nature*. 2012:1-7.
60. Langevin F, Crossan GP, Rosado IV, Arends MJ, Patel KJ. Fancd2 counteracts the toxic effects of naturally produced aldehydes in mice. *Nature*. 2011;475(7354):53-58.
61. Voulgaridou G-P, Anestopoulos I, Franco R, Panayiotidis MI, Pappa A. DNA damage induced by endogenous aldehydes: Current state of knowledge. *Mutat Res*. 2011;711(1-2):13-27.
62. Brooks PJ, Enoch M-A, Goldman D, Li T-K, Yokoyama A. The Alcohol Flushing Response: An Unrecognized Risk Factor for Esophageal Cancer from Alcohol Consumption. *Plos Med*. 2009;6(3):e50.
63. Crabb DW, Matsumoto M, Chang D, You M. Overview of the role of alcohol dehydrogenase and aldehyde dehydrogenase and their variants in the genesis of alcohol-related pathology. *Proc Nutr Soc*. 2004;63(1):49-63.
64. Hira A, Yabe H, Yoshida K, et al. Variant ALDH2 is associated with accelerated progression of bone marrow failure in Japanese Fanconi anemia patients. *Blood*. 2013;122(18):3206-3209.
65. Antoshechkin AG. On intracellular formation of ethanol and its possible role in energy metabolism. *Alcohol Alcohol*. 2001;36(6):608.
66. Kottemann MC, Smogorzewska A. Fanconi anaemia and the repair of Watson and Crick DNA crosslinks. *Nature*. 2013;493(7432):356-363.
67. Pang Q, Andreassen PR. Fanconi anemia proteins and endogenous stresses. *Mutat Res*. 2009;668(1-2):42-53.

68. Stone MP, Cho Y-J, Huang H, et al. Interstrand DNA cross-links induced by alpha,beta-unsaturated aldehydes derived from lipid peroxidation and environmental sources. *Acc Chem Res*. 2008;41(7):793-804.
69. Schindler D, Hoehn H. Fanconi anemia mutation causes cellular susceptibility to ambient oxygen. *Am J Hum Genet*. 1988;43(4):429-435.
70. Pagano G, Aiello Talamanca A, Castello G, Pallardo FV, Zatterale A, Degan P. Oxidative stress in Fanconi anaemia: from cells and molecules toward prospects in clinical management. *Biol Chem*. 2011.
71. Zhang X, Sejas DP, Qiu Y, Williams DA, Pang Q. Inflammatory ROS promote and cooperate with the Fanconi anemia mutation for hematopoietic senescence. *J Cell Sci*. 2007;120(Pt 9):1572-1583.
72. Cohen-Haguenauer O, Péault B, Bauche C, et al. In vivo repopulation ability of genetically corrected bone marrow cells from Fanconi anemia patients. *Proc Natl Acad Sci USA*. 2006;103(7):2340-2345.
73. Auerbach AD, Liu Q, Ghosh R, Pollack MS, Douglas GW, Broxmeyer HE. Prenatal identification of potential donors for umbilical cord blood transplantation for Fanconi anemia. *Transfusion*. 1990;30(8):682-687.
74. Abu-Issa R, Eichele G, Youssoufian H. Expression of the Fanconi anemia group A gene (Fanca) during mouse embryogenesis. *Blood*. 1999;94(2):818-824.
75. Tulpule A, Lensch MW, Miller JD, et al. Knockdown of Fanconi anemia genes in human embryonic stem cells reveals early developmental defects in the hematopoietic lineage. *Blood*. 2010;115(17):3453-3462.
76. Ceccaldi R, Parmar K, Mouly E, et al. Bone Marrow Failure in Fanconi Anemia Is Triggered by an Exacerbated p53/p21 DNA Damage Response that Impairs Hematopoietic Stem and Progenitor Cells. *Cell Stem Cell*. 2012:1-20.
77. Jaffredo T, Nottingham W, Liddiard K, Bollerot K, Pouget C, de Bruijn M. From hemangioblast to hematopoietic stem cell: an endothelial connection? *Exp Hematol*. 2005;33(9):1029-1040.
78. Sturgeon CM, Ditadi A, Clarke RL, Keller G. Defining the path to hematopoietic stem cells. *Nat Biotechnol*. 2013;31(5):416-418.
79. Medvinsky A, Dzierzak E. Definitive hematopoiesis is autonomously initiated by the AGM region. *Cell*. 1996;86(6):897-906.
80. de Bruijn MF, Speck NA, Peeters MC, Dzierzak E. Definitive hematopoietic stem cells first develop within the major arterial regions of the mouse embryo. *EMBO J*. 2000;19(11):2465-2474.
81. Gekas C, Dieterlen-Lièvre F, Orkin SH, Mikkola HKA. The placenta is a niche for hematopoietic stem cells. *Dev Cell*. 2005;8(3):365-375.
82. Müller AM, Medvinsky A, Strouboulis J, Grosveld F, Dzierzak E. Development of hematopoietic stem cell activity in the mouse embryo. *Immunity*. 1994;1(4):291-301.
83. Mikkola HK, Orkin SH. The journey of developing hematopoietic stem cells. *Development*. 2006;133(19):3733-3744.
84. Christensen JL, Wright DE, Wagers AJ, Weissman IL. Circulation and chemotaxis of fetal hematopoietic stem cells. *Plos Biol*. 2004;2(3):E75.
85. Yokota T, Huang J, Tavian M, et al. Tracing the first waves of lymphopoiesis in mice. *Development*. 2006;133(10):2041-2051.

86. Morrison SJ, Hemmati HD, Wandycz AM, Weissman IL. The purification and characterization of fetal liver hematopoietic stem cells. *Proc Natl Acad Sci USA*. 1995;92(22):10302-10306.
87. Shiojiri N. Development and differentiation of bile ducts in the mammalian liver. *Microsc Res Tech*. 1997;39(4):328-335.
88. Wolber FM, Leonard E, Michael S, Orschell-Traycoff CM, Yoder MC, Srour EF. Roles of spleen and liver in development of the murine hematopoietic system. *Exp Hematol*. 2002;30(9):1010-1019.
89. Weiss L. A scanning electron microscopic study of the spleen. *Blood*. 1974;43(5):665-691.
90. O'Malley D, Kim YS, Perkins SL, Baldridge L, Juliar BE, Orazi A. Morphologic and immunohistochemical evaluation of splenic hematopoietic proliferations in neoplastic and benign disorders. *Mod Pathol*. 2005;18(12):1550-1561.
91. Purton LE, Scadden DT. The hematopoietic stem cell niche. *StemBook*. 2008:1-14.
92. Kim I, He S, Yilmaz OH, Kiel MJ, Morrison SJ. Enhanced purification of fetal liver hematopoietic stem cells using SLAM family receptors. *Blood*. 2006;108(2):737-744.
93. Ikuta K, Kina T, MacNeil I, et al. A developmental switch in thymic lymphocyte maturation potential occurs at the level of hematopoietic stem cells. *Cell*. 1990;62(5):863-874.
94. Ikuta K, Weissman IL. Evidence that hematopoietic stem cells express mouse c-kit but do not depend on steel factor for their generation. *Proc Natl Acad Sci USA*. 1992;89(4):1502-1506.
95. Kiel MJ, Yilmaz OH, Iwashita T, Yilmaz OH, Terhorst C, Morrison SJ. SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. *Cell*. 2005;121(7):1109-1121.
96. Christensen JL, Weissman IL. Flk-2 is a marker in hematopoietic stem cell differentiation: a simple method to isolate long-term stem cells. *Proc Natl Acad Sci USA*. 2001;98(25):14541-14546.
97. Kim I, Yilmaz OH, Morrison SJ. CD144 (VE-cadherin) is transiently expressed by fetal liver hematopoietic stem cells. *Blood*. 2005;106(3):903-905.
98. Petrenko O, Beavis A, Klaine M, Kittappa R, Godin I, Lemischka IR. The molecular characterization of the fetal stem cell marker AA4. *Immunity*. 1999;10(6):691-700.
99. Jordan CT, McKearn JP, Lemischka IR. Cellular and developmental properties of fetal hematopoietic stem cells. *Cell*. 1990;61(6):953-963.
100. Jordan CT, Astle CM, Zawadzki J, Mackarehtschian K, Lemischka IR, Harrison DE. Long-term repopulating abilities of enriched fetal liver stem cells measured by competitive repopulation. *Exp Hematol*. 1995;23(9):1011-1015.
101. Fleming WH, Alpern EJ, Uchida N, Ikuta K, Spangrude GJ, Weissman IL. Functional heterogeneity is associated with the cell cycle status of murine hematopoietic stem cells. *J Cell Biol*. 1993;122(4):897-902.

102. Wilson A, Laurenti E, Oser G, et al. Hematopoietic stem cells reversibly switch from dormancy to self-renewal during homeostasis and repair. *Cell*. 2008;135(6):1118-1129.
103. Wilson A, Laurenti E, Oser G, et al. Hematopoietic Stem Cells Reversibly Switch from Dormancy to Self-Renewal during Homeostasis and Repair. *Cell*. 2008;135(6):1118-1129.
104. Takizawa H, Regoes RR, Boddupalli CS, Bonhoeffer S, Manz MG. Dynamic variation in cycling of hematopoietic stem cells in steady state and inflammation. *J Exp Med*. 2011.
105. Rossi DJ, Seita J, Czechowicz A, Bhattacharya D, Bryder D, Weissman IL. Hematopoietic stem cell quiescence attenuates DNA damage response and permits DNA damage accumulation during aging. *Cell Cycle*. 2007;6(19):2371-2376.
106. Mandal Pankaj K, Rossi Derrick J. DNA-Damage-Induced Differentiation in Hematopoietic Stem Cells. *Cell*. 2012;148(5):847-848.
107. Takubo K, Nagamatsu G, Kobayashi Chiharu I, et al. Regulation of Glycolysis by Pdk Functions as a Metabolic Checkpoint for Cell Cycle Quiescence in Hematopoietic Stem Cells. *Cell Stem Cell*. 2013;12(1):49-61.
108. Ito K, Carracedo A, Weiss D, et al. A PML–PPAR-δ pathway for fatty acid oxidation regulates hematopoietic stem cell maintenance. *Nat Med*. 2012;18(9):1350-1358.
109. Odaka Y, Nakano M, Tanaka T, et al. The influence of a high-fat dietary environment in the fetal period on postnatal metabolic and immune function. *Obesity (Silver Spring)*. 2010;18(9):1688-1694.
110. Bouchard L, Thibault S, Guay S-P, et al. Leptin Gene Epigenetic Adaptation to Impaired Glucose Metabolism During Pregnancy. *Diabetes Care*. 2010;33(11):2436-2441.
111. Bouchard L, Hivert M-F, Guay S-P, St-Pierre J, Perron P, Brisson D. Placental Adiponectin Gene DNA Methylation Levels Are Associated With Mothers' Blood Glucose Concentration. *Diabetes*. 2012;61(5):1272-1280.
112. Nicholas LM, Rattanatray L, Maclaughlin SM, et al. Differential effects of maternal obesity and weight loss in the periconceptional period on the epigenetic regulation of hepatic insulin-signaling pathways in the offspring. *FASEB J*. 2013;27(9):3786-3796.
113. Ruchat S-M, Hivert M-F, Bouchard L. Epigenetic programming of obesity and diabetes by in utero exposure to gestational diabetes mellitus. *Nutr Rev*. 2013;71:S88-S94.
114. Sookoian S, Gianotti TF, Burgueño AL, Pirola CJ. Fetal metabolic programming and epigenetic modifications: a systems biology approach. *Pediatr Res*. 2013;73(4-2):531-542.
115. Handel AE, Ebers GC, Ramagopalan SV. Epigenetics: molecular mechanisms and implications for disease. *Trends Mol Med*. 2010;16(1):7-16.
116. Bernstein BE, Meissner A, Lander ES. The mammalian epigenome. *Cell*. 2007;128(4):669-681.

117. Haines TR, Rodenhiser DI, Ainsworth PJ. Allele-Specific Non-CpG Methylation of the Nf1 Gene during Early Mouse Development. *Developmental Biology*. 2001;240(2):585-598.
118. Ramsahoye BH, Biniszkiewicz D, Lyko F, Clark V, Bird AP, Jaenisch R. Non-CpG methylation is prevalent in embryonic stem cells and may be mediated by DNA methyltransferase 3a. *Proc Natl Acad Sci USA*. 2000;97(10):5237-5242.
119. Aagaard-Tillery KM, Grove K, Bishop J, et al. Developmental origins of disease and determinants of chromatin structure: maternal diet modifies the primate fetal epigenome. *J Mol Endocrinol*. 2008;41(2):91-102.
120. Schuler N, Rübe CE. Accumulation of DNA Damage-Induced Chromatin Alterations in Tissue-Specific Stem Cells: The Driving Force of Aging? *PLoS ONE*. 2013;8(5):e63932.
121. Polo SE, Almouzni G. DNA damage leaves its mark on chromatin. *Cell Cycle*. 2007;6(19):2355-2359.
122. Igosheva N, Abramov AY, Poston L, et al. Maternal Diet-Induced Obesity Alters Mitochondrial Activity and Redox Status in Mouse Oocytes and Zygotes. *PLoS ONE*. 2010;5(4):e10074.
123. Mantel C, Messina-Graham S, Moh A, et al. Mouse hematopoietic cell-targeted STAT3 deletion: stem/progenitor cell defects, mitochondrial dysfunction, ROS overproduction, and a rapid aging-like phenotype. *Blood*. 2012:1-34.
124. Binder NK, Hannan NJ, Gardner DK. Paternal Diet-Induced Obesity Retards Early Mouse Embryo Development, Mitochondrial Activity and Pregnancy Health. *PLoS ONE*. 2012;7(12):e52304.
125. Grant WF, Nicol LE, Thorn SR, Grove KL, Friedman JE, Marks DL. Perinatal exposure to a high-fat diet is associated with reduced hepatic sympathetic innervation in one-year old male Japanese macaques. *PLoS One*. 2012;7(10):e48119.
126. Tischkowitz M, Dokal I. Fanconi anaemia and leukaemia - clinical and molecular aspects. *Br J Haematol*. 2004;126(2):176-191.
127. Haneline LS, Gobbett TA, Ramani R, et al. Loss of FancC function results in decreased hematopoietic stem cell repopulating ability. *Blood*. 1999;94(1):1-8.
128. Barroca V, Mouthon MA, Lewandowski D, et al. Impaired functionality and homing of Fancg-deficient hematopoietic stem cells. *Hum Mol Genet*. 2012;21(1):121-135.
129. Parmar K, Kim J, Sykes SM, et al. Hematopoietic Stem Cell Defects in Mice with Deficiency of Fancd2 or Usp1. *Stem Cells*. 2010;28(7):1186-1195.
130. Chen M, Tomkins DJ, Auerbach W, et al. Inactivation of Fac in mice produces inducible chromosomal instability and reduced fertility reminiscent of Fanconi anaemia. *Nat Genet*. 1996;12(4):448-451.
131. Houghtaling S, Timmers C, Noll M, et al. Epithelial cancer in Fanconi anemia complementation group D2 (Fancd2) knockout mice. *Genes Dev*. 2003;17(16):2021-2035.
132. Carreau M. Not-so-novel phenotypes in the Fanconi anemia group D2 mouse model. *Blood*. 2004;103(6):2430-2430.

133. Whitney MA, Royle G, Low MJ, et al. Germ cell defects and hematopoietic hypersensitivity to gamma-interferon in mice with a targeted disruption of the Fanconi anemia C gene. *Blood*. 1996;88(1):49-58.
134. Haneline LS, Broxmeyer HE, Cooper S, et al. Multiple inhibitory cytokines induce deregulated progenitor growth and apoptosis in hematopoietic cells from Fac-/- mice. *Blood*. 1998;91(11):4092-4098.
135. Du W, Adam Z, Rani R, Zhang X, Pang Q. Oxidative stress in Fanconi anemia hematopoiesis and disease progression. *Antioxid Redox Signal*. 2008;10(11):1909-1921.
136. Rani R, Li J, Pang Q. Differential p53 Engagement in Response to Oxidative and Oncogenic Stresses in Fanconi Anemia Mice. *Cancer Res*. 2008;68(23):9693-9702.
137. Zhang Q-S, Marquez-Loza L, Eaton L, et al. Fancd2-/- mice have hematopoietic defects that can be partially corrected by resveratrol. *Blood*. 2010;116(24):5140-5148.
138. Carreau M, Gan OI, Liu L, Doedens M, Dick JE, Buchwald M. Hematopoietic compartment of Fanconi anemia group C null mice contains fewer lineage-negative CD34+ primitive hematopoietic cells and shows reduced reconstruction ability. *Exp Hematol*. 1999;27(11):1667-1674.
139. Skinner AM, O'Neill SL, Grompe M, Kurre P. CXCR4 induction in hematopoietic progenitor cells from Fanca(-/-), -c(-/-), and -d2(-/-) mice. *Exp Hematol*. 2008;36(3):273-282.
140. Kent DG, Copley MR, Benz C, et al. Prospective isolation and molecular characterization of hematopoietic stem cells with durable self-renewal potential. *Blood*. 2009;113(25):6342-6350.
141. Faivre L, Guardiola P, Lewis C, et al. Association of complementation group and mutation type with clinical outcome in fanconi anemia. European Fanconi Anemia Research Group. *Blood*. 2000;96(13):4064-4070.
142. De Haan G, Nijhof W, Van Zant G. Mouse strain-dependent changes in frequency and proliferation of hematopoietic stem cells during aging: correlation between lifespan and cycling activity. *Blood*. 1997;89(5):1543-1550.
143. Parmar K, D'Andrea A, Niedernhofer LJ. Mouse models of Fanconi anemia. *Mutat Res*. 2009;668(1-2):133-140.
144. Carreau M, Gan OI, Liu L, et al. Bone marrow failure in the Fanconi anemia group C mouse model after DNA damage. *Blood*. 1998;91(8):2737-2744.
145. Rathbun RK, Faulkner GR, Ostroski MH, et al. Inactivation of the Fanconi anemia group C gene augments interferon-gamma-induced apoptotic responses in hematopoietic cells. *Blood*. 1997;90(3):974-985.
146. Sejas DP, Rani R, Qiu Y, et al. Inflammatory reactive oxygen species-mediated hemopoietic suppression in Fancc-deficient mice. *J Immunol*. 2007;178(8):5277-5287.
147. Kamimae-Lanning AN, Goloviznina NA, Kurre P. Fetal origins of hematopoietic failure in a murine model of Fanconi anemia. *Blood*. 2013;121(11):2008-2012.

148. Schlacher K, Wu H, Jasin M. A distinct replication fork protection pathway connects Fanconi anemia tumor suppressors to RAD51-BRCA1/2. *Cancer Cell*. 2012;22(1):106-116.
149. De Haan G, Van Zant G. Dynamic changes in mouse hematopoietic stem cell numbers during aging. *Blood*. 1999;93(10):3294-3301.
150. Zhang S, Yajima H, Huynh H, et al. Congenital bone marrow failure in DNA-PKcs mutant mice associated with deficiencies in DNA repair. *J Cell Biol*. 2011;193(2):295-305.
151. Ruzankina Y, Pinzon-Guzman C, Asare A, et al. Deletion of the developmentally essential gene ATR in adult mice leads to age-related phenotypes and stem cell loss. *Cell Stem Cell*. 2007;1(1):113-126.
152. Niedernhofer LJ. DNA repair is crucial for maintaining hematopoietic stem cell function. *DNA Repair (Amst)*. 2008;7(3):523-529.
153. Prasher JM, Lalai AS, Heijmans-Antonissen C, et al. Reduced hematopoietic reserves in DNA interstrand crosslink repair-deficient Ercc1−/− mice. *EMBO J*. 2005;24(4):861-871.
154. Copley MR, Babovic S, Benz C, et al. The Lin28b--let-7--Hmga2 axis determines the higher self-renewal potential of fetal haematopoietic stem cells. *Nat Cell Biol*. 2013;15(7):1-12.
155. Nishino J, Kim I, Chada K, Morrison SJ. Hmga2 Promotes Neural Stem Cell Self-Renewal in Young but Not Old Mice by Reducing p16Ink4a and p19Arf Expression. *Cell*. 2008;135(2):227-239.
156. Li AYJ, Boo LM, Wang S-Y, et al. Suppression of Nonhomologous End Joining Repair by Overexpression of HMGA2. *Cancer Res*. 2009;69(14):5699-5706.
157. Boo LM, Lin HH, Chung V, et al. High mobility group A2 potentiates genotoxic stress in part through the modulation of basal and DNA damage-dependent phosphatidylinositol 3-kinase-related protein kinase activation. *Cancer Res*. 2005;65(15):6622-6630.
158. Arruda LK, Sole D, Baena-Cagnani CE, Naspitz CK. Risk factors for asthma and atopy. *Curr Opin Allergy Clin Immunol*. 2005;5(2):153-159.
159. Hopkin JM. Mechanisms of enhanced prevalence of asthma and atopy in developed countries. *Curr Opin Immunol*. 1997;9(6):788-792.
160. Cizza G, Brown RJ, Rother KI. Rising incidence and challenges of childhood diabetes. A mini review. *J Endocrinol Invest*. 2012;35(5):541-546.
161. Forlenza GP, Rewers M. The epidemic of type 1 diabetes: what is it telling us? *Curr Opin Endocrinol Diabetes Obes*. 2011;18(4):248-251.
162. Fasano A, Berti I, Gerarduzzi T, et al. Prevalence of celiac disease in at-risk and not-at-risk groups in the United States: a large multicenter study. *Arch Intern Med*. 2003;163(3):286-292.
163. Taylor PD, Poston L. Developmental programming of obesity in mammals. *Experimental Physiology*. 2007;92(2):287-298.
164. Reynolds RM, Allan KM, Raja EA, et al. Maternal obesity during pregnancy and premature mortality from cardiovascular event in adult offspring: follow-up of 1 323 275 person years. *BMJ*. 2013;347:f4539.

165. Luzzo KM, Wang Q, Purcell SH, et al. High Fat Diet Induced Developmental Defects in the Mouse: Oocyte Meiotic Aneuploidy and Fetal Growth Retardation/Brain Defects. *PLoS ONE*. 2012;7(11):e49217.
166. Alfaradhi MZ, Ozanne SE. Developmental programming in response to maternal overnutrition. *Front Gene*. 2011;2:27.
167. Jones KD, Berkley JA, Warner JO. Perinatal nutrition and immunity to infection. *Pediatr Allergy Immunol*. 2010;21(4 Pt 1):564-576.
168. Palmer AC. Nutritionally mediated programming of the developing immune system. *Adv Nutr*. 2011;2(5):377-395.
169. Wagner N-M, Brandhorst G, Czepluch F, et al. Circulating regulatory T cells are reduced in obesity and may identify subjects at increased metabolic and cardiovascular risk. *Obesity (Silver Spring)*. 2013;21(3):461-468.
170. Mold JE, Michaelsson J, Burt TD, et al. Maternal alloantigens promote the development of tolerogenic fetal regulatory T cells in utero. *Science*. 2008;322(5907):1562-1565.
171. Trottier MD, Naaz A, Li Y, Fraker PJ. Inaugural Article: Enhancement of hematopoiesis and lymphopoiesis in diet-induced obese mice. *Proc Natl Acad Sci USA*. 2012;109(20):7622-7629.
172. Gomes AL, Carvalho T, Serpa J, Torre C, Dias S. Hypercholesterolemia promotes bone marrow cell mobilization by perturbing the SDF-1:CXCR4 axis. *Blood*. 2010;115(19):3886-3894.
173. Mold JE, McCune JM. Immunological Tolerance During Fetal Development: From Mouse to Man. *Advances in Immunology*. 2012;115:73-111.
174. Harris JM, Esain V, Frechette GM, et al. Glucose metabolism impacts the spatiotemporal onset and magnitude of HSC induction in vivo. *Blood*. 2013;121(13):2483-2493.
175. Warr MR, Binnewies M, Flach J, et al. FOXO3A directs a protective autophagy program in haematopoietic stem cells. *Nature*. 2013:1-7.
176. Yahata T, Takanashi T, Muguruma Y, et al. Accumulation of oxidative DNA damage restricts the self-renewal capacity of human hematopoietic stem cells. *Blood*. 2011:1-38.
177. Krasnow SM, Nguyen ML, Marks DL. Increased maternal fat consumption during pregnancy alters body composition in neonatal mice. *Am J Physiol Endocrinol Metab*. 2011;301(6):E1243-1253.
178. Sreekumar R, Unnikrishnan J, Fu A, et al. Impact of high-fat diet and antioxidant supplement on mitochondrial functions and gene transcripts in rat muscle. *Am J Physiol Endocrinol Metab*. 2002;282(5):E1055-1061.
179. Loft S, Thorling EB, Poulsen HE. High fat diet induced oxidative DNA damage estimated by 8-oxo-7,8-dihydro-2-deoxyguanosine excretion in rats. *Free Radic Res*. 1998;29(6):595-600.
180. de Assis AM, Rieger DK, Longoni A, et al. High fat and highly thermolyzed fat diets promote insulin resistance and increase DNA damage in rats. *Exp Biol Med (Maywood)*. 2009;234(11):1296-1304.
181. Reeves R, Adair JE. Role of high mobility group (HMG) chromatin proteins in DNA repair. *DNA Repair (Amst)*. 2005;4(8):926-938.

182. Summer H, Li O, Bao Q, et al. HMGA2 exhibits dRP/AP site cleavage activity and protects cancer cells from DNA-damage-induced cytotoxicity during chemotherapy. *Nucleic Acids Research*. 2009;37(13):4371-4384.
183. Anand A, Chada K. In vivo modulation of Hmgic reduces obesity. *Nat Genet*. 2000;24(4):377-380.
184. Watkins ML, Rasmussen SA, Honein MA, Botto LD, Moore CA. Maternal obesity and risk for birth defects. *Pediatrics*. 2003;111(5 Pt 2):1152-1158.
185. Blomberg MI, Källén B. Maternal obesity and morbid obesity: the risk for birth defects in the offspring. *Birth Defects Res Part A Clin Mol Teratol*. 2010;88(1):35-40.
186. Jungheim ES, Schoeller EL, Marquard KL, Louden ED, Schaffer JE, Moley KH. Diet-Induced Obesity Model: Abnormal Oocytes and Persistent Growth Abnormalities in the Offspring. *Endocrinology*. 2010;151(8):4039-4046.
187. Sampath H, Vartanian V, Rollins MR, Sakumi K, Nakabeppu Y, Lloyd RS. 8-Oxoguanine DNA Glycosylase (OGG1) Deficiency Increases Susceptibility to Obesity and Metabolic Dysfunction. *PLoS ONE*. 2012;7(12):e51697.
188. Wang B. 53BP1, a Mediator of the DNA Damage Checkpoint. *Science*. 2002;298(5597):1435-1438.
189. Markowski DN, Thies HW, Gottlieb A, Wenk H, Wischnewsky M, Bullerdiek J. HMGA2 expression in white adipose tissue linking cellular senescence with diabetes. *Genes Nutr*. 2013;8(5):449-456.
190. Vahratian A. Prevalence of overweight and obesity among women of childbearing age: results from the 2002 National Survey of Family Growth. *Matern Child Health J*. 2009;13(2):268-273.
191. Devereux G. The increase in the prevalence of asthma and allergy: food for thought. *Nat Rev Immunol*. 2006;6(11):869-874.
192. Lumia M, Luukkainen P, Tapanainen H, et al. Dietary fatty acid composition during pregnancy and the risk of asthma in the offspring. *Pediatr Allergy Immunol*. 2011;22(8):827-835.
193. Miyake Y, Sasaki S, Tanaka K, Ohfuji S, Hirota Y. Maternal fat consumption during pregnancy and risk of wheeze and eczema in Japanese infants aged 16-24 months: the Osaka Maternal and Child Health Study. *Thorax*. 2009;64(9):815-821.
194. Brekke HK, Ludvigsson J. Daily vegetable intake during pregnancy negatively associated to islet autoimmunity in the offspring--the ABIS study. *Pediatr Diabetes*. 2010;11(4):244-250.
195. Bogdan C, Röllinghoff M, Diefenbach A. Reactive oxygen and reactive nitrogen intermediates in innate and specific immunity. *Curr Opin Immunol*. 2000;12(1):64-76.
196. Valavanidis A, Vlachogianni T, Fiotakis C. 8-hydroxy-2' -deoxyguanosine (8-OHdG): A Critical Biomarker of Oxidative Stress and Carcinogenesis. *J of Env Sc & Hlth, Part C*. 2009;27(2):120-139.
197. Marnett LJ. Lipid peroxidation-DNA damage by malondialdehyde. *Mutat Res*. 1999;424(1-2):83-95.

198. Niedernhofer LJ, Daniels JS, Rouzer CA, Greene RE, Marnett LJ. Malondialdehyde, a product of lipid peroxidation, is mutagenic in human cells. *J Biol Chem*. 2003;278(33):31426-31433.
199. Yuzefovych LV, Musiyenko SI, Wilson GL, Rachek LI. Mitochondrial DNA Damage and Dysfunction, and Oxidative Stress Are Associated with Endoplasmic Reticulum Stress, Protein Degradation and Apoptosis in High Fat Diet-Induced Insulin Resistance Mice. *PLoS ONE*. 2013;8(1):e54059.
200. Djuric Z, Lewis SM, Lu MH, Mayhugh M, Tang N, Hart RW. Effect of varying dietary fat levels on rat growth and oxidative DNA damage. *Nutr Cancer*. 2001;39(2):214-219.
201. Milyavsky M, Gan OI, Trottier M, et al. A distinctive DNA damage response in human hematopoietic stem cells reveals an apoptosis-independent role for p53 in self-renewal. *Cell Stem Cell*. 2010;7(2):186-197.
202. Cipolleschi MG, Dello Sbarba P, Olivotto M. The role of hypoxia in the maintenance of hematopoietic stem cells. *Blood*. 1993;82(7):2031-2037.
203. Zhang X, Li J, Sejas DP, Pang Q. Hypoxia-reoxygenation induces premature senescence in FA bone marrow hematopoietic cells. *Blood*. 2005;106(1):75-85.
204. Grant WF, Gillingham MB, Batra AK, et al. Maternal high fat diet is associated with decreased plasma n-3 fatty acids and fetal hepatic apoptosis in nonhuman primates. *PLoS ONE*. 2011;6(2):e17261.
205. Body weight information: JAX® Mice Strain C57BL/6J. Vol. 2013; 2013.
206. Giri N, Batista DL, Alter BP, Stratakis CA. Endocrine abnormalities in patients with Fanconi anemia. *J Clin Endocrinol Metab*. 2007;92(7):2624-2631.
207. Pettersson US, Waldén TB, Carlsson P-O, Jansson L, Phillipson M. Female mice are protected against high-fat diet induced metabolic syndrome and increase the regulatory T cell population in adipose tissue. *PLoS ONE*. 2012;7(9):e46057.
208. Herrera E, Amusquivar E. Lipid metabolism in the fetus and the newborn. *Diabetes Metab Res Rev*. 2000;16(3):202-210.
209. Ceriello A, dello Russo P, Amstad P, Cerutti P. High glucose induces antioxidant enzymes in human endothelial cells in culture. Evidence linking hyperglycemia and oxidative stress. *Diabetes*. 1996;45(4):471-477.
210. Marfella R, Quagliaro L, Nappo F, Ceriello A, Giugliano D. Acute hyperglycemia induces an oxidative stress in healthy subjects. *J Clin Invest*. 2001;108(4):635-636.
211. Parrilla R, Ayuso-Parrilla MS, Williamson JR. Use of endogenous triglycerides to support gluconeogenesis in the perfused isolated rat liver. *Pflugers Arch*. 1976;366(2-3):211-216.
212. Niedernhofer LJ, Lalai AS, Hoeijmakers JHJ. Fanconi anemia (cross)linked to DNA repair. *Cell*. 2005;123(7):1191-1198.
213. Patel KJ, Joenje H. Fanconi anemia and DNA replication repair. *DNA Repair (Amst)*. 2007;6(7):885-890.
214. Speck NA, Iruela-Arispe ML. Conditional Cre/LoxP strategies for the study of hematopoietic stem cell formation. *Blood Cells, Molecules, and Diseases*. 2009;43(1):6-11.

215. Yamamoto R, Morita Y, Ooehara J, et al. Clonal analysis unveils self-renewing lineage-restricted progenitors generated directly from hematopoietic stem cells. *Cell*. 2013;154(5):1112-1126.
216. Hu X, Shen H, Tian C, et al. Kinetics of normal hematopoietic stem and progenitor cells in a Notch1-induced leukemia model. *Blood*. 2009;114(18):3783-3792.
217. Mothersill C, Seymour RJ, Seymour CB. Bystander effects in repair-deficient cell lines. *Radiation Research*. 2004;161(3):256-263.
218. Shen H, Yu H, Liang PH, et al. An acute negative bystander effect of -irradiated recipients on transplanted hematopoietic stem cells. *Blood*. 2012;119(15):3629-3637.
219. Wilson PF, Nagasawa H, Kohlgruber AC, et al. Hypersensitivity of human and rodent Fanconianemia (FA) cells to bystander effect-induced DNA damage. *2011 Low Dose Radiation Research Investigator's Workshop*. 2011:1-2.
220. Xun CQ, Thompson JS, Jennings CD, Brown SA, Widmer MB. Effect of total body irradiation, busulfan-cyclophosphamide, or cyclophosphamide conditioning on inflammatory cytokine release and development of acute and chronic graft-versus-host disease in H-2-incompatible transplanted SCID mice. *Blood*. 1994;83(8):2360-2367.
221. Yusuf RZ, Scadden DT. Homing of hematopoietic cells to the bone marrow. *Journal of visualized experiments : JoVE*. 2009(25).
222. Burstein Y, Rashbaum WK, Hatch WC, et al. Alterations in human fetal hematopoiesis are associated with maternal HIV infection. *Pediatric Research*. 1992;32(2):155-159.
223. Simsek T, Kocabas F, Zheng J, et al. The distinct metabolic profile of hematopoietic stem cells reflects their location in a hypoxic niche. *Cell Stem Cell*. 2010;7(3):380-390.
224. Suda T, Takubo K, Semenza GL. Metabolic regulation of hematopoietic stem cells in the hypoxic niche. *Cell Stem Cell*. 2011;9(4):298-310.
225. Lautt WW. Hepatic Circulation: Physiology and Pathophysiology. San Rafael, CA: Morgan & Claypool Life Sciences; 2009.
226. Mckinney-Freeman S, Cahan P, Li H, et al. The Transcriptional Landscape of Hematopoietic Stem Cell Ontogeny. *Stem Cell*. 2012;11(5):701-714.
227. Bonnefont-Rousselot D. Glucose and reactive oxygen species. *Curr Opin Clin Nutr Metab Care*. 2002;5(5):561-568.
228. Ejdesjo A, Wentzel P, Eriksson UJ. Influence of maternal metabolism and parental genetics on fetal maldevelopment in diabetic rat pregnancy. *AJP: Endocrinology and Metabolism*. 2012;302(10):E1198-E1209.
229. Wentzel P, Ejdesjö A, Eriksson UJ. Maternal diabetes in vivo and high glucose in vitro diminish GAPDH activity in rat embryos. *Diabetes*. 2003;52(5):1222-1228.
230. Yakes FM, Van Houten B. Mitochondrial DNA damage is more extensive and persists longer than nuclear DNA damage in human cells following oxidative stress. *Proc Natl Acad Sci USA*. 1997;94(2):514-519.

231. Hoek JB, Cahill A, Pastorino JG. Alcohol and mitochondria: a dysfunctional relationship. *Gastroenterology*. 2002;122(7):2049-2063.
232. Larsen NB, Rasmussen M, Rasmussen LJ. Nuclear and mitochondrial DNA repair: similar pathways? *Mitochondrion*. 2005;5(2):89-108.
233. Maniccia AW, Lewis C, Begum N, et al. Mitochondrial localization, ELK-1 transcriptional regulation and growth inhibitory functions of BRCA1, BRCA1a, and BRCA1b proteins. *J Cell Physiol*. 2009;219(3):634-641.
234. Mukhopadhyay SS, Leung KS, Hicks MJ, Hastings PJ, Youssoufian H, Plon SE. Defective mitochondrial peroxiredoxin-3 results in sensitivity to oxidative stress in Fanconi anemia. *J Cell Biol*. 2006;175(2):225-235.
235. Liu P, Demple B. DNA repair in mammalian mitochondria: Much more than we thought? *Environ Mol Mutagen*. 2010;51(5):417-426.
236. Brooks PJ, Zakhari S. Acetaldehyde and the genome: Beyond nuclear DNA adducts and carcinogenesis. *Environ Mol Mutagen*. 2013.
237. Kumari U, Ya Jun W, Huat Bay B, Lyakhovich A. Evidence of mitochondrial dysfunction and impaired ROS detoxifying machinery in Fanconi Anemia cells. *Oncogene*. 2013.
238. Cappelli E, Ravera S, Vaccaro D, et al. Mitochondrial respiratory complex I defects in Fanconi anemia. *Trends in molecular medicine*. 2013;19(9):513-514.
239. Pagano G, Talamanca AA, Castello G, et al. From clinical description, to in vitro and animal studies, and backward to patients Oxidative stress and mitochondrial dysfunction in Fanconi anemia. *Free Radic Biol Med*. 2013:1-8.
240. Bonnard C, Durand A, Peyrol S, et al. Mitochondrial dysfunction results from oxidative stress in the skeletal muscle of diet-induced insulin-resistant mice. *J Clin Invest*. 2008;118(2):789-800.
241. Sparks LM, Xie H, Koza RA, et al. A high-fat diet coordinately downregulates genes required for mitochondrial oxidative phosphorylation in skeletal muscle. *Diabetes*. 2005;54(7):1926-1933.
242. Ponte F, Sousa R, Fernandes AP, et al. Improvement of genetic stability in lymphocytes from Fanconi anemia patients through the combined effect of alpha-lipoic acid and N-acetylcysteine. *Orphanet journal of rare diseases*. 2012;7(1):28.
243. Zhang Q-s, Eaton L, Snyder ER, et al. Tempol protects against oxidative damage and delays epithelial tumor onset in Fanconi anemia mice. *Cancer Res*. 2008;68(5):1601-1608.
244. Li J, Sipple J, Maynard S, et al. Fanconi Anemia Links Reactive Oxygen Species to Insulin Resistance and Obesity. *Antioxid Redox Signal*. 2012.
245. Liang C, DeCourcy K, Prater MR. High-saturated-fat diet induces gestational diabetes and placental vasculopathy in C57BL/6 mice. *Metab Clin Exp*. 2010;59(7):943-950.
246. Challier JC, Basu S, Bintein T, et al. Obesity in pregnancy stimulates macrophage accumulation and inflammation in the placenta. *Placenta*. 2008;29(3):274-281.
247. Lappas M, Mitton A, Mittion A, Permezel M. In response to oxidative stress, the expression of inflammatory cytokines and antioxidant enzymes are impaired in placenta, but not adipose tissue, of women with gestational diabetes. *J Endocrinol*. 2010;204(1):75-84.

248. Li H-P, Chen X, Li M-Q. Gestational diabetes induces chronic hypoxia stress and excessive inflammatory response in murine placenta. *Int J Clin Exp Pathol*. 2013;6(4):650-659.
249. Alvarez-Silva M. Mouse placenta is a major hematopoietic organ. *Development*. 2003;130(22):5437-5444.
250. Lee LK, Ueno M, Van Handel B, Mikkola HK. Placenta as a newly identified source of hematopoietic stem cells. *Curr Opin Hematol*. 2010;17(4):313-318.
251. Yi C-X, Fleur SEl, Fliers E, Kalsbeek A. The role of the autonomic nervous liver innervation in the control of energy metabolism. *BBA - Molecular Basis of Disease*. 2010;1802(4):416-431.
252. Méndez-Ferrer S, Lucas D, Battista M, Frenette PS. Haematopoietic stem cell release is regulated by circadian oscillations. *Nature*. 2008;452(7186):442-447.
253. Pulliam-Leath AC, Ciccone SL, Nalepa G, et al. Genetic disruption of both Fancc and Fancg in mice recapitulates the hematopoietic manifestations of Fanconi anemia. *Blood*. 2010.
254. Hu L, Huang W, Hjort E, Eklund EA. Increased Fanconi C expression contributes to the emergency granulopoiesis response. *J Clin Invest*. 2013;123(9):3952-3966.
255. Pfannkuche K, Summer H, Li O, Hescheler J, Dröge P. The High Mobility Group Protein HMGA2: A Co-Regulator of Chromatin Structure and Pluripotency in Stem Cells? *Stem Cell Rev and Rep*. 2009;5(3):224-230.