The Role of Reactive Oxygen and Nitrogen Species in the Development of Fanconi Anemia, an Inherited Bone Marrow Failure Disorder

by

SUZANA HADJUR

B.Sc., The University of Western Ontario (Honors), 1997

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES
(Department of Biology; Genetics Graduate Program)

We accept this thesis as conforming to the required standard:

THE UNIVERSITY OF BRITISH COLUMBIA
September 6, 2002

© Suzana Hadjur, 2002
In presenting this thesis in partial fulfilment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by the head of my department or by his or her representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

Department of Genetics Graduate Program

The University of British Columbia
Vancouver, Canada

Date 8 October 2002

DE-6 (2/88)
The objective of this thesis was to investigate the role of endogenous reactive oxygen and nitrogen species in the pathogenesis of Fanconi’s Anemia (FA) using Fanconi Anemia Complementation Group C (Fancc) - deficient mice. This objective was examined in two distinct ways. First, through the generation and characterization of a novel mouse model for FA with intrinsic oxidant stress and secondly, through the observation that nitric oxide (NO) may have a role in cytokine - mediated inhibition of hematopoiesis in FA.

FA is an autosomal recessive disorder, which primarily affects children and young adults, resulting in morbidity and mortality due to BM failure or acute myelogenous leukemia (AML). Currently, eight complementation groups have been identified and six FA genes have been cloned. Although knockout mice have been generated for each of the genes cloned, they do not exhibit the primary hematopoietic defect of FA. Thus, no spontaneously occurring mouse model for FA exists with which to better define the pathogenesis of this disease. Several lines of evidence have pointed to abnormal regulation of intracellular reactive oxygen species (ROS) in individuals with FA. To investigate the possible role of the Fancc protein in the regulation of an in vivo redox state, mice were generated having combined deficiencies in the genes encoding the cytoplasmic antioxidant, Cu/Zn superoxide dismutase (Sod1) and Fancc. Fancc\textsuperscript{−/−}Sod1\textsuperscript{−/−} mice developed hepatic lipid accumulation, peripheral blood bicytopenia, marrow hypocellularity, little to no growth of committed progenitor cells in vitro, and decreased frequencies of long-term progenitors. This novel murine model of FA partially replicates the hematopoietic defect of this disease and may be useful in defining novel therapies.

The second theme of this thesis involved the observation that NO may have a role in FA BM failure. Cytokine inhibition of hematopoietic progenitor colony growth from Fancc\textsuperscript{−/−} mice was completely rescued in the presence of an iNOS inhibitor, L-NMMA. Fancc\textsuperscript{−/−} progenitor cells were hypersensitive to NO generating drugs in vitro while primary macrophages had elevated expression of iNOS and NO production in response to IFN\textgamma and IFN\textgamma/LPS. To date, no information exists regarding FA and NO and these studies have opened a new avenue of investigation in FA research.
# Table of Contents

**Abstract** ........................................................................................................... ii

**Table of Contents** .............................................................................................. iii

List of Tables ........................................................................................................... v

List of Figures ......................................................................................................... vi

List of Abbreviations .............................................................................................. viii

Acknowledgements ............................................................................................... ix

Dedication ................................................................................................................ ix

Publications arising from the work in this thesis ..................................................... x

**Chapter One - Introduction** .............................................................................. 1

1.1 Review of Current Literature ............................................................................. 1

Clinical and Cellular Phenotypes ........................................................................... 1

Complementation Analysis and Cloning of the FA Genes ..................................... 2

FA Molecular Interactions ....................................................................................... 5

Role of the FA Proteins ............................................................................................ 7

FA and DNA Repair ................................................................................................. 7

FA and Redox Regulation ....................................................................................... 9

Animal Models ......................................................................................................... 11

Bone Marrow Failure in FA .................................................................................... 13

1.2 Thesis Goals ..................................................................................................... 17

**Chapter Two - Generation and Analysis of Mice with Combined Deficiencies of the Genes Encoding Fanconi Anemia Complementation Group C (Fancc) and Cu/Zn Superoxide Dismutase (Sod1).** ....................................................... 18

2.1 Introduction ...................................................................................................... 18

2.2 Materials and Methods ..................................................................................... 21

2.3 Results ............................................................................................................. 25

*Fancc<sup>−/−</sup>Sod1<sup>−/−</sup>* mice develop zonal microvesicular hepatic steatosis .......... 25

Primary *Fancc<sup>−/−</sup>Sod1<sup>−/−</sup>* liver cell cultures generate increased levels of superoxide .................................................. 26

Increased expression of Mn SOD and HO-1 in *Fancc<sup>−/−</sup>Sod1<sup>−/−</sup>* livers. .......... 27

Peripheral blood and bone marrow abnormalities of *Fancc<sup>−/−</sup>Sod1<sup>−/−</sup>* mice .... 31

*Fancc<sup>−/−</sup>Sod1<sup>−/−</sup>* total marrow cells fail to show increased apoptosis or chromosomal aberrations .................................................. 30

*In vitro* hematopoietic colony growth is severely impaired in *Fancc<sup>−/−</sup>Sod1<sup>−/−</sup>* mice 30

Primitive progenitor numbers are normal in *Fancc<sup>−/−</sup>Sod1<sup>−/−</sup>* mice ................. 31

Aging study reveals persistent aberrant hematopoiesis in *Fancc<sup>−/−</sup>Sod1<sup>−/−</sup>* mice. 32

2.4 Discussion ....................................................................................................... 33

**Chapter Three - Absence of Cu/Zn Superoxide Dismutase (Sod1) Limits Long-Term Progenitor Cell Self-Renewal in Fanconi Anemia Complementation Group C (Fancc)-deficient Mice.** ................................................................................. 38

3.1 Introduction ...................................................................................................... 38

3.2 Materials and Methods ..................................................................................... 40

3.3 Results ............................................................................................................. 46
CAFC frequencies are significantly decreased from Fancc<sup>−/−</sup>Sod1<sup>−/−</sup> mice and can be partially rescued in hypoxia. 

Column purified early HPC from Fancc<sup>−/−</sup>Sod1<sup>−/−</sup> mice have reduced growth rates and increased apoptosis. 46
HPC can be partially rescued when grown in the presence of hypoxia or antioxidants. 49
Inability to generate cell lines from Fancc<sup>−/−</sup>Sod1<sup>−/−</sup> mice. 51

3.4 DISCUSSION 52

CHAPTER FOUR - Fancc<sup>−/−</sup>Sod1<sup>−/−</sup> mice have a less severe phenotype compared to Fancc<sup>−/−</sup>Sod1<sup>−/−</sup> Mice 57

4.1 INTRODUCTION 57

4.2 MATERIALS AND METHODS 59

4.3 RESULTS 60
Histological analysis of Fancc<sup>−/−</sup>Sod1<sup>−/−</sup> mice. 60
BM cellularity and body weights are slightly decreased in Fancc<sup>−/−</sup>Sod1<sup>−/−</sup> mice. 60
Colony Forming Assays reveal defective growth from Fancc<sup>−/−</sup>Sod1<sup>−/−</sup> committed progenitors. 61

4.4 DISCUSSION 62

CHAPTER FIVE - A Novel Role for Nitric Oxide in Mediating Cytokine - Induced Growth Inhibition of Fancc-deficient Bone Marrow Cells. 65

5.1 INTRODUCTION 65

5.2 MATERIALS AND METHODS 67

5.3 RESULTS 71
Cytokine - inhibited colony growth of Fancc<sup>−/−</sup> BM cells is rescued with L-NMMA. 71
Fancc<sup>−/−</sup> BM cells are hypersensitive to NO donors. 72
Apoptosis of IFNγ-treated HPC from Fancc<sup>−/−</sup> mice is inhibited by L-NMMA. 73
Fancc<sup>−/−</sup> macrophages have elevated expression of iNOS. 74
Fancc<sup>−/−</sup> macrophages have increased nitrite production. 75
Phosphorylated Stat1 is increased in Fancc<sup>−/−</sup> macrophages stimulated with IFNγ 76

5.4 DISCUSSION 77

CHAPTER SIX - DISCUSSION 81

6.1 Results Summary 81

6.2 A Role for FA Proteins in Redox Regulation. 83

6.3 A Role for NO in FA BM Aplasia 85

BIBLIOGRAPHY 90

TABLES and FIGURES 104
List of Tables

Table I. Clinical and Cellular Phenotypes in Fanconi Anemia............................................ p.104
Table II. Fanconi Anemia Complementation Groups........................................................ p.105
Table III. Current Mouse Models for Fanconi Anemia......................................................... p.108
Table IV. Peripheral Blood Values from 8 - 10 week old mice............................................ p.115
Table V. Total BM Cellularity and absolute number of cell types from 8-10 week old mice___ p.117
Table VI. PI/Annexin V FACS Analysis from total BM samples........................................ p.119
Table VII. Reagents used to try to rescue growth of methylcellulose colonies from \textit{Fancc}^+\textit{Sod1}^+ mice......................................................................................................................... p.121
Table VIII. CAFC Frequencies in 20% and 5% Oxygen....................................................... p.124
List of Figures

Fig. 1 Mutations identified in Fanconi anemia genes______________________________________________p.106
Fig. 2 Current knowledge of protein interactions and function of FA proteins_____________________________p.107
Fig. 3 Major sources of reactive species found within cells________________________________________________p.109
Fig. 4 Body weights of 8 wk old FancA"Sod1" mice__________________________________________________________________p.110
Fig. 5 Histologic examination reveals zonal hepatic microvesicular steatosis in FancA"Sod1" mouse livers__________________________________________________________p.111
Fig. 6 Electron microscopy of hepatocytes from FancA"Sod1" mice__________________________________________p.112
Fig. 7 Primary hepatocytes from FancA"Sod1" mice demonstrate increased superoxide levels____p.113
Fig. 8 Liver specific expression of MnSOD and HO-1 is increased in FancA"Sod1" mice____p.114
Fig. 9 Hypocellularity and increased fat accumulation in FancA"Sod1" bone marrows____p.116
Fig. 10 FACS analysis of Lin" cells for progenitor markers_____________________________________________p.118
Fig. 11 Colony forming assays reveal abnormal progenitor growth in FancA"Sod1" mice____p.120
Fig. 12 FACS analysis of Lin" cells from FancA"Sod1" marrows after column selection____p.122
Fig. 13 Colony Forming Assays from 6-8 month old mice______________________________________________p.123
Fig. 14 Representative CAFC colonies at day 7 in 20% and 5% O2________________________________________p.125-6
Fig. 15 Morphological changes in CAFC colonies from FancA"Sod1" cultures______________________________p.127
Fig. 16 HPC growth in liquid media reveals abnormal proliferation and increased apoptosis____p.128
Fig. 17 Hypoxia partially rescues FancA"Sod1" HPC growth______________________________________________p.129
Fig. 18 TIRON partially rescues FancA"Sod1" HPC growth______________________________________________p.130
Fig. 19 MnTMPyP dose response and partial rescue of HPC proliferation in vitro________________________p.131
Fig. 20 Primary mast cell cultures from FancA"Sod1" marrows fail to grow in vitro____________________p.132
Fig. 21 Histologic examination of FancA"Sod1" mouse livers_____________________________________________p.133
Fig. 22 Body weights are significantly decreased in FancA"Sod1" mice____________________________________p.134
Fig. 23 Marrow cellularity is decreased in FancA"Sod1" mice____________________________________________p.135
Fig. 24 Colony forming assays reveal abnormal progenitor growth in FancA'Sod1'' mice p.136
Fig. 25 Frequency of BM progenitors from FancA'Sod1'' mice is abnormal p.137
Fig. 26 Inhibition of Fanc'' colony formation by IFNγ is reversed by L-NMMA p.138
Fig. 27 Reduced colony formation by TNFα and MIP1α is reversed by L-NMMA p.139
Fig. 28 Fanc'' BM progenitors show increased sensitivity to NO-generating drugs p.140
Fig. 29 Inhibition of Fanc'' HPC growth by IFNγ is reversed by L-NMMA p.141
Fig. 30 Elevated iNOS expression in stimulated Fanc'' peritoneal macrophages p.142
Fig. 31 Elevated iNOS expression in Fanc'' BM-derived macrophages stimulated with IFNγ p.143
Fig. 32 Increased NO production by Fanc'' macrophages p.144
Fig. 33 Stat1 phosphorylation is augmented in IFNγ-stimulated Fanc'' macrophages p.145
Fig. 34 Hif1α expression is increased in IFNγ-stimulated Fanc'' macrophages p.146
Fig. 35 NO and superoxide anion; Converging Pathways to FA? p.147
### List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT</td>
<td>Alanine Amino Transferase</td>
</tr>
<tr>
<td>AML</td>
<td>Acute Myeloid Leukemia</td>
</tr>
<tr>
<td>BM</td>
<td>Bone Marrow</td>
</tr>
<tr>
<td>BRCA</td>
<td>Breast Cancer</td>
</tr>
<tr>
<td>CAFC</td>
<td>Cobblestone Area Forming Cell</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony Forming Units</td>
</tr>
<tr>
<td>Cu/Zn SOD</td>
<td>Copper/Zinc Superoxide Dismutase</td>
</tr>
<tr>
<td>DEB</td>
<td>Diepoxybutane</td>
</tr>
<tr>
<td>DSB</td>
<td>Double Strand Breaks</td>
</tr>
<tr>
<td>FA</td>
<td>Fanconi Anemia</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence Activated Cell Sorter</td>
</tr>
<tr>
<td>GSTP1</td>
<td>Glutathione S-Transferase Protein 1</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>Hydrogen Peroxide</td>
</tr>
<tr>
<td>HO-1</td>
<td>Heme Oxygenase 1</td>
</tr>
<tr>
<td>HPC</td>
<td>Hematopoietic Progenitor Cell</td>
</tr>
<tr>
<td>IFNγ</td>
<td>Interferon - γ</td>
</tr>
<tr>
<td>MIP1α</td>
<td>Macrophage Inflammatory Protein 1 - α</td>
</tr>
<tr>
<td>MMC</td>
<td>Mitomycin C</td>
</tr>
<tr>
<td>Mn SOD</td>
<td>Mangenese Superoxide Dismutase</td>
</tr>
<tr>
<td>NLS</td>
<td>Nuclear Localization Signal</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric Oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric Oxide Synthase</td>
</tr>
<tr>
<td>ONOO⁻</td>
<td>Peroxynitrite</td>
</tr>
<tr>
<td>ORO</td>
<td>Oil Red O Stain</td>
</tr>
<tr>
<td>O₂⁻</td>
<td>Superoxide anion</td>
</tr>
<tr>
<td>RED</td>
<td>NADPH Cytochrome P-450 Reductase</td>
</tr>
<tr>
<td>RNS</td>
<td>Reactive Nitrogen Species</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>SCF</td>
<td>Stem Cell Factor</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide Dismutase</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor Necrosis Factor - α</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal</td>
</tr>
</tbody>
</table>
Acknowledgements

I would like to extend my most sincere thanks to my supervisor Dr. Frank Jirik, who believed in my abilities as a graduate student and who provided constant encouragement. He gave me the confidence to become a scientist. I thank the entire Jirik Lab, both in Vancouver and in Calgary, especially Jennifer Moody, and all the members of the respective animal units. A very special thank you to Mark Cordy for his endless support, love and positivity.

Dedication

This thesis is dedicated to my parents. It is because they had the courage to leave their homeland that I was allowed this opportunity.
Publications arising from the work in this thesis


Work contained within this thesis not completed by the author

- Metaphase chromosome preparation and breakage analysis: Results, page 30
1.1 REVIEW OF CURRENT LITERATURE

Clinical and Cellular Phenotypes

Fanconi Anemia (FA) is an autosomal recessive disorder clinically characterized by progressive pancytopenia, developmental abnormalities of the skeleton, kidneys and heart, hypogonadism (resulting in reduced fertility) and an increased risk of malignancy. FA has a wide spectrum of severity ranging from congenital abnormalities and acute myeloid leukemia (AML) in the first decade of life to mild anemia and oral cancer in the fifth decade of life. The mean age of onset of bone marrow (BM) aplasia is 8 years and the mean life expectancy of FA patients is 20 years with the major causes of fatality being BM failure (aplastic anemia) or malignancies, primarily AML and squamous cell carcinomas. The disease has a frequency in the population of 1-5 per million and is found in all ethnic groups, with an estimated heterozygous mutation carrier frequency between 0.3 and 1%. Currently, there is no cure for FA. Pancytopenic FA patients become transfusion dependent and BM transplants are the only way to permanently correct the hematological phenotype.

FA was originally classified as a chromosomal instability disorder due to the observation that metaphase spreads from FA patient lymphocytes exhibited spontaneous chromosomal breaks, interchanges and arrest during the G2 phase of the cell cycle. When FA cells are challenged with DNA cross-linking agents such as mitomycin C (MMC), diepoxybutane (DEB) and cisplatin there is a marked increase in chromosomal aberrations, which include the formation of radial and quadrilateral chromosomes as well as increased numbers of chromosomal breaks and interchanges. This dramatic cross-linker sensitivity of FA cells serves as a tool for disease diagnosis. FA cells are also hypersensitive to elevated oxygen concentrations. Cells grow slowly at elevated oxygen levels (35%) and tend to arrest at G2, while at low oxygen concentrations (≤ 5%), growth is normal and accompanied by decreased chromosomal aberrations.
In fact there is indirect evidence that the effect of MMC on FA cells is mediated through oxidant stress.

Several other complex genetic diseases have been identified which have chromosomal instability and an increased predisposition to cancer. The genes mutated in these diseases are thought to have a role in genome surveillance, otherwise known as 'care-taker' genes. Examples include xeroderma pigmentosa (XP), hereditary non-polyposis colorectal cancer (HNPCC) and ataxia telangiectasia (AT). These disorders all involve genes that are required for double strand break repair response (nucleotide excision repair, mismatch repair and ATM, respectively) and thus the inability to maintain genome integrity leads to the accumulation of mutations and eventual malignant transformation of the cell. Due to the chromosomal instability and increased cancer risk in FA patients, FA genes were immediately assumed to be involved in the repair or recognition of DNA damage.

Complementation Analysis and Cloning of the FA Genes

Genetic complementation analysis was employed with cultured FA lymphoblasts to determine the number of genes involved in the disease. This method took advantage of the fact that FA cells are characterized by their high sensitivity to MMC (10-100 times over wildtype controls). A panel of somatic cell hybrids was generated by fusing together lymphoblast cell lines derived from different FA patients and the DEB and MMC sensitivities of each hybrid and parental line was assayed in order to determine the extent of genetic complementation. If the MMC-sensitive phenotype persisted then the mutations occurred in the same FA gene and the complementation group was identical, however if the MMC-sensitive phenotype was corrected, then the two cell lines were from different complementation groups and likely represented different genes.

The FANCC cDNA that corresponded to the human FA-C complementation group was cloned using essentially the same functional complementation method. The FA-C cell line,
selected because of its extreme sensitivity to MMC and DEB, was transfected with a cDNA library and the FA-C cells were selected for the complementing cDNA by repeated exposure to high doses of MMC and following outgrowth of clones, to repeated doses of DEB. The resultant clones represented the potential FANCC gene. The plasmid carrying the wildtype cDNA was shown to directly correct the MMC sensitivity of its particular complementation group (FA-C) and not other complementation groups. Subsequently, pathogenic mutations were identified within this gene.

Clearly, the rarity of FA mutations in the population has been an obstacle in the cloning of the genes. Despite this, complementation studies have identified that FA is genetically heterogeneous, with at least eight FA complementation groups (A, B, C, D1, D2, E, F and G) identified to date (Table II). The genes corresponding to the groups (A) FANCA, (C) FANCC, (D2) FANCD2, (E) FANCE, (F) FANCF and (G) FANCG, have now been cloned using a variety of techniques including positional and complementation cloning and microcell-mediated chromosome transfer strategies. Complementation group D appears to be heterogeneous since the FANCD2 gene has mutations that have been described in only a subset of FA-D patients, suggesting that another gene must be involved, temporarily called FANCD1.

Fig. I is a cartoon showing each of the six cloned FA genes and pathogenic mutations identified within each gene. Due to founder effects, the prevalence of mutations within a particular FA gene may vary with ethnic background. For example, most South African FA patients belong to group A and deletion mutations in exons 11-17 and 12-31 of the FANCA gene are frequently found in this population. Complementation group C is most common among the Ashkenazi-Jewish population. The most common mutations in FANCC are the splice-site mutation (IVS4 + 4A → T) and the frameshift mutation (322delG) and these are found most often in the Ashkenazi-Jewish and in the Dutch populations, respectively. Interestingly, mutations in FANCA account for approximately 80% of all FA patients. There are upwards of 100 unique-site pathogenic mutations in the FANCA gene alone and greater then one-third of these mutations are deletions, likely due to the large number of Alu repeats found within the gene. As more
mutation information is collected, genotype - phenotype correlations for the FA genes have begun to emerge. FA-C patients are generally considered to have the most severe form of the disease. Patients with mutations in FANCC and FANCF show onset of hematological problems at a much earlier age, and have an increased risk of malignancy compared to patients with mutations in FANCA, which are considered to have an overall milder form of the disease. Furthermore, disease severity is also correlated to particular mutations within an FA sub-type. For example, genotype - phenotype analyses of FA-C patients divided these into three distinct groups; a) patients with the IVS4 + 4A → T mutation, b) patients with at least one exon 14 mutation, and c) patients with at least one exon 1 mutation. The median age of hematological onset was 2.7, 2.4 and 7.6 years respectively for each group and the mean age of survival was 6.7, 7.8 and 14.3 years, respectively.

Initial attempts to identify the role of the FA proteins within the cell focused on sequence homology and expression studies. Homologous regions within the predicted FA proteins do exist in lower organisms, however these are not significantly similar to functional motifs in non-vertebrate, yeast or bacterial sequences. Significant conservation of protein and gene sequence in FANCA, FANCC, FANCG and FANCF is observed within vertebrates (Table II), while FANCD2 is relatively well conserved and appears to have orthologs in lower organisms, including Drosophila melanogaster, Caenorhabditis elegans and Arabidopsis thaliana. Unfortunately, the role of the proteins in these organisms is not known. Interestingly, FANCG was found to be identical to XRCC9, a gene that is defective in specific radiation sensitive CHO cell lines. The significance of this observation is unknown.

Studies have revealed that the mouse orthologs of FANCA and FANCC are ubiquitously expressed at low levels in adult tissues. During embryogenesis, Fancc is expressed in whisker follicles, brain, kidney, lung, gut, stomach and in osteogenic and hematopoietic lineages. The expression patterns of Fanca are very similar to Fancc with the major differences being high expression in lymphoid tissue (spleen, thymus and lymph nodes), testis and ovary and no
expression in the lung and gut \textsuperscript{28,29}. \textit{Fancg} has similar expression patterns to the \textit{Fanca} knockout mice, primarily in the spleen, thymus and the testis \textsuperscript{30}.

FA Molecular Interactions

Since homology and expression studies were not useful in elucidating the function of the FA proteins, researchers focused on subcellular localization and protein interactions. Cell fractionation and confocal microscopy studies have indicated that FANCA, FANCC and FANCG are nuclear as well as cytoplasmic proteins \textsuperscript{31,32}. The majority of the FANCC protein is cytoplasmic and this is in keeping with the observation that FANCC is required in the cytoplasm in order to complement the MMC sensitivity of FA-C cells \textsuperscript{33}. The remainder of the known FA proteins are thought to be nuclear, although some results suggest that a small fraction of FANCE and FANCF can also be cytoplasmic \textsuperscript{34}.

Using yeast two-hybrid, co-immunoprecipitation and immunofluorescence studies, a substantial body of evidence has shown that most of the FA proteins interact, directly or indirectly in a common functional pathway. These methods have shown that there are direct interactions between FANCA and FANCG and between FANCC and FANCE in the cytoplasm of normal cells. Furthermore, all five FA proteins (FANCA, FANCC, FANCE, FANCF and FANCG) can be co-purified out together as a complex from nuclear extracts \textsuperscript{32,35-38}. Binding affinities vary depending on the FA protein interactions in question. For example, FANCA binds very strongly to FANCG while FANCC binds with lower affinity to FANCA, and this is strongest in the presence of FANCG. These observations suggest that in normal cells, the FA proteins interact sequentially in a linear pathway within the cytoplasm forming an initial complex that then translocates into the nucleus where it becomes part of a larger complex. It is important to note that: a) an intact FA complex is found only in the nucleus and not in the cytoplasm; b) complex formation does not occur in response to DNA damage or to the cell cycle \textsuperscript{39} and; c) the formation of an intact FA complex is disrupted in cells from all FA complementation groups except FA-D \textsuperscript{39}.
A recent study has shown that the FA complex has been found specifically within the nuclear matrix and in condensed chromatin and when FANCG becomes phosphorylated the complex exits the chromatin sites. 

Both FANCA and most recently, FANCG have been identified as phosphoproteins, however, the stimulus for the phosphorylation events, and the kinase(s) remain unidentified. In mutant FA cell lines (except FANCD2), FANCA is not phosphorylated, suggesting that phosphorylation may be important for the assembly of the FA proteins or for the proper function of the complex. The phosphorylation of FANCA was shown to be regulated by a cytoplasmic serine protein kinase, later identified as Akt. Once FANCA becomes phosphorylated, the FA proteins move into the nucleus, presumably via the NLS of FANCA. A mutation within the phosphorylation domain or in the NLS region of FANCA prevents nuclear inclusion of the complex and does not correct MMC sensitivity of FA-A cells. FANCB cells contain only cytoplasmic FANCA protein, suggesting that the FANCB protein is needed for FANCA and/or FA complex nuclear localization. FANCD1 cells have an intact FA complex and a monoubiquitinated form of FANCD2, therefore it is believed to act downstream of the FANCD2 protein. In FA complementation groups A, B, C, E, F and G, the formation of the complex is disrupted, thus all proteins are required for the proper assembly and translocation of the nuclear complex. However, in FANCD2 mutant cells, the FA complex is intact suggesting that the FANCD2 protein functions downstream of complex formation and is not required for the assembly or stability of the complex. Taken together all of these studies support the hypothesis that the FA proteins sense a signal in the cytoplasm and are eventually translocated into the nucleus in order to maintain chromosomal integrity. Several key questions remain, such as what is the stimulus that precipitates FA complex formation, what is the eventual downstream effect of nuclear inclusion and does the FA complex have a direct or indirect role in damage repair?
Role of the FA Proteins

FA and DNA Repair

While it is quite clear that the FA complex forms and translocates into the nucleus in response to a cellular signal, the exact molecular role for the FA proteins is still actively debated. Two major theories exist in FA research: one hypothesis proposes that the FA proteins constitute a DNA damage recognition/repair pathway, whose impairment is manifested by chromosomal instability and increased sensitivity to inter-strand DNA cross linking agents such as MMC, DEB and cisplatin44. While some FA cell lines (primarily FANCD2) have been shown to have modest sensitivities to ionizing radiation (IR) as well39, it is worthy to note that FA cells are not particularly sensitive to ultra-violet (UV) or to monofunctional alkylating agents such as ethyl methane sulfonate (EMS) or methyl methane sulfonate (MMS)45. Based on these sensitivity studies, the primary defect in FA cells involves the removal of DNA inter-strand cross-links. The mechanism for the removal of DNA inter-strand cross-links from mammalian cells is not well understood, however in lower eukaryotes and bacteria these lesions are removed by homologous recombination (HR) or non-homologous end-joining (NHEJ) repair pathways39. Defects in both of these repair mechanisms have been described for FA cells. HR frequencies and activity were found to be increased in nuclear extracts from primary FA fibroblasts46, while the fidelity of end-joining of specific DSB was lost in FA cells47. Mutation analyses done on FA lymphoblasts found hypo-mutability at the HPRT locus, however the spectrum of mutations revealed a significant number of deletions48. Deletion events represent up to 65% of spontaneous mutations occurring at the HPRT locus in FA lymphoblasts of the D2 complementation group compared to 18% in wildtype cells49. A heptamer motif exists at the 3' breakpoint of these deletions and suggests that site-directed activity may be involved. These results suggest that the FA proteins may have a role in the repair of DSB through HR or NHEJ mechanisms.
A major step was made in support of a role for FA proteins in DNA repair when the FANCD2 gene was cloned. Subsequent functional studies showed that the FA complex is required in the nucleus for the ubiquitination of the FANCD2 protein, shown to interact with the BRCA1 DNA repair pathway. FANCD2 is exclusively nuclear in location and is present in wildtype cells in two isoforms, FANCD2-short and FANCD2-long. The long isoform is monoubiquitinated and this form is absent in cells from FA complementation groups A, B, C, E, F and G. Interestingly, these complementation groups do not have an intact FA nuclear complex and therefore, it is assumed that the FA complex has a role, direct or indirect, in either the addition of ubiquitin groups to FANCD2 or the recruitment of a ubiquitin ligase to perform this task. The ubiquitination of FANCD2 occurs in response to DNA inter-strand cross-linkers such as MMC and immunofluorescence studies have shown that FANCD2 can be found at nuclear foci in conjunction with BRCA1 when cells are challenged with these agents. Nuclear foci are formed by other DNA repair proteins upon induction of DNA inter-strand croslinks, including BRCA1, BRCA2 and Rad51. Using the novel technique of micro-irradiation of human fibroblast cells and fluorescent markers for various repair proteins, FANCD2 has been shown to directly associate with damaged DNA, in particular double strand breaks (DSB) and proteins involved in genetic recombination repair. This important study was the first to show the FA proteins interacting with either damaged DNA or other repair proteins in vivo, and that FANCD2 does associate with BRCA1 and BLM (components of recombination repair). However, this association is not perfect since nuclear DSB foci can be found without the FANCD2 protein present even though BRCA1 and BLM are there, thus BRCA1 and FANCD2 do not always co-localize. This study also showed that the localization of FANCD2 with DSB does require FANCA, presumably for the proper ubiquitination of the FANCD2 protein.
FA and Redox Regulation

The other hypothesis for the role of the FA proteins suggests that FA might result, at least in part, from an abnormal regulation of cellular redox state and/or of the cellular response to oxidative stress. For example, increased production of ROS by FA cells, such as leukocytes and fibroblasts, has also been reported, suggesting that FA proteins might regulate the generation of these species \[^{51,52}\]. Furthermore, reduction of MMC in FA cells has been shown to lead to the production of reactive oxygen species (ROS) that indirectly may generate cross-links and other types of oxidative lesions \[^{53}\]. Many studies have presented evidence in defense of this hypothesis. The addition of cellular antioxidants, such as superoxide dismutases (SOD) to the culture medium of FA cells was reported to attenuate chromosomal breakage as well as MMC cytotoxicity \[^{54,55}\], an effect also observed in FA cells over-expressing thioredoxin, another cellular antioxidant \[^{56}\]. SOD administration has been used in pilot studies as a potential therapeutic agent for FA BM recovery with apparently promising results \[^{57}\].

To further support the redox dysregulation hypothesis, some of the FA proteins have been shown to interact with various non-FA cytoplasmic and nuclear proteins involved in regulating oxidant stress. These protein interactions have also indicated that FA proteins may have numerous additional roles within the cell besides DNA repair, and might conceivably explain the fact that multiple congenital defects can occur in a patient with FA. Specifically, FANCC is known to interact with the molecular chaperone GRP94 \[^{58}\], NADPH cytochrome P-450 reductase \[^{59}\], the zinc-finger FAZF \[^{60}\] and most recently with the Phase II detoxification enzyme, GSTP1 \[^{61}\].

The NADPH cytochrome P-450 reductase (RED) system is a potential source of endogenous superoxide. The cytochrome P-450 enzymes are a superfamily of heme-containing proteins that are expressed in all tissues and catalyze the oxidation of many endogenous and xenobiotic chemicals \[^{62}\]. P-450 metabolism usually requires two protein components, P-450 and RED. These enzymes are embedded in the microsomal membrane and RED is responsible for shuttling electrons to P-450 from NADPH \[^{62}\]. Not only were chromosomal breaks in FA cells
reduced to background levels by cytochrome P-450 inhibition, but evidence of a direct physical interaction between FANCC and RED was reported, where FANCC modulated the activity of RED. These results led to the hypothesis that FANCC might protect cells from ROS via regulation of RED activity. Interestingly, the cytochrome P-450 system has also been implicated in association with FANCG. CYP2E1 is a member of the P-450 superfamily responsible for the production of ROS, the bioactivation of carcinogens, and the removal of lipid-peroxidation products. Constitutively elevated levels of CYP2E1 protein were found in FA-G lymphoblast lines which was reduced when the cells were complemented with wildtype FANCG. This study also showed that there was a dose dependent increase in oxidized DNA bases in mutant FA-G lines post MMC treatment.

FANCC also interacts with glutathione S-transferase P1-1 (GSTP1), a phase II detoxification enzyme responsible for the reduction of glutathione (GSH) and various xenobiotics. GSTP1 and GSH are required by the cell for the detoxification of exogenous and endogenous electrophilic compounds and in doing so prevent protein oxidation overload. ROS can damage proteins through the oxidation of sulfhydryl groups which results in the formation of disulfide bonds and the eventual inactivation of the protein. The study by Cumming et al. revealed that FANCC prevents the formation of these inactivating disulfide bonds within GSTP1. Thus, FANCC indirectly regulates cellular oxidant stress by maintaining an active form of GSTP1, which in turn functions to detoxify xenobiotics and reduce GSH proteins. The overexpression of FANCC maintains GSTP1, and thus indirectly, GSH in an oxidized or 'open' state, preventing apoptosis in hematopoietic cells following growth factor deprivation. Surprisingly, FANCC lacks homology with any conventional disulfide reductases (there is no active - site motif C-X-X-C), however other proteins have been described with reductase function that also lack conventional active - site motifs. FANCC may be part of an undiscovered subset of disulfide reductases within the cytoplasm and may have a role in the general regulation of redox sensitive proteins. The lack of FANCC in FA-C cells may result in the oxidation of an increased number
of protective cytoplasmic proteins which in turn cannot function properly to clear oxidative stress, leading to an increased susceptibility to apoptosis.

The observation that FA proteins may have potential functions outside of the FA complex is not specific to FANCC. Yeast two-hybrid analysis using FANCA as a bait revealed a novel interaction between FANCA and brm-related gene 1 (BRG1) product. BRG1 is a component of the SWI/SNF complex which is involved in chromatin remodelling. BRG1 and FANCA have been shown to co-localise in the nucleus of the cell, however chromatin re-modelling is normal in FA-A cells, suggesting that FANCA may have a role in opening chromatin at specific sites for the transcription of specific genes. The FANCA protein may also have roles in redox regulation since amino acid residues located within the N-terminal region of FANCA have been identified that are homologous to heme peroxidases. Mutations of highly conserved sites within the putative peroxidase domain of FANCA abolished the ability of FANCA (mut) to correct the MMC sensitivity of FA-A cells, however this mutant did not compromise either the stability of the FANCA protein or the interaction of FANCA with FANCG.

Animal Models

Mice have been generated with targeted mutations of Fancc, Fanca, Fancg and Fancd2. With the exception of Fancd2, all mouse models have similar phenotypes, demonstrating compromised gametogenesis, and an increase in the number of chromosomal aberrations, both spontaneously and following exposure of cultured embryonic fibroblasts and primary splenocytes to MMC. The targeted strains do not spontaneously display developmental or hematological defects typical of human FA, and do not have a predisposition to malignancy (for a comprehensive list of FA mouse models see Table III). Peripheral blood counts of FA knockout mice remain normal over time and there is no significant difference in committed progenitor growth over time in most knockout mice. Two Fancc-null murine strains have been developed with slightly
different targeting mutations, disruption in exon 8\textsuperscript{69} and disruption in exon 9 of \textit{Fancc}\textsuperscript{70}. Both \textit{Fancc}\textsuperscript{−−} strains undergo progressive hematopoietic failure and eventual death (within 3-8 weeks) when MMC is administered \textit{in vivo}\textsuperscript{75} and when challenged with inhibitory cytokines \textit{in vitro}\textsuperscript{76}. Interestingly, only the \textit{Fancc}\textsuperscript{−−} mice generated by Whitney \textit{et al.} develop age-dependent decreases in the number of committed progenitors from the BM, suggesting that a disruption of exon 9 leads to a more severe phenotype. While \textit{Fancg}\textsuperscript{−−} mice have the same phenotypes as the \textit{Fanca} and \textit{Fancc} null mice, preliminary studies of \textit{Fancg}\textsuperscript{−−} mice reveal that the cellular sensitivities to MMS, UV and X-rays observed in the CHO cell lines have not been duplicated from embryonic fibroblasts isolated from these mice\textsuperscript{73}. The phenotype of \textit{Fancd2} mutant mice is slightly more severe than the phenotype of the remaining FA mouse models. Gonads of both sexes show significantly decreased numbers of germ cells over wildtype, \textit{Fanca}\textsuperscript{−−} and \textit{Fancc}\textsuperscript{−−} mice\textsuperscript{74}. Moreover, embryonic fibroblasts from \textit{Fancd2}\textsuperscript{−−} mice have increased sensitivity to cross-linkers as well as a modest sensitivity to IR \textit{in vitro} that becomes significantly more pronounced \textit{in vivo}\textsuperscript{74}. This difference is in keeping with the fact that the FANCD2 protein product has a role in the cell that seems to be quite different from the other FA proteins. However, despite this difference, even \textit{Fancd2}\textsuperscript{−−} mice do not demonstrate spontaneous BM aplasia.

The lack of an FA-like BM failure phenotype in mutant mice is surprising since all murine FA cDNAs are able to fully complement the MMC sensitivity in human lymphoblast cells, suggesting that the proteins are able to function properly \textit{in vitro}. The reason(s) for this interspecies difference is unknown, but it has limited the utility of the mutant mice as potential models of FA. A stressful insult or an affected modifier gene may be required in order to uncover an FA-like hematological defect in these mice. Thus, double mutant mice have been generated to better understand the role of the FA proteins in human BM failure. To date, three ‘double mutant’ models have been described in the literature. Since TNFα is known to be elevated in the serum of FA patients and is known to prime the fas pathway, \textit{Fancc}\textsuperscript{−−} mice were generated to overexpress human TNFα (FNT mice) and the effect on hematopoiesis was examined\textsuperscript{77}. FNT mice had
normal peripheral CBC and marrow cellularity, and while whole marrow from FNT mice had increased levels of CD95\(^+\) cells, myeloid progenitors assayed in methylcellulose cultures were not spontaneously sensitive to the \textit{in vivo} effects of overexpressed TNF\(\alpha\). Erythroid progenitors from FNT mice did however show decreased growth in methylcellulose cultures both spontaneously and upon treatment with fas ligand \(^77\). Mice with targeted mutations in different FA genes have also been generated. Not surprisingly, \textit{Fanca/Fancg} double mutant mice have a phenotype that is identical to the single knockout mice, reduced fertility with no hematological defects or tumor development \(^78\). The overexpression of human FANCC has been shown to protect murine hematopoietic progenitor cells (HPC) from Fas-mediated apoptosis \(^79\). Committed progenitors from these mice were resistant to the killing effects of \textit{fas} in methylcellulose assays and \textit{Lin}\(^-\) HPC treated with \textit{fas} had significantly less apoptosis compared to littermate controls \(^79\). These results indicated that FANCC was required for the proper protection of progenitor cells from \textit{fas}-induced apoptosis.

**Bone Marrow Failure in FA**

Despite the fact that the FA genes are expressed in most tissues, the BM is the only organ that consistently fails in FA patients. Thus, there is a preferential defect within the BM compartment of FA patients that leads to aplasia and eventual death. Studies of BM aspirates from FA patients indicate that there is a dramatic reduction (up to a 15-fold decrease) in the frequency of committed hematopoietic progenitors from both the erythroid (BFU-E) and the myeloid (CFU-GM) lineages of FA patients. Occasionally, committed progenitors from FA patients do not grow \textit{in vitro} at all, even when cultured in the presence of SCF \(^80,81\). This reduction is seen in pancytopenic as well as asymptomatic (non-anemic) FA patients \(^82,83\). Interestingly, the colonies enumerated in the above named studies are consistently decreased in
size as well as frequency. These results indicate that there is a defect in the proliferation of lineage constricted progenitors.

Long-term BM cultures (LTC) derived from FA patients reveal that hematopoiesis is impaired at the multipotent progenitor level as well. Frequencies of early LTC progenitors from FA BM samples in vitro have not been directly determined, rather CFU-GM was determined from the non-adherent nucleated cells in LTC. Two independent studies indicate that CFU-GM from LTC cultures were markedly decreased or null from FA patients and while adherent stromal layers consistently formed from FA BM samples, they took significantly longer to achieve confluency than normal controls. These studies indicated that while progenitors exist in the marrow of FA patients, their proliferative potential is affected, or they cannot respond properly to growth factor stimulation.

In addition to defective progenitors, it is possible that there is also a defect in the proper production of cytokines from FA cells, although the data has been somewhat conflicting and dependent on the cell type analyzed. FA lymphocyte cell lines revealed a consistent increase in TNFα production and decreased production of IL-6. When these cell lines were treated with anti-TNFα antibodies or supplemented with IL-6, their MMC sensitivity was corrected. These results are in keeping with the observation that both TNFα and flt3 ligand are elevated in the serum of FA patients. In contrast to the these results, LTC cultures from primary FA BM samples reveal a significant decrease in TNFα and IL1β production over a 4 week time period in vitro and no difference in IL-6 production.

The above data all suggest that loss of FA protein function results in quantitative or qualitative injury to hematopoietic progenitor cells or to the progenitor environment. In keeping with this hypothesis, a specific role for FANCC in the survival and/or proliferation of HPC has been established in both murine and human cells. Committed progenitor cells from Fancc−/− animals were found to be less responsive to stem cell factor (SCF) stimulation, suggesting that
the lack of Fancc may lead to an inability to respond to growth factor stimulation. Furthermore, in addition to initial reports which showed that Fancc−/− HPC cells were hypersensitive to MMC and to inhibitory cytokines, Fancc−/− mice were also used in competitive repopulation assays to determine the role of Fancc in short and long-term reconstitution of the BM compartment. Competitive repopulation assays involve the co-transplantation of test cells (Fancc−/−) with congenic competitor (wildtype) cells into irradiated mice and reconstitution of the marrow is measured. These experiments showed that Fancc−/− cells had 7-12 fold decreased repopulation ability compared to wildtype controls. Despite these results, no hematologic malignancy has ever been observed in Fancc−/− mice, perhaps owing to the lack of a sufficient insult to exacerbate the phenotype.

The observation that Fancc−/− progenitor cells are hypersensitive to low doses of the inhibitory cytokines IFNγ, TNFα, and MIP1α suggested that these cytokines may be mediating apoptotic responses in FANCC progenitor cells. In fact, progenitors from FANCC patients were shown to induce fas and interferon response factor 1 (IRF-1) gene expression at significantly lower doses of IFNγ compared to normal controls. An increase in CD95 (fas receptor) expression was also found on CD34+ hematopoietic progenitors from Fancc−/− mice. These apoptotic responses were found to be mediated by caspase 8-dependent activation of caspase 3 family members. The suppressive effects of IFNγ and TNFα on clonal growth of Fancc−/− BM cells in vitro can be augmented with the addition of agonistic anti-fas antibodies, and the reverse is also true, neutralizing anti-fas antibodies abolish the inhibitory effects of these cytokines on clonogenic growth. Similarly, caspase 3 inhibitors can rescue IFNγ-mediated committed progenitor growth from Fancc−/− BM cells and human FA-C CD34+ cells.

Although FANCC cells are known to be hypersensitive to the effects of IFNγ, paradoxically, the activation of STAT1 in response to IFNγ in EBV-transformed FANCC lymphoblast cell lines appears to be suppressed. It was later reported that this STAT1
abnormality is dependent on the type of mutation that occurs within the FANCC gene. Furthermore, Fancc−/− mouse embryonic fibroblasts were found to have increased levels of active RNA-dependent protein kinase (PKR) after treatment with dsRNA and IFNγ. When catalytically inactive forms of the PKR were overexpressed in Fancc−/− embryonic fibroblasts, the cells were protected from apoptosis. This provided a mechanism through which the apoptotic response could be mediated.
1.2 THESIS GOALS

The main goals of this thesis were:

1) To generate, through breeding protocols, a novel model of FA that has an in vivo pro-oxidant state. Using this genetic model of FA (Fancc and Sodl double mutant animals), we investigated the role of intrinsically elevated superoxide anion on the pathogenesis of the disease, specifically on hematopoietic failure.

2) To determine the potential role of the Fanca protein in the regulation of cellular redox potential through the generation of Fanca and Sodl double mutant mice. The phenotype of these mice was compared to the Fancc and Sodl double mutants to examine if ROS-mediated FA pathogenesis is a Fancc-specific effect of a more general FA defect.

3) To investigate the potential role of nitric oxide in the response of Fancc+ BM cells to growth inhibitory cytokines.
CHAPTER TWO - Generation and Analysis of Mice with Combined Deficiencies of the Genes Encoding Fanconi Anemia Complementation Group C (Fancc) and Cu/Zn Superoxide Dismutase (Sod1).

2.1 INTRODUCTION

A number of hypotheses regarding the nature of the primary defect in FA have been suggested, including the proposal that FA proteins constitute a DNA damage recognition and signaling pathway, whose impairment is manifested by chromosomal instability and increased sensitivity to interstrand DNA cross linking agents. While a reduced ability to process DNA cross-links is clearly evident, it has also been proposed that an abnormal reduction of MMC in FA cells leads to the production of reactive species that in turn generate cross-links and other types of oxidative lesions. Thus, FA might also result, at least in part, from an abnormal regulation of cell redox state and/or of the cellular response to oxidative stress. In support of this notion, SOD1 addition to the culture medium of FA cells was reported to attenuate chromosomal breakage as well as MMC cytotoxicity, an effect also observed in FA cells over-expressing thioredoxin. In keeping with an inability to regulate either production, or the consequences of ROS, some FA cells have been shown to be hypersensitive to oxygen. Thus, cells grew slowly at elevated oxygen levels (e.g. 35%) and tended to arrest at G2, while at low oxygen concentrations (e.g. ≤ 5%), growth was normal and accompanied by decreased chromosomal aberrations. Increased production of ROS by FA cells, such as leukocytes and fibroblasts, has also been reported, suggesting that FANCC might regulate the generation of these species. Furthermore, a high level of 8-hydroxy-2-deoxyguanine residues have been detected in the DNA of FA cells which is a marker of oxidative DNA damage and is thought to reflect oxidative stress in FA cells. A potential endogenous source of superoxide, the NADPH cytochrome P-450 reductase (RED) system, has also been implicated in FA. Chromosomal breaks in FA cells were reduced by cytochrome P-450 inhibition, and a direct physical interaction between FANCC and RED has been
reported\textsuperscript{59,63}, leading to the hypothesis that FANCC might protect cells from ROS via regulation of RED activity. Furthermore, a recent paper describes a role for FANCC in the redox regulation of GSTP1, a Phase II detoxification enzyme\textsuperscript{61}. The overexpression of FANCC and GSTP1 results in enhanced survival of a growth factor dependent hematopoietic cell line.

Aerobic organisms generate energy through the conversion of molecular oxygen (O\textsubscript{2}) to H\textsubscript{2}O and ATP via the process of oxidative phosphorylation. It has been estimated that approximately 2-4\% of the oxygen used during mitochondrial respiration results in aberrant production of superoxide anion (O\textsubscript{2}\textsuperscript{-}), one of several reactive forms of oxygen (ROS) (Fig. 3 for a list of reactive species)\textsuperscript{97}. ROS, are free radicals associated with the oxygen atom, some of which have strong reactivity with other molecules\textsuperscript{97}. In addition to respiration, ROS are also generated in the cytosol and extracellular spaces and their presence can be both beneficial as well as harmful to the cell. For example, macrophages use the respiratory burst oxidase to generate O\textsubscript{2}\textsuperscript{-} within cellular membranes to kill microorganisms and many ROS are critical components of cellular signal transduction pathways\textsuperscript{98,99}. However a balance must exist since excessive production of ROS is also capable of causing cellular damage to protein, lipids and DNA, which can ultimately lead to mutagenesis and cell death\textsuperscript{97}.

In order to maintain an equilibrium and to combat toxic O\textsubscript{2}\textsuperscript{-}, organisms have evolved three forms of superoxide dismutase as an antioxidant defense, cytosolic Cu/Zn SOD (Sod1), mitochondrial MnSOD (Sod2), and extracellular EC SOD (Sod3)\textsuperscript{100}. SODs are homodimeric metalloenzymes that catalyze the dismutation of O\textsubscript{2}\textsuperscript{-} to hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) and O\textsubscript{2}\textsuperscript{100}. These enzymes are ubiquitously expressed and their importance is highlighted by their appearance in all aerobic organisms. Organisms genetically engineered to be null for one of the SOD enzymes have defined phenotypes. Bacteria and yeast the SOD1 gene have severe growth defects under an aerobic environment\textsuperscript{101-103} while Drosophila have severely shortened lifespans and are very sensitive to paraquat and radiation\textsuperscript{104,105}. Mice lacking MnSOD die within 2 weeks
of birth due to cardiomyopathy, degenerative brain neuron injury and severe mitochondrial
damage\textsuperscript{106,107}.

Sod1 constitutes the major portion of total Sod activity within most tissues and is considered
to be the primary \( \text{O}_2^\bullet^- \) scavenger within the cytosol. Surprisingly however, mice with a targeted
disruption of the gene encoding the cytosolic Cu/Zn SOD (\textit{Sod1}) exhibit normal growth and
development, age at similar rates as wildtype mice and do not have an increase in the expression of
secondary antioxidant genes \textsuperscript{108-110}. However, \textit{Sod1}\textsuperscript{-/-} mice do show a distinctive motor
axonopathy \textsuperscript{108,111}, as well as impaired gametogenesis \textsuperscript{109}. The limited spontaneous pathology of
\textit{Sod1}\textsuperscript{-/-} mice suggested that while this enzyme might function to modulate superoxide-mediated
effects in some tissues under basal conditions, that it was of critical importance during exposures
to specific pro-oxidant stimuli. In keeping with this, \textit{Sod1}\textsuperscript{-/-} embryonic fibroblasts exposed to the
superoxide-generating herbicide, paraquat, exhibited a pronounced sensitivity compared to both
\textit{Sod1}\textsuperscript{+/+} and \textit{Sod2}\textsuperscript{+/+} controls \textsuperscript{112,113}. Other studies have shown a clear role for dominant mutations
of Sod1 in familial amyotrophic lateral sclerosis (FALS) \textsuperscript{114}, as well as other neurodegenerative
diseases, aging and cancer \textsuperscript{115}.

We hypothesized that a lack of Sod1 might reveal a role for alterations in redox state with
respect to the development of an FA-like syndrome in Fancc deficient mice. To examine this
possibility, we generated mice with combined deficiencies of both the \textit{Fancc} and \textit{Sod1} genes.
\textit{Fancc}\textsuperscript{-/-}\textit{Sod1}\textsuperscript{-/-} mice developed not only a novel liver pathology, but also demonstrated BM
hypocellularity and peripheral blood bicytopenia (RBC and leukocytes) accompanied by a
profound reduction of the clonogenic growth of hematopoietic precursors \textit{in vitro}, that persisted in
aged (6-8 month) \textit{Fancc}\textsuperscript{-/-}\textit{Sod1}\textsuperscript{-/-} mice.
2.2 MATERIALS AND METHODS

Generation of Fancc<sup>−/−</sup>Sod1<sup>−/−</sup> mice and histological analysis

Fancc<sup>−/−</sup> mice<sup>69</sup> were crossed with Sod1<sup>−/−</sup> mice<sup>108</sup> until mice that were heterozygous at both loci were obtained (three generations of matings). Locus-specific PCR was used to genotype mice. Brother-sister matings of Fancc<sup>−/−</sup>Sod1<sup>−/−</sup> mice were carried out to produce litters having Fancc<sup>−/−</sup>Sod1<sup>−/−</sup> mice. Mice, 8-10 weeks of age, were of a mixed genetic background and thus littermate controls were used in all experiments. Viral antibody-free mice were housed in the Center for Molecular Medicine and Therapeutics barrier facility according to protocols approved by the Animal Care Committee at the University of British Columbia. For light microscopy, tissue samples were either frozen, or fixed in 4% paraformaldehyde solution and embedded in paraffin and bone sections were first de-calcified before processing. For paraffin-embedded sections; hematoxylin and eosin (H&E); periodic acid-Schiff (PAS) with and without diastase; and Masson's trichrome were used. Frozen sections were stained with oil-red-O (ORO). For electron microscopy, liver blocks were fixed in cold 3% gluteraldehyde and stored at 4 °C. Samples were rinsed twice in Millonig's buffer (pH 7.4) and were subsequently fixed in 1% osmium tetroxide in Palade's solution for 1.5 hours at 4 °C. Samples were stained en bloc for 15 minutes with 2 % aqueous uranyl acetate and then dehydrated before embedding. Sections were examined with a Phillips 400 electron microscope.

Blood collection, serum ALT measurement and peripheral blood counts

Following avertin overdose, blood was obtained by cardiac puncture, and either allowed to clot at room temperature, and/or added to microtainer tubes, pre-treated with EDTA, for blood counts. Clotted blood was centrifuged at 14,000 rpm for 5 min, serum was removed and frozen at -80 °C. Serum alanine aminotransferase (ALT) levels were determined using a Beckman
SynchroN CX7. Peripheral blood (PB) counts were performed using a Sysmex 9500 analyzer. RBC, WBC, PLT, hemoglobin (HGB) and mean cell volume (MCV) values were determined.

_Tissue isolation and BM cell Preparation_

Mice were sacrificed at 8-10 weeks of age by intraperitoneal (ip) injection of 4% avertin (0.01 ml/g). Samples were harvested from the same region of the liver in all mice. Single-cell suspensions were prepared by pressing samples through a wire mesh into cold serum-free RPMI 1640 (Life Technologies). Cells were then passed through a 40 μ nylon filter to remove clumps and debris. Liver cells were pelleted at 1500 rpm, and resuspended in cold RPMI. Total BM cells were collected by flushing femurs from mice with cold Hank’s Balanced Salt Solution containing 5% FCS. Cell viability, > 90% in all samples, was determined by trypan blue exclusion.

_Superoxide quantitation_

Isolated liver cells were resuspended, in triplicate, at a density of 5.0 x 10^5/ml in serum-free RPMI and centrifuged at 1500 rpm, before being resuspended in 100 μl Superoxide Assay Medium (Calbiochem, San Diego, CA). Each culture was then placed into a well of an opaque 96-well polystyrene flat-bottomed microtiter plate (VWR Canlab) kept on ice until analysis. Then, 5.0 μl of 4.0 mM luminol solution (Calbiochem, San Diego, CA), diluted in 95 μl of Superoxide Assay Medium, was added simultaneously to all samples. Chemiluminescence was measured at one minute after luminol addition using a MLX Microtiter Plate, Luminometer (Dynex Technologies, Chantilly, VA). The average intensity of the triplicates was recorded as Relative Light Units (RLU). Purified SOD (Calbiochem, San Diego, CA) was added to the cells as a specificity control to show that chemiluminescence was due to superoxide anion.
Immunoblotting and densitometry

Flash-frozen liver samples were lysed in Nonidet P-40 lysis buffer (1% Nonidet P-40, 150 mM NaCl, 50 mM Tris (pH 7.5) and 10% glycerol) in the presence of multiple protease inhibitors (Boehringer Mannheim, Indianapolis, IN and BDH, Toronto, Canada). Lysates were centrifuged for 15 min at 14,000 rpm. Liver protein concentration was determined by Bradford-method-based assay. Lysate volume corresponding to 250 μg of total protein was diluted 3:1 with Laemmli sample buffer. Samples were boiled for 5 min prior to electrophoresis. Total cell lysates were separated by SDS-PAGE at 150V and transferred to nitrocellulose paper by electroblotting at 100 V for 1 hr at RT in a solution containing 192 mM glycine, 25 mM Tris and 20% methanol. Filters were blocked overnight at 4 °C in TBST (10 mM Tris (pH8.0), 150 mM NaCl and 0.05% Tween-20) containing 5% BSA. Filters were then incubated for 60 min at room temperature in TBST with 1% BSA with one of the following monoclonal antibodies (StressGen Biotechnologies, Victoria, BC): anti-MnSOD (1/5000), anti-HO-1 (1/2000), or anti-β-tubulin (1/250). After three TBST washes, filters were incubated for 1 h with a horseradish peroxidase-conjugated secondary antibody (Dako Diagnostics, Mississauga, ON). Proteins were detected by chemiluminescence (Amersham, Arlington Heights, IL) using Biomax MR film (Eastman Kodak, Rochester, NY). Densitometry was performed using a GS300 reader (Hoefer Scientific Instruments, San Francisco, CA), and results were analyzed using the GS370 1-D Data System, version 2.0 for Macintosh.

Flow cytometry

A total of 1 x 10^6 cells was resuspended in 500 μL PBS + 2% FCS (FACS buffer), blocked on ice with 1 μg of anti-FcγRIIb (2.4G2, Pharmingen, Mississauga, ON) for 20 min, and then stained with either 0.5 μg anti-CD11b-FITC (for liver samples) or one of the following FITC-conjugated antibodies for 30 min on ice (BM cells); PGP1; B220; Ly6G (Gr-1); 7-4 (PMN); CD11b; CD14 and TER-119; and (primitive populations); Sca1, ckit, CD34 (Pharmingen,
Mississauga, ON). Cells were washed 3x with FACS Buffer and resuspended in 500 μL of FACS buffer before analysis on a FACSsort (Becton Dickinson, Mountain View, CA) flow cytometer equipped with CellQuest software (Becton Dickinson). The viable cells that remained unstained represented hepatocytes, while the CD11b+ population included Kupffer cells and contaminating peripheral blood phagocytes. For BM samples, the percent staining was multiplied by the total cellularity (obtained from one femur) to determine the absolute number of each cell type.

**Chromosome analysis**

For Fancc⁺/⁺Sod1⁺/⁺, Fancc⁺/⁻Sod1⁺/⁻ and Fancc⁻/⁻Sod1⁻/⁻ marrows, an aliquot of RPMI + 5% FCS containing 1.5 x 10⁶ resuspended BM cells was added to a tube containing 1 ml Trypsin-EDTA (Irvine Scientific) and 0.75 M KCl. The tubes were incubated at 37 °C for 25 min, spun for 10 min at 1000 rpm and the pellet carefully resuspended in Carnoy's fixative (3 parts methanol: 1 part glacial acetic acid). The fixative was changed two more times and the slides made by air-drying. Approximately 10 metaphases per sample were examined for evidence of chromosomal breaks, gaps or detectable rearrangements.

**Methylcellulose colony forming assays and lineage depletion of total BM cells**

Whole BM cells were plated in 1.1 ml of 1% methylcellulose media supplemented with 10% FCS, 2 mM L-glutamine, 10⁻⁴ M 2-mercaptopethanol and the following recombinant growth factors: for myeloid assays, methylcellulose was supplemented with 1% bovine serum albumin, 10 μg/ml bovine pancreatic insulin, 200 μg/ml human transferrin, 3 units/ml recombinant human erythropoietin, 10 ng/ml recombinant mouse IL-3, 10 ng/ml recombinant human interleukin-6 and 50 ng/ml recombinant mouse stem cell factor (SCF). For Pre-B assays 10 ng/ml recombinant human IL-7 was used (StemCell Technologies, Vancouver, BC). Cells were dispensed using a blunt-ended needle and cultured at a density of 1.7 x 10⁵ and 5.5 x 10⁴ cells per 35 mm dish for
Pre-B and Myeloid colonies respectively (each sample done in duplicate). Dishes were incubated for 6 (for Pre-B) or 12 (for myeloid) days at 37 °C, 5 % CO₂ in air, ≥ 95 % humidity. Colonies (> 20 cells) were counted on a gridded stage using an inverted light microscope. Lineage depleted samples were collected by resuspending the cells at 5.0 x 10⁷ nucleated cells/ml in PBS with 2% FBS, plus 5% rat serum for 15 min at 4 °C. Samples were first incubated with an antibody cocktail (CD5, CD11b, CD45R, GR1, 7-4 and TER-119) and subsequently with an anti-biotin tetrameric antibody (both antibody cocktails from StemCell Technologies, Vancouver, BC) complex (each step for 15 min at 4 °C), then a magnetic colloid was added for cell separation as recommended (StemCell Technologies, Vancouver, BC). To isolate Lin' populations, the suspension was applied to a primed 0.3 inch magnetic column and washed 3x with PBS containing 2% FBS. The cells in the flow-through were enumerated and trypan blue exclusion used to determine viability (> 95%).

Statistical Methods

The Student's t test (Microsoft Excel) was used when analyzing the results. p ≤ 0.05 was considered significant.

2.3 RESULTS

Fance'/Sod1−/− mice develop zonal microvesicular hepatic steatosis

No developmental defects or gross skeletal abnormalities were seen in Fance'/Sod1−/− mice. Body weights of Fance−/−, Sod1−/−, Fance−/Sod1−/−, Fance−/Sod1+/−, and Fance−/Sod1+/+ mice, both male and female, were not statistically different from one another (Fig. 4). Liver and spleen weights were not increased in any of the mutants as compared to Fance−/Sod1+/+ controls. However, necroscopy and histological analysis of Fance−/Sod1−/− mice revealed abnormalities of the liver and BM.
On inspection, livers of Fancc<sup>−/−</sup>Sod1<sup>+/−</sup> mice (n=6) were pale and exhibited a yellow reticular surface pattern. Liver sections were examined by light and electron microscopy, with a typical sample shown in Fig. 5. Liver sections from a, e) Fancc<sup>−/−</sup>, b, f) Sod1<sup>+/−</sup>, c, g) Fancc<sup>+/−</sup>Sod1<sup>+/−</sup> and d, h) Fancc<sup>−/−</sup>Sod1<sup>+/+</sup> mice were stained with Masson’s trichrome (a-d). While periportal (zones 1 and 2) hepatocytes were unremarkable, zone 3 cells were distended by numerous PAS negative (data not shown) cytoplasmic vacuoles that did not displace the nuclei. No inflammatory cell infiltrates were present in the liver, and trichrome stain did not reveal evidence of hepatic fibrosis or increased collagen deposition. Oil red O staining (Fig. 5 e-h) demonstrated microvesicular steatosis (Fig. 1h) in zone 3 hepatocytes of Fancc<sup>−/−</sup>Sod1<sup>+/+</sup> mice. Fancc<sup>−/−</sup> mice (Fig. 5e) revealed no increase in lipid staining over controls, while Sod1<sup>+/−</sup> mice (Fig. 5f) revealed modest amounts of oil red O-positive droplets distributed in a non-zonal pattern. Transmission electron microscopy (EM), performed on Fancc<sup>+/−</sup>Sod1<sup>+/−</sup> (Fig. 6, a,c) and Fancc<sup>−/−</sup>Sod1<sup>+/+</sup> (Fig. 6, b,d) liver samples, revealed no morphological abnormalities of Kupffer cells or endothelial sinusoidal cells, and aside from the obvious lipid filled vacuoles, the structure of hepatocyte smooth endoplasmic reticulum and mitochondria was unremarkable (Fig. 6 c,d).

To search for evidence of hepatocyte injury, serum alanine aminotransferase (ALT) and TUNEL assays were employed. ALT levels were as follows: Sod1<sup>+/−</sup> (55.3 ± 8.67), Fancc<sup>−/−</sup> (45.5 ± 2.87), Fancc<sup>+/−</sup>Sod1<sup>+/−</sup> (41.3 ± 7.98), Fancc<sup>+/−</sup>Sod1<sup>+/+</sup> (22.7 ± 4.2) and Fancc<sup>−/−</sup>Sod1<sup>+/+</sup> (126.8 ± 52.0). While serum ALT from Fancc<sup>−/−</sup>Sod1<sup>+/+</sup> mice was increased (~3-fold) over littermate controls, suggesting that low levels of hepatocyte damage may have been present, this data is not statistically significant (p=0.12). TUNEL assay of liver sections revealed no difference with respect to the rare apoptotic cells seen when Fancc<sup>−/−</sup>Sod1<sup>+/+</sup> and littermate mice were compared.

Primary Fancc<sup>−/−</sup>Sod1<sup>+/+</sup> liver cell cultures generate increased levels of superoxide

To determine whether oxidant stress was present in Fancc<sup>−/−</sup>Sod1<sup>+/+</sup> livers, we assayed spontaneous superoxide production from primary liver cell cultures. Luminol, which undergoes
chemiluminescence when oxidized by superoxide, enabled quantitation of the relative amounts of this species. The average intensity of the samples was recorded as relative light units (RLU), with the RLU values being proportional to the level of superoxide in the samples. Fig. 7 shows the average RLU values for 5 mice per group, each mouse sample assayed in triplicate, taken immediately after luminol addition. In all samples, the luminol signal was ablated when SOD protein was added to the culture medium. Fanc"+/Sod1"/+; Fanc"+; and Fanc"−/Sod1"+/− controls all had statistically similar RLU values of 0.33, 0.38 and 0.44, respectively. Sod1"+ samples showed a marginally elevated RLU of 0.55 that was statistically different from Fanc"+/Sod1"+ mice (p=0.02). In contrast, there was a 4.8-fold increase in the RLU value obtained from Fanc"−/Sod1"− cells (1.62) compared to Fanc"+/Sod1"+ controls (p=0.0008). While hepatocytes were likely the source of the increased levels of superoxide in Fanc"−/Sod1"− cells, we are unable to define the potential contribution of Kupffer cell-derived superoxide. FACS analysis demonstrated that the percentage of CD11b+ cells was similar in all samples.

Increased expression of Mn SOD and HO-1 in Fanc"−/Sod1"− livers.

Heme-oxygenase-1 (HO-1) is induced by various cellular stressors, including ROS. Similarly, MnSOD can also be induced by ROS, including superoxide anion. Thus, an increased level of MnSOD and/or HO-1 in total liver cell lysates, as assessed by immunoblotting with anti-MnSOD and HO-1 antibodies, would be predicted to accompany the putative pro-oxidant state in Fanc"−/Sod1"− hepatocytes. Fig. 8 is a collection of representative Western blots showing protein levels of HO-1 and MnSOD in liver lysates from A) suppliers control, B) Fanc"+/Sod1"+; C) Fanc"−/Sod1"+; and D) Fanc"−/Sod1"− samples. Densitometric analysis of a number of immunoblotting experiments was carried out, with the ratio of protein band intensities for MnSOD or HO-1 normalized according to band intensities following stripping and re-immunoblotting of the same filters with an anti-β-tubulin Ab. The results indicated that there were increased levels of MnSOD and HO-1 of ~4-fold, and ~10-fold, respectively, in Fanc"−/Sod1"−
livers, as compared to \textit{Fancc}^{+/+} \textit{Sodl}^{+/+} littermates, consistent with \textit{in vivo} oxidative stress, however this difference was not significant.

**Peripheral blood and bone marrow abnormalities of \textit{Fancc}^{+/-} \textit{Sodl}^{+/-} mice**

To search for evidence of marrow dysfunction we evaluated peripheral blood (PB) cells from \textit{Fancc}^{+/-} \textit{Sodl}^{+/-}, \textit{Fancc}^{+/-} \textit{Sodl}^{+/-}, \textit{Fancc}^{+/-}, \textit{Sodl}^{+/-} and \textit{Fancc}^{+/-} \textit{Sodl}^{+/-} mice (both males and females) (Table IV). Significant decreases were observed in the RBC (p=0.005) and WBC (p=0.03) compartments of \textit{Fancc}^{+/-} \textit{Sodl}^{+/-} mice, as compared to \textit{Fancc}^{+/-} \textit{Sodl}^{+/-} littermates. WBC values from \textit{Fancc}^{+/-}, \textit{Sodl}^{+/-} and \textit{Fancc}^{+/-} \textit{Sodl}^{+/-} mice, however, were not significantly different from one another (p=0.18 and p=0.12, respectively). \textit{Fancc}^{+/-} \textit{Sodl}^{+/-} differentials (n=4) revealed that the WBC decrease was due to a reduction in both neutrophils and lymphocytes. There was no indication of a granulocyte maturation arrest in any of the samples. PB smears revealed that lymphocytes from \textit{Fancc}^{+/-} \textit{Sodl}^{+/-} blood were often larger with more immature nuclear chromatin than either \textit{Fancc}^{+/-} \textit{Sodl}^{+/-} or \textit{Fancc}^{+/-} \textit{Sodl}^{+/-} controls. Red cell MCV was significantly increased (p=1.4 x 10^{-5}), and there were higher numbers of polychromatic erythrocytes in \textit{Fancc}^{+/-} \textit{Sodl}^{+/-} mice compared to controls (data not shown). \textit{Fancc}^{+/-} mice demonstrated a trend towards reduced WBC, however, this decrease was not significant (p=0.07). Platelet counts were normal in \textit{Fancc}^{+/-} \textit{Sodl}^{+/-} mice, consistent with the normal megakaryocyte numbers observed in the marrow. Interestingly, 8 wk old, but not older (platelet count 587 ± 12.9) \textit{Fancc}^{+/-} mice showed a significant (p=0.007) decrease in platelet numbers. Furthermore, there were reductions in both RBC (p=0.03) and HGB (p=0.04) in \textit{Sodl}^{+/-} mice, as compared to \textit{Fancc}^{+/-} \textit{Sodl}^{+/-} controls. Peripheral counts were obtained from mice up to the age of 3 months. With age, WBC values from \textit{Fancc}^{+/-} mice (10.5 ± 1.5) increased to \textit{Fancc}^{+/-} \textit{Sodl}^{+/-} levels (10.21 ± 0.86), ceasing to be statistically similar to WBC values from \textit{Fancc}^{+/-} \textit{Sodl}^{+/-} mice (5.9 ± 0.86). Thus, in contrast to \textit{Fancc}^{+/-} \textit{Sodl}^{+/-} mice, the reductions of WBC and RBC seen in 8-10 wk old \textit{Fancc}^{+/-} mice normalize over time.
The marrows of Fanc\textsuperscript{+}Sod1\textsuperscript{+}, Fanc\textsuperscript{+}, Sod1\textsuperscript{+} and Fanc\textsuperscript{+}Sod1\textsuperscript{+} femora were then assessed (representative examples are shown in Fig. 9). Decreased cellularity was present in Fanc\textsuperscript{+}Sod1\textsuperscript{+}, suggested by increased fat cell numbers, particularly in the long bone metaphyses. In 5/5 Fanc\textsuperscript{+}Sod1\textsuperscript{+} femurs analyzed, a large increase in the amount of BM fat was present, while in only 1/4 Fanc\textsuperscript{+}Sod1\textsuperscript{+} and 1/4 Fanc\textsuperscript{+}Sod1\textsuperscript{+} controls there was an increase in fat, and when present this was less pronounced than that of Fanc\textsuperscript{+}Sod1\textsuperscript{+} mice. To quantitate these observations, total BM cell numbers per femur were obtained from Fanc\textsuperscript{+}Sod1\textsuperscript{+}, Fanc\textsuperscript{+}, Sod1\textsuperscript{+}, Fanc\textsuperscript{+}Sod1\textsuperscript{+}, Fanc\textsuperscript{+}Sod1\textsuperscript{+}, Fanc\textsuperscript{+}, Sod1\textsuperscript{+}, and Fanc\textsuperscript{+}Sod1\textsuperscript{+} mice (Table V; n is as shown). Cellularity was decreased in Fanc\textsuperscript{+}Sod1\textsuperscript{+} mice (2.33 x 10\textsuperscript{7} ± 0.19) compared to Fanc\textsuperscript{+}Sod1\textsuperscript{+} controls (3.98 x 10\textsuperscript{7} ± 0.62) by 58%, however, this was not significant (p=0.06). There was also no statistical difference in total BM cellularity between Fanc\textsuperscript{+}Sod1\textsuperscript{+}, Fanc\textsuperscript{+}, and Sod1\textsuperscript{+} controls. To determine whether any specific marrow cell type might be differentially affected, flow cytometry was carried out on total BM samples with the following monoclonal antibodies: PGP1, B220, Ly6G, 7/4 (PMN), CD11b, CD14, and Ter-119. As shown in Table V, the average number of Fanc\textsuperscript{+}Sod1\textsuperscript{+} cells of each type was decreased by at least 40%, as compared to Fanc\textsuperscript{+}Sod1\textsuperscript{+} controls. To investigate whether these reductions were mirrored by reduced numbers of committed (Lin\textsuperscript{+}) progenitors, Lin\textsuperscript{+}Sca1\textsuperscript{+}, Lin\textsuperscript{+}cKit\textsuperscript{+} and Lin\textsuperscript{+}CD34\textsuperscript{+} values were obtained from Fanc\textsuperscript{+}Sod1\textsuperscript{+} and Fanc\textsuperscript{+}Sod1\textsuperscript{+} mice (n=3). The average absolute number of these phenotypes for Fanc\textsuperscript{+}Sod1\textsuperscript{+} mice was 1.8 x 10\textsuperscript{6}, 3.6 x 10\textsuperscript{6} and 1.7 x 10\textsuperscript{6}, and for Fanc\textsuperscript{+}Sod1\textsuperscript{+} mice, was 0.9 x 10\textsuperscript{6}, 1.26 x 10\textsuperscript{6} and 0.8 x 10\textsuperscript{6}, respectively, demonstrating that committed progenitor populations were similarly decreased in Fanc\textsuperscript{+}Sod1\textsuperscript{+} marrows (Fig. 10). There was no evidence of increased extramedullary hematopoiesis, as spleens obtained from two sets of animals, revealed no change in cellularity: Fanc\textsuperscript{+}Sod1\textsuperscript{+} (9.65 x 10\textsuperscript{7} ± 0.45) and Fanc\textsuperscript{+}Sod1\textsuperscript{+} (13 x 10\textsuperscript{7} ± 3.85). Furthermore, the average number of Fanc\textsuperscript{+}Sod1\textsuperscript{+} cells of each type from the spleen was the same when compared to Fanc\textsuperscript{+}Sod1\textsuperscript{+} controls.
Fancc<sup>−/−</sup>Sod1<sup>−/−</sup> total marrow cells fail to show increased apoptosis or chromosomal aberrations

Since BM hypocellularity in Fancc<sup>−/−</sup>Sod1<sup>−/−</sup> mice might have been due to an increased level of apoptosis, total marrow samples were analyzed by flow cytometry and propidium iodide / annexinV (PI/A) staining. However, this revealed no gross increase in apoptotic cells in Fancc<sup>−/−</sup>Sod1<sup>−/−</sup> mice, as compared to Fancc<sup>−/−</sup>Sod1<sup>−/+</sup> controls (Table VI). While suggesting that increased apoptosis might not be the explanation for the BM hypocellularity, the possibility of increased apoptosis within a progenitor subset was not excluded by this procedure. As gross cytogenetic abnormalities would impair hematopoietic cell development, we evaluated metaphase chromosome spreads from Fancc<sup>−/−</sup>Sod1<sup>−/−</sup>, Fancc<sup>−/−</sup>Sod1<sup>−/+</sup>, and Fancc<sup>−/−</sup>Sod1<sup>−/+</sup> BM cells. However, there was no evidence of increased chromosomal aberrations (breaks, gaps or detectable rearrangements) in Fancc<sup>−/−</sup>Sod1<sup>−/−</sup> mice (n=2) as compared to control mice (n=3) upon examination of 10 metaphase cells per mouse.

In vitro hematopoietic colony growth is severely impaired in Fancc<sup>−/−</sup>Sod1<sup>−/−</sup> mice

As BM hypocellularity would also result from inadequate growth of hematopoietic progenitors in Fancc<sup>−/−</sup>Sod1<sup>−/−</sup> mice, we examined the in vitro clonogenic potential of committed myeloid (CFU-GM) and lymphoid (CFU-pre-B) progenitors from Sod1<sup>−/−</sup>, Fancc<sup>−/−</sup>, Fancc<sup>−/−</sup>Sod1<sup>−/+</sup>, Fancc<sup>−/−</sup>Sod1<sup>−/+</sup>, and Fancc<sup>−/−</sup>Sod1<sup>−/+</sup> mice. Fig. 1la represents the average number of progenitors/femur ± SEM from myeloid (dark bars) and pre-B (hatched bars) methylcellulose assays for n=6-8 animals per genotype, with each experiment done in duplicate (for Sod1<sup>−/−</sup> pre-B cultures, n=4). This data clearly shows that the numbers of colonies from myeloid and pre-B progenitors/femur from Fancc<sup>−/−</sup>Sod1<sup>−/−</sup> mice (p=0.0002 for both) was severely decreased, as compared to Fancc<sup>−/−</sup>Sod1<sup>−/+</sup> controls. This data indicates that the number of myeloid and pre-B progenitors/femur from Fancc<sup>−/−</sup>Sod1<sup>−/−</sup> mice was approximately 75-fold lower than from Fancc<sup>−/−</sup>Sod1<sup>−/+</sup> controls. The number of colonies obtained from Sod1<sup>−/−</sup> and Fancc<sup>−/−</sup> marrows was
also significantly reduced (p=0.04 and p=0.01, respectively) for both the myeloid and pre-B assays when compared to \( Fanc^c/Sod1^{+/c} \) and \( Fanc^c/Sod1^{+/-} \) controls.

*In vitro* colony forming assays provide additional information about the quality of committed progenitors since both the size of the colonies as well as the frequency of different cell types arising from a myeloid progenitor can be evaluated. For example, most colonies scored from \( Fanc^c/Sod1^{+/-} \) mice just met the criteria for colony size (> 20 cells/colony), as compared to colonies from \( Fanc^c/Sod1^{+/-} \) controls which were highly cellular. Furthermore, the colonies described in Fig. 11a were scored by cell morphology into CFU-GEMM, CFU-GM/G/M and BFU-E groups. Fig. 11b represents the frequency of progenitors/10^5 BM cells. We found that colonies enumerated from \( Fanc^c/Sod1^{+/-} \) samples were mostly erythroid in origin, 57.4 ± 20.44% (p=0.12) with very few CFU-GM/G/M, 8.78 ± 8.0% and CFU-GEMM, 0.46 ± 0.21% colonies being present (p=0.002; p=0.003, respectively). \( Sod1^{+/-}, Fanc^c, Fanc^c/Sod1^{+/-}, Fanc^c/Sod1^{+/-} \) progenitors, on the other hand, all gave rise to the different cell types at similar frequencies.

It was important to determine whether we could rescue colony growth from \( Fanc^c/Sod1^{+/-} \) progenitors in methylcellulose since this would give us an indication of what was causing the lack of growth. We plated whole BM cells from \( Fanc^<c/Sod1^{+/-} \) and \( Fanc^c/Sod1^{+/-} \) mice in the following conditions and scored for colony rescue: a) increased numbers of cells to 2-, 5-, or 10-times the original cell number, b) growth in 5% O2, c) a pan-caspase inhibitor (ZVAD-fmk), d) various antioxidants, e) and increasing concentrations of SCF (Table VII). None of the above tests augmented colony growth. Each rescue experiment represents 3 mice for each of \( Fanc^c/Sod1^{+/-} \) and \( Fanc^c/Sod1^{+/-} \), with each experiment performed in duplicate.

**Primitive progenitor numbers are normal in \( Fanc^c/Sod1^{+/-} \) mice**

Although BM hypocellularity could result from reduced levels of early progenitors, \( Fanc^c/Sod1^{+/-} \) marrows did not exhibit a significant reduction in the lineage-negative (Lin) compartment. The absolute number of Lin- cells, as determined by flow cytometry of non-
fractionated total BM samples, was similar for \textit{Fancc}^{+/-}\textit{Sodl}^{+/-} (1.7 x 10^5 \pm 0.3/femur) and \textit{Fancc}^{-}\textit{Sodl}^{-} mice (1.3 x 10^5 \pm 0.7/femur); n=3 for each genotype. Furthermore, the absolute number of Lin' cells (obtained after Lin' cell depletion-column experiments) from \textit{Fancc}^{+/-}\textit{Sodl}^{+/-}-controls (7.6 x 10^5 \pm 1.52) was similar to that of \textit{Fancc}^{-}\textit{Sodl}^{-} mice (5.2 x 10^5 \pm 1.05), n=6 (values represent cell numbers obtained from both femurs and tibiae per mouse). Flow cytometry of Lin' cells, obtained following Lin' depletion, using monoclonal antibodies against CD34, Sca1 and c-kit revealed no significant differences in absolute number (Fig. 12). The values below represent experiments using 5 animals per group and are the average absolute number \pm SEM. Thus, the absolute number of Lin'Sca1+c-kit' and Lin'Sca1+c-kit' cells from \textit{Fancc}^{+/-}\textit{Sodl}^{+/-} mice was 1.3 x 10^4 \pm 0.3 and 5.1 x 10^4 \pm 2.0, while for \textit{Fancc}^{+/-}\textit{Sodl}^{+/-}-controls were 2.0 x 10^4 \pm 0.9 and 3.4 x 10^4 \pm 0.9, respectively. The similarity is also observed in the Lin'CD34'Sca1' compartment, where both \textit{Fancc}^{+/-}\textit{Sodl}^{+/-} mice and \textit{Fancc}^{+/-}\textit{Sodl}^{+/-}-controls had 13 x 10^5 \pm 0.7 cells. We conclude that the absolute number of cells within the Lin' compartment of \textit{Fancc}^{+/-}\textit{Sodl}^{+/-} BM is similar to controls and that subpopulations within the Lin' compartment of these mice are also similar to controls, as assessed by immunophenotyping.

**Aging study reveals persistant aberrant hematopoiesis in \textit{Fancc}^{+/-}\textit{Sodl}^{+/-} mice.**

Cohorts of mice (wildtype controls, \textit{Fancc}^{+/-}, \textit{Sodl}^{+/-} and \textit{Fancc}^{+/-}\textit{Sodl}^{+/-}) were aged up to 8 months at which point the mice were sacrificed and committed progenitor growth was analyzed (n = 3-4 per genotype). We hypothesized that the lack of progenitor growth observed in the 8 week old \textit{Fancc}^{+/-}\textit{Sodl}^{+/-} mice may persist in this older population and eventually lead to complete BM aplasia. We observed a significant decrease (p=0.03) in BM cellularity/femur of approximately 43% from \textit{Fancc}^{+/-}\textit{Sodl}^{+/-} mice (1.6 x 10^7 \pm 0.1) compared to wildtype mice (3.7 x 10^7 \pm 0.4), consistent with previous BM cellularity data from 8 week old mice (see Table V). To measure the level of apoptosis within the whole BM samples, BM cells were analyzed by flow cytometry for propidium iodide/annexinV (PI/A) staining. We did not detect a significant increase in apoptotic
cells (A7/P1+) from Fancc\textsuperscript{−/−}Sodl\textsuperscript{−/−} mice (11.7 ± 0.8 %), as compared to Fancc\textsuperscript{+/+}Sodl\textsuperscript{+/+} controls (8.9 ± 1.0 %). We also assessed clonogenic growth potential from committed BM progenitors from 6-8 month old mice and our results were very similar to those obtained for younger 8 wk old mice. Fig. 13 represents the average number of progenitors/femur ± SEM from myeloid (dark bars) and pre-B (hatched bars) methylcellulose assays for n=3-4 animals per genotype, with each experiment done in duplicate. This data clearly shows that the numbers of colonies from myeloid and pre-B progenitors/femur from Fancc\textsuperscript{−/−}Sodl\textsuperscript{−/−} mice (p=0.0003 and p=0.005, respectively) was severely decreased, as compared to Fancc\textsuperscript{+/+}Sodl\textsuperscript{+/+} controls. The number of progenitors obtained from Sodl\textsuperscript{−/−} marrows was also significantly reduced for both the myeloid and pre-B assays (p=0.04 and p=0.03, respectively) when compared to Fancc\textsuperscript{−/+}Sodl\textsuperscript{−/+} controls, while myeloid and pre-B progenitors from Fancc\textsuperscript{−/−} marrows were never significantly decreased compared to wildtype, however this did not reach statistical significance. This data clearly indicates that the BM hypocellularity and lack of committed progenitor growth that was observed from the BM of 8 week old mice persisted in the marrows of older Fancc\textsuperscript{−/−}Sodl\textsuperscript{−/−} mice and suggests that there is a defect in the ability of progenitors to proliferate and populate the marrow \textit{in vivo} in Fancc\textsuperscript{−/−}Sodl\textsuperscript{−/−} mice.

2.4 DISCUSSION

We have shown that mice having combined deficiencies of Fancc and the primary cytosolic superoxide-detoxifying enzyme, Sodl, exhibit two novel phenotypes: fatty liver and an impairment of hematopoietic cell development.

Fancc\textsuperscript{−/−}Sodl\textsuperscript{−/−} liver pathology was characterized primarily by zone 3 microvesicular steatosis, possibly a manifestation of \textit{in vivo} superoxide toxicity as evidenced by increased superoxide production. In keeping with superoxide-mediated pathology, we found increases in the oxidative stress-inducible enzymes, MnSOD and HO-1 within Fancc\textsuperscript{−/−}Sodl\textsuperscript{−/−} livers. While microvesicular
fatty liver is often encountered as a result of mitochondrial dysfunction, it can also result from impaired egress of lipids from hepatocytes as seen following specific hepatotoxin exposures. ROS, such as superoxide anion, either alone, or in combination with nitric oxide (NO) yielding peroxynitrite (ONOO⁻), are able to react with a variety of cellular macromolecules, including membrane lipids. Lipid peroxidation and accumulated hydroxy fatty acids within membranes of the endoplasmic reticulum, for example, can interfere with transport of lipids and/or components of VLDL particles that are responsible for removing lipids from hepatocytes. Interestingly, and possibly in keeping with superoxide-mediated organelle, and possibly plasma membrane damage, the livers of mice lacking MnSOD (Sod2) also demonstrated microvesicular steatosis and increased levels of serum ALT. It should also be noted that ROS can function as second messengers, modulating the activities of intracellular signaling molecules and transcription factors. Thus, liver pathology in Fancc⁻Sod1⁻ mice might stem from abnormal gene expression patterns secondary to elevated superoxide levels or reduced dismutation of this species into hydrogen peroxide.

Increased superoxide production by Fancc⁻Sod1⁻ liver cell cultures was interesting given the reports of elevated ROS generation by FA cells, and the reported protective effects of SOD where extrinsic SOD reduced the high rates of chromosomal breakage in FA cells and also diminished MMC cytotoxicity. Reduced SOD1 levels have been reported in FA erythrocytes. While there are no definitive reports that implicate SOD1 as a direct modifier of the human FA disease, genetic evidence suggesting that modifier genes may exist in FA is emerging. Clinical analysis of Japanese FA patients carrying a classic ‘severe’ mutation in FANCC, paradoxically have a mild phenotype, suggesting that modifier genes may be involved in determining severity of the clinical phenotype. Unlike the Fancc⁻Sod1⁻ cross, hepatic steatosis has not been reported human FA. Assuming that a similar pathogenetic mechanism were involved in FA, this discordance might be explained by the known species-specific differences in CYP P-450 genes, or in differences in xenobiotic exposures.
Since lipid accumulation in hepatocytes can be accompanied by necrosis and inflammation, we searched for evidence of hepatocyte damage, and cell infiltrates. Only the modest elevations of serum ALT were suggestive of hepatocyte damage, and this occurred in the absence of overt necrosis or pathological collagen deposition. Activation of Kupffer cells, which also leads to ROS, NO, as well as pro-inflammatory cytokine production, can injure hepatocytes, and is often accompanied by neutrophil infiltration. The lack of infiltrates, the normal percentages of CD11b+ cells in Fancc-Sod1- liver samples, and the zonal liver pathology, however, would suggest a defect intrinsic to the hepatocytes of Fancc-Sod1- mice.

The second phenotype of Fancc-Sod1- mice was that of marrow hypoplasia, accompanied by a striking impairment of in vitro hematopoietic colony formation. Given the normal levels of primitive precursors (as assessed by immunophenotyping), this was suggestive of a growth and/or survival defect in committed progenitor populations. Similar to Fancc-Sod1- mice, in vitro colony generation by FA marrows is impaired at both the multipotential and differentiated progenitor levels in vitro, with the mean CFU-GM values for human FA being approximately 15-fold lower than controls. In contrast to humans with FANCC mutations, however, Fancc- mice do not show spontaneous permanent cytopenias or decreased clonogenic potential. Like their human counterparts, however, hematopoietic cells from Fancc- show increased sensitivity to IFNγ, TNFα and MIP-1α, as well as deregulated apoptosis, and Fancc- BM cells exhibit a decrease (7-12 fold) in short-term and long-term multilineage repopulating ability. The mild thrombocytopenia in young (8-10 wk), but not older (3 mo) Fancc- mice had not been reported previously, and is likely attributable to differences in the genetic backgrounds of the mice. This variable may also account for the modest reductions in myeloid and lymphoid colony formation in our Fancc- mice. Although, unlike FA, platelet counts of young Fancc-Sod1- mice were normal, it is possible that thrombocytopenia would occur over time if marrow failure is progressive in these mice. Interestingly, increased mean corpuscular volume of Fancc-Sod1- red cells was
analogous to the macrocytosis commonly observed in FA patients\textsuperscript{3}, however this morphology has many causes, including liver dysfunction\textsuperscript{126}.

The finding of impaired hematopoiesis in Fancc\textsuperscript{-/-}Sod1\textsuperscript{-/-} mice is most strongly supported by our functional studies of in vitro growth of committed progenitors. CFU-GEMM, CFU-GM, and CFU-preB from Fancc\textsuperscript{-/-}Sod1\textsuperscript{-/-} marrows failed to grow and this observation was also made in older (6-8 month) Fancc\textsuperscript{-/-}Sod1\textsuperscript{-/-} mice as well. Furthermore, despite normal levels of the earliest (lineage-negative) progenitors, there were considerably lower numbers of lineage-positive progenitors/femur in Fancc\textsuperscript{-/-}Sod1\textsuperscript{-/-} mice. In addition, the ability of marrow cells to produce colonies of normal size and containing the usual range of cell types was compromised. In keeping with a markedly reduced proliferative potential (and/or an increased rate of apoptosis) CFU numbers in vitro was not increased by plating more cells (up to 10-fold), adding antioxidants, caspase inhibitors or growth in hypoxia. Thus, poor Fancc\textsuperscript{-/-}Sod1\textsuperscript{-/-} colony formation did not appear to result from the plating of lower progenitor numbers, but instead pointed to a progenitor cell growth or survival defect. Although Fancc\textsuperscript{-/-}Sod1\textsuperscript{-/-} total marrow samples did not reveal evidence of overt apoptosis, increased death restricted to a progenitor subset(s) would readily explain the lack of growth in the Fancc\textsuperscript{-/-}Sod1\textsuperscript{-/-} CFAs. This might be attributable to superoxide-mediated genotoxicity superimposed on a background of reduced DNA repair capacity due to the lack of Fancc. While oxygen-dependent toxicity did not appear to play a role in CFA inhibition (growth in 5% oxygen did not ‘rescue’ growth), it is possible that committed progenitors have pro-oxidant intracellular environments that result in toxicity even at reduced oxygen tensions, that 5% oxygen was still not sufficient to rescue growth, and that 1 - 0.1% oxygen would be necessary to see such an affect. It is also possible that marrow Fancc\textsuperscript{-/-}Sod1\textsuperscript{-/-} progenitors are damaged by ambient oxygen during harvesting and initial CFA plating procedures.

Alternatively, as ROS appear to be required for the normal proliferative response to various growth factors\textsuperscript{127,128}, it is possible that Sod1 deficiency led to loss of a positive growth signal. There is evidence that ROS, such as superoxide and hydrogen peroxide, can act as second
messengers for a variety of stimuli, including growth factors. GM-CSF stimulation, for example, lead to rapid increases in cellular hydrogen peroxide levels, accompanied by elevated levels of tyrosine phosphorylation. The latter may be due to the transient inhibition of protein-tyrosine phosphatases by this species, an event favoring protein-tyrosine kinase-dependent signaling. Furthermore, alterations in redox potential impact a wide range of cellular processes. The balance between ROS and antioxidant systems can thus regulate cellular responses to external stimuli, for example, interfering with hydrogen peroxide generation attenuated the proliferative response of hematopoietic cells to colony stimulating factors. A lack of Sod1 would be predicted to inhibit growth factor mediated cell growth by reducing conversion of superoxide into hydrogen peroxide. Thus, hematopoietic progenitors from Fancc\(^{-}\)Sod1\(^{-}\) mice might be intrinsically hypo-responsive to growth factor stimulation. Perhaps in keeping with this, we observed a consistent reduction in colony formation in vitro when Sod1\(^{-}\) marrow samples were plated. It is also notable that the abnormalities of Fancc\(^{-}\)Sod1\(^{-}\) mice are analogous to those of W/W\(v\) mice that lack normal stem cell factor receptor kinase (c-kit) activity and demonstrate a marked suppression of CFU growth in vitro. Interestingly, there is evidence that FANCC is required for normal STAT1 activation following growth factor stimulation. This intriguing finding raises the possibility that a 'two hit' signaling abnormality might account for the hematopoietic defect of Fancc\(^{-}\)Sod1\(^{-}\) mice: namely, that a decreased level of growth factor-induced ROS (specifically, hydrogen peroxide), together with a defect in Stat1-mediated signaling, act synergistically to inhibit proliferation and/or survival of hematopoietic progenitors.
CHAPTER THREE - Absence of Cu/Zn Superoxide Dismutase (Sod1) Limits Long-Term Progenitor Cell Self-Renewal in Fanconi Anemia Complementation Group C (Fancc)-deficient Mice.

3.1 INTRODUCTION

Chapter 2 described a hematopoietic defect in Fancc$^{-}\text{Sod1}^{+}$ mice that manifested itself as peripheral blood bicytopenia, whole BM hypocellularity, and a complete lack of committed progenitor growth in methylcellulose. These results indicate that excess endogenous superoxide anion results in decreased committed progenitor growth from Fancc$^{-}\text{Sod1}^{+}$ mice and suggests a mechanism by which human FANCC HPC with elevated levels of superoxide anion have abnormal growth. As a result of these observations, we were interested in enumerating a) earlier hematopoietic progenitors in functional assays and b) progenitor growth recovery, to identify if decreased growth is due to a block in this progenitor compartment, or if the defect lies in an earlier population of progenitors.

The cause of human FA BM failure is unknown and previous to our Fancc$^{-}\text{Sod1}^{+}$ strain, spontaneous BM failure has not been observed in any FA mouse models (see Table III for current list of mouse models). This gave us a unique opportunity to examine the nature of bicytopenia in these mice and to generate some hypotheses about the nature of aplasia in FA. One of the possibilities for FA BM failure is that it results from absent or defective hematopoietic precursors. In FA patients (and our Fancc$^{-}\text{Sod1}^{+}$ mice), progenitors do exist, however the growth of CFU-GM, CFU-GEMM and BFU-E is completely absent and the addition of SCF does nothing to correct progenitor outgrowth. This effect is seen both in aplastic anemia patients as well as patients that have not yet developed BM failure. Studies measuring the growth of BM cells from FA patients in long-term cultures (LTBMC) have been difficult to interpret. One study found that differentiated myeloid cells were generated in normal numbers and that the cells were able to
initiate secondary LTBMC cultures. It is important to note that these cultures were grown in 5% oxygen since FA cells are known to have a significant growth advantage in lower oxygen conditions. This study is important because it clearly shows that early hematopoietic progenitors are in fact present in appropriate numbers in FA patients with concurrent BM failure. A second study investigating LTBMC from FA patients (in 20% oxygen conditions) revealed that adherent stromal layers did develop in all FA cases, however, stromal growth rate was slower than that of controls, and impaired CFU-GM was observed in all FA patients. CFU-GM was measured from patient BM aspirates as well as cells harvested from the suspension of LTBMC. To our knowledge, there has been no study done that has measured the frequency of long-term culture initiating cells (LTC-IC), the earliest hemopoietic progenitor cells, in vitro from FA patients or murine models.

The Cobblestone Area Forming Cell (CAFC) assay can be used as an alternative to the traditional LTBMC assays that measure LTC-IC frequencies in vitro. The CAFC assay was developed as a quantitative in vitro assay that allows the direct measurement of the frequency of both short-term (CFU-S) and long-term (MRA) hematopoietic engraftment ability. The most primitive hematopoietic cells are preferentially located within the adherent layer, and can be seen as colonies. Previous reports indicate that in vivo CFU-S are accurately estimated by day 7 CAFC frequencies while MRA are measured by day 28 CAFC frequencies.

The major goal of this work was two-fold; first to define the hematopoietic progenitor compartment that was defective in Fanc+cSod1− mice and was responsible for the peripheral bicytopenia and BM hypocellularity observed in these mice. Secondly, to try to rescue this growth defect using various antioxidants and hypoxic conditions in an attempt to identify the nature of decreased growth. Using the CAFC assay, we measured the frequency of the earliest hematopoietic progenitors (LTC-IC) in vitro from Fanc+cSod1− mice and found that while CAFC did exist in the marrow of these mice, that there was a profound decrease in frequency with time that was only partially rescued with growth in 5% oxygen. Furthermore, lineage depleted, column
- purified progenitors were assessed for growth in liquid media and found to have stunted growth potential \textit{in vitro} as well as a concomitant increase in apoptosis.

If one of the roles of the Fancc protein is to regulate oxidant states within the cell, and the loss of the Fancc protein leads to ROS -mediated cellular toxicity, then forcing a pro-oxidant state (through the introduction of modifier genes) onto a \textit{Fancc} - null background should result in a phenotype reminiscent of FA. If this were successful then we would be able to infer that the BM failure seen in these mice might be a good reflection of the events occurring in human FA patients. It would also be useful in aiding the identification of the causes for BM death, and possibly ways to correct this phenotype. Based on this logic and our results herein, we suggest that BM failure in FA results from the death of the earliest progenitors in the marrow. This may, in part, be due to ROS - mediated (or other reactive species) toxicity, as the specific scavenging of superoxide anion species partially corrects the reduced growth of HPC from \textit{Fancc}/\textit{Sod1}^{-/-} mice.

3.2 MATERIALS AND METHODS

\textit{BM Cell Preparation and Column Purified Progenitor Cell Isolation}

Mice were sacrificed at 8 weeks of age by CO\textsubscript{2} asphyxiation. Total BM cells were collected by flushing femurs with cold αMEM containing 5% FCS. Cell viability, > 90% in all samples, was determined by trypan blue exclusion. Lineage depleted samples were collected by resuspending the cells at 5.0 x 10\textsuperscript{7} nucleated cells/ml in PBS with 2% FBS, plus 5% rat serum for 15 min at 4 °C. Samples were first incubated with an antibody cocktail (CD5, CD11b, CD45R, GR1, 7-4 and TER-119) and subsequently with an anti-biotin tetrameric antibody (both antibody cocktails from StemCell Technologies, Vancouver, BC) complex (each step for 15 min at 4 °C), then a magnetic colloid was added for cell separation as recommended (StemCell Technologies, Vancouver, BC). To isolate Lin\textsuperscript{−} populations, the suspension was applied to a primed 0.3 inch
magnetic column and washed 3x with PBS containing 2% FBS. The cells in the flow-through were enumerated and trypan blue exclusion used to determine viability (> 95%). Column purified progenitors were plated in Iscove's modified Dulbecco's media (IMDM, Stemcell Technologies) supplemented with 15% FBS (lot # 6250, StemCell Technologies), 50 ng/ml SCF, 10 ng/ml IL3 and 10 ng/ml IL6 and allowed to grow in a humidified chamber in 5% CO₂ in air (+/- 5% O₂ in air).

Chemicals

4,5-Dihydroxy-1,3-Benzene-Disulfonic Acid (TIRON) was purchased from Sigma Company (Cat no. D 7389) and [Mn(III)tetrakis (1-methyl-4-pyridyl) porphyrin pentachloride] (MnTMPyP) was purchased from Calbiochem (Cat no. 475872).

Clonogenic Assays for committed hematopoietic progenitor cells

Whole BM cells were plated in 1.1 ml of 1% methylcellulose media supplemented with 10% FCS, 2 mM L-glutamine, 10⁻⁴ M 2-mercaptoethanol and the following recombinant growth factors: for myeloid assays, methylcellulose was supplemented with 1% bovine serum albumin, 10 μg/ml bovine pancreatic insulin, 200 μg/ml human transferrin, 3 units/ml recombinant human erythropoietin, 10 ng/ml recombinant mouse IL-3, 10 ng/ml recombinant human interleukin-6 and 50 ng/ml recombinant mouse stem cell factor (SCF). For Pre-B assays 10 ng/ml recombinant human IL-7 was used (StemCell Technologies, Vancouver, BC). Cells were dispensed using a blunt-ended needle and cultured at a density of 1.7 x 10⁵ and 5.5 x 10⁴ cells per 35 mm dish for Pre-B and Myeloid colonies respectively (each sample done in duplicate). Dishes were incubated for 6 (for Pre-B) or 12 (for myeloid) days at 37 °C, 5% CO₂ in air, ≥ 95% humidity. Colonies (> 20 cells) were counted on a gridded stage using an inverted light microscope.
Long term Cobblestone Area Forming Cell Assays (CAFC)

Primary BM stromal feeder layers were prepared from 4-6 week old female Bl/6 mice. 3.0 x 10^5 BM cells were plated into 96 well dishes in 0.2 ml Myelocult media (StemCell Technologies, Vancouver BC) supplemented with freshly prepared 10^-6 M hydrocortisone hemisuccinate (StemCell Technologies). The cultures were grown until they reached 80% confluence in a 33°C incubator in 5% CO_2 in air (> 95% humidity). Confluency was achieved by 2-3 weeks of culture with weekly half-media changes. One day before BM overlay, the cultures were irradiated with 15 Gy using a ^{137}Cs source that emitted 280 rad/min and the cells were allowed to recover overnight (as per the protocol of StemCell Technologies). There were no CAFC colonies observed in the irradiated cultures without BM overlay up to 6 weeks post-irradiation. Fresh, non-fractionated BM cultures were obtained from the femurs of mice sacrificed by CO_2 asphyxiation. The marrow was flushed into cold αMEM + 5% FBS. The BM cells were diluted in MyeloCult + 10^-6 M hydrocortisone starting at a density of 3.0 x 10^5 c/ 0.2 ml media and five further dilutions were made, each titrated down by half to a final dilution of 937 c/0.2 ml of media. Each dilution from each mouse was repeated 6 times on the same plate and 32 wells were scored from each oxygen concentration (n = 5 mice/genotype). All of the media was removed from the irradiated stromal cultures and the BM cells were plated on top of the adherent irradiated stromal cells (StemCell Technologies). Weekly half media changes were done for 4 weeks with fresh MyeloCult + 10^-6 M hydrocortisone and scoring of CAFC colonies was done before addition of fresh media. Scoring was done by scanning each well at 400 x magnification using an inverted scope on phase contrast. A well was scored positive if it contained at least one CAFC, which represented a cluster of cells with at least 5 cells grouped together [13]. A CAFC was defined as cells that were large and homogenous, not refractive and in the same plane as the stromal layer. The frequency of CAFCs were calculated using the frequency of negative wells at each cell dilution in a Poission-based limiting dilution analysis.
Poission statistical software was provided by StemCell Technologies and was used to calculate significant differences.

*Column Purified Progenitor Growth Curves*

Column purified progenitors were grown in IMDM + 15% FBS, 50 ng/ml SCF, 10 ng/ml IL-3 and 10 ng/ml IL-6 in a 37°C incubator with 5% CO₂ in air (+/- 5% O₂), ≥ 95 % humidity. Growth was measured using trypan blue exclusion. For studies without drug addition, cells were plated at a density of 2.0 x 10⁵ in 1 ml of media, in triplicate in a 24 well plate (n=5 for each genotype). For TIRON and MnTMPyP studies, chemicals were diluted directly into IMDM media (+ growth factors) at the concentrations indicated and cells were plated at a density of 4.0 x 10⁴ in 0.2 ml of media, in triplicate in 96 well plates (n=3 for each genotype).

*Flow cytometry for TUNEL Assays of Column Purified Progenitors*

Cells were collected on day 13 for TUNEL analysis. Samples were washed twice in cold PBS + 1% BSA and were transferred to a U-bottom 96 well plate. Cells were resuspended in 0.1 ml of freshly prepared 4% paraformaldehyde, pH 7.4 and allowed to incubate at room temperature for 1h on an orbital shaker. The plate was centrifuged using a flat-bed spinner at 300 g for 10 min to remove fixative. Cells were washed once in cold 0.2 ml/well PBS + 1% BSA and resuspended in 0.1 ml/well permeabilization solution (0.2% Triton X in PBS) for 10 min on ice. Cells were washed twice in cold PBS and resuspended in 25 μl/well of TUNEL reaction mixture, which was made up of 2.5 μl TdT enzyme + 22.5 μl dUTP-FITC labeling solution (Boehringer Mannheim, Cat no. 1684795). Labeling was done for 1h in a 37°C incubator in the dark. Cells were washed twice with cold PBS (0.2 ml/wash) and resuspended in a final volume of 0.3 ml in PBS. The samples were collected using a Becton Dickinson FACS machine equipped with Cell Quest Software and the samples were analyzed using FloJo Software.
Primary Mast Cell Cultures

The femurs and tibiae from wildtype and Fancc<sup>−/−</sup>Sod1<sup>−/−</sup> mice were flushed and cell density was determined by trypan blue staining. The BM cells were grown at a density of 1-2 x 10<sup>6</sup> c/ml for the first week in a 24-well tissue culture plate in Iscove’s media supplemented with 15% FCS (#6250, StemCell Technologies), 10 ng/ml IL-3, 10 ng/ml IL-6, and 50 ng/ml SCF. In the second week, the adherent stromal cells were discarded and the suspension cells were centrifuged, washed and resuspended in new media (the same as above, but without IL-6). The cells were then maintained and expanded at 2.0 x 10<sup>5</sup> c/ml and were not split until the sixth week in culture at which point the cells began to grow slowly and were homogenous mast cell cultures.

Murine Embryonic Fibroblast Cultures

Female mice were set-up in cages with proven stud males and were checked for vaginal plugs in the morning of each day. Once a plug was found, this was considered day 1 and the pregnancy was timed so that the female is sacrificed on day 14 by CO<sub>2</sub> asphyxiation. The uterus was dissected carefully from the female, so as not to puncture the gut, and placed into a sterile petri-dish within a tissue culture hood. The individual embryos were removed from the uterus into 100 mm sterile dishes using freshly cleaned scissors and tweezers sterilized with 70% ethanol between embryos. The embryos were minced as finely as possible using re-flamed scissors and 1.5 ml of warmed trypsin was added to the plate and incubated at 37 °C for 5 minutes to fully break up the embryo. 8.5 ml of warm α-MEM with 20% FCS was added to the plate and transferred to a 50 ml Falcon tube. The petri-dish was washed with the same medium to collect all of the cells and then added to the same tube. The suspension was then passed through a syringe fitted with a 15 gauge and subsequently an 18 gauge needle to finely break up the embryo pieces. The suspension cells were counted with acetic acid and plated at a density of 5.0 x 10<sup>6</sup> c/100 mm dish (this yields approximately 10 dishes per embryo). The media was changed every 3-4 days until the cells became confluent, which took 1-2 weeks. The confluent cells were transfected by
calcium phosphate precipitation. Briefly, on day one, cells were plated at a density of 5-9 x 10^5 c/60 mm dish in α-MEM + 10% FCS. On day two, the plasmid DNA was prepared using the following components; 0.25 ml CCT (0.25M CaCl_2, 40 μg/ml salmon sperm carrier DNA, 0.1 x TE, pH 7.05), 5 μg SV40 large T antigen plasmid, 0.5 μg selectable pSV_2neo plasmid and 0.25 ml 2x HBS (280 mM NaCl, 50 mM Hepes, 1.5 mM Na_2HPO_4, pH 7.10). The HBS was added dropwise while bubbling air into the CCT/plasmid mixture through a plugged small pasteur pipette at a rate of 1 bubble/sec. After addition of the HBS, the precipitate was left to form for 30-40 minutes. Once the precipitate formed, 0.5 ml was added to the 60 mm dish, swirled and incubated at 37 °C for 16 hours or overnight. On day 3, the media was removed, the monolayer was rinsed and 5 ml of fresh α-MEM + 10% FCS was added to the cells. Five hours later, the cells were trypsinized and split into 6 x 60mm dishes containing 5 ml α-MEM + 10% FCS + 400 μg/ml G418. The media was changed every 3-4 days until the selected clones were grown out and confluent.

**Bone Marrow Derived Macrophage Cell Lines**

The BM from two femurs (per mouse) were flushed, pooled together and resuspended into 4.5 mls of DMEM + 10% FCS. To this suspension, 4.5 mls of J2 viral supernatant (3T3 cells stably transfected with retroviruses carrying the v-raf and v-myc oncogenes) and 5 μg/ml protamine sulfate are added and plated onto a 10 cm² petri-dish and left overnight. The viral supernatant was first collected from fully confluent viral producer cells (for maximal viral titer) and filter sterilized using a 0.45 micron filter. The following day the adherent cells were discarded and the suspension cells were collected into a fresh Falcon tube and resuspended in 4.5 mls DMEM + 10% FCS, 4.5 mls fresh viral supernatant and fresh 5 μg/ml protamine sulfate. The cells were plated and left overnight for a second time. On the third day, the cells were collected again into a fresh Falcon tube, washed in PBS once and resuspended into 12 mls of DMEM + 10% FCS.
The cells were aliquoted into 6 well tissue culture dishes in decreasing concentrations and left to culture for 5-6 weeks. During this time, the media was changed once a week until the sixth week when the culture became confluent and the media began to turn yellow, at this point the cells were terminally differentiated. Cytodex beads were added to the cultures and left for 48 hours at which point the beads were transferred to a new tissue culture dish and the cells were allowed to move off of the beads and adhere to the dish. Once the cultures grew out into confluent culture, the cells were then cloned into base methylcellulose media and frozen in aliquots as polyclonal and clonal cultures.

Statistical Methods

The Student's $t$ test (Microsoft Excel) was used when analyzing the results other than those obtained for CAFC frequencies. $p < 0.05$ was considered significant.

3.3 RESULTS

CAFC frequencies are significantly decreased from $Fance^{-}Sod1^{-}$ mice and can be partially rescued in hypoxia.

We were interested in defining the exact progenitor compartment within which the hematopoietic defect occurs. LTC-IC assays could not be used to examine the frequency of the earliest progenitor populations because cells from $Fance^{-}Sod1^{-}$ mice did not survive in vitro for the duration of the assay (28 days). Thus, the alternative was to examine the frequency of the earliest progenitor populations on a daily basis from the initial day of culture using the CAFC assay system. The frequency of CAFC/$10^5$ BM cells from wildtype, $Sod1^{+/+}$, $Fance^{-}$ and $Fance^{-}Sod1^{-}$ mice was determined on days 3, 7, 14, 21 and 28 and cultures were grown either in 20% or 5% oxygen. Table VIII represents the CAFC frequencies from 5 mice, each set done with 6 repeats of 6 dilutions of BM cells/mouse. The data clearly shows that progenitors from $Fance^{-}$.
Sod1+ mice only exist *ex vivo* for a maximum of two weeks in 20% O₂ and these CAFC frequencies are significantly decreased in Fancc+ Sod1+ mice at every day measured compared to wildtype, Fancc+ and Sod1+ controls in 20% O₂. When cultures were grown in 5% O₂, there was a partial increase in CAFC frequency in Fancc+ Sod1+ mice and CAFC now existed *ex vivo* for the duration of the assay, albeit at constant declining frequencies. CAFC frequencies from Fancc+ Sod1+ mice at 5% O₂ were still statistically different from wildtype at all time points, however they were no longer different from Sod1+ and Fancc+ controls at days 3, 7 and 14. This data suggests that early progenitor cells do exist in the marrow of Fancc+ Sod1+ mice, albeit at lower frequencies than in wildtype marrows.

A benefit of using the CAFC assay system to measure progenitor frequencies and proliferation is that qualitative observations of CAFC can be made at the time of scoring. Fig. 14 is a representative collection of pictures of CAFC colonies from wildtype, Sod1+, Fancc+ and Fancc+ Sod1+ cultures taken at day 7 in a) 20% and b) 5% O₂. The CAFC colonies can be seen as a group of refractive, homogenous cells located under the stromal layer. It is clear from these pictures that there is a difference in the quality of CAFC from Fancc+ Sod1+ mice. CAFC colonies from wildtype cultures were always large when they appeared in culture, a result that was also seen in Fancc+ cultures. Thus, the decrease in CAFC frequency seen in wildtype and Fancc+ cultures was due to a normal reduction in CAFC frequency over time and not to a change in the size of the actual colonies. CAFC colonies from Sod1+ BM were consistently smaller than either the wildtype or Fancc+ colonies, but were always significantly larger than CAFC colonies from Fancc+ Sod1+ cultures. This result was partially ameliorated by a reduction in oxygen concentration, since Fancc+ Sod1+ CAFC grown in 5% O₂ were larger (Fig. 14b).

Fig. 15 is a collection of CAFC colonies from wildtype (a, b) and Fancc+ Sod1+ (c – f) cultures taken at day 7 (a, c, e) and day 10 (b, d, f) in either 20% (a – d) or 5% (e, f) O₂ on high magnification. These figures show morphological changes in the CAFC colonies from Fancc+ Sod1+ cultures. As we had already observed, wildtype CAFC were always large when they
appeared in culture, whether it is an early CAFC (day 7) or a later CAFC (day 10) colony and this observation was identical in 5% O₂. Conversely, CAFC progenitors from Fancε⁺Sod1⁺ cultures barely constituted a significant colony with only 4 cells being present (Fig. 15 c), on day 7 in 20% oxygen) and the progenitors within a given colony were irregular in volume and shape (Fig. 15 f). It is also clear from Fig. 15 f) that Fancε⁺Sod1⁺ CAFC grown in 5% O₂ were significantly larger and more homogenous then those grown in 20% O₂ (Fig. 15 d). These results indicate that while early progenitors do exist in the marrow of Fancε⁺Sod1⁺ mice, they appear to be exquisitely sensitive to the toxic effects of 20% oxygen, exhibiting abnormal proliferative capacities.

Column purified early HPC from Fancε⁺Sod1⁺ mice have reduced growth rates and increased apoptosis.

To further explain the reduced growth of both the committed progenitors in methylcellulose as well as the earlier CAFC progenitors in long-term BM cultures, as well as to isolate a population of cells that can be assessed biochemically, early hematopoietic progenitors (HPC) were purified by negative selection and grown in the presence of SCF, IL-3 and IL-6. HPC growth was measured by direct cell counts of wildtype, Fancε⁺, Sod1⁺ and Fancε⁺Sod1⁺ cells on days 3, 6, 9 and 13 post-isolation (Fig. 16a), (n=5 for each genotype, each count done in triplicate). Both Fancε⁺ and Sod1⁺ HPC had similarly decreased growth rates compared to wildtype controls, however this difference was not statistically significant at any time point measured. Not surprisingly, Fancε⁺Sod1⁺ HPC were found to have significantly reduced growth rates in liquid media in vitro compared to wildtype controls at all time points measured (p < 0.01). We wanted to assess whether the reduced growth of HPC from Fancε⁺Sod1⁺ mice was due to increased apoptosis or decreased response to growth factor stimulation. Thus, HPCs were assessed for the presence of apoptotic nuclei on day 13 post-isolation by the FACS TUNEL assay (Fig. 16b). There was a significant increase in the number of positively staining d-UTP nuclei.
from Fancc<sup>-</sup>Sod1<sup>-</sup> HPC (26.7 ± 1.1 %) on day 13 compared to wildtype HPC (12.2 ± 1.5 %) controls (p = 0.001). We hypothesize then that the lack of proliferation seen in BM cells isolated from Fancc<sup>-</sup>Sod1<sup>-</sup> mice and grown in vitro was, at least partially, due to increased apoptosis of progenitor cells.

HPC can be partially rescued when grown in the presence of hypoxia or antioxidants.

Since we hypothesized that the increased apoptosis was a ROS-mediated effect, we next attempted to identify the ROS responsible for this effect. HPCs were thus grown in the presence of the cell permeable superoxide anion scavenger, 4,5-dihydroxy-1,3-benzene-disulfonic acid (TIRON); a Sod1 mimetic, [Mn(III)tetrakis (1-methyl-4-pyridyl) porphyrin pentachloride] (MnTMPyP), or hypoxia (5% O<sub>2</sub>). Similar to the partial rescue of CAFC colonies grown in 5% O<sub>2</sub>, Fancc<sup>-</sup>Sod1<sup>-</sup> HPC were partially rescued when the cultures were grown in hypoxic conditions, n=3-4 per genotype, each done in triplicate (Fig. 17). Fancc<sup>-</sup>Sod1<sup>-</sup> HPC grow at 43% of wildtype in 20% O<sub>2</sub> and this difference is statistically significant (p=0.009) while hypoxia rescues HPC growth to 66% of wildtype and the difference is no longer statistically different (p=0.08). This data, from a different subset of cells, nicely mirrors the observations we accumulated from the CAFC colonies and suggests that the HPC are undergoing excessive apoptosis, likely due to ROS - mediated toxicity.

We also attempted to rescue HPC growth by the specific scavenging of superoxide anion, to try to determine whether this species was involved. A dose response of TIRON on HPC cultures grown for 9 days in vitro shows that 0.001 mM TIRON maximizes growth from all genotypes, n=3-4 per genotype, with each count done in triplicate (Fig. 18). At this dose, TIRON increases HPC growth over basal (no drug) conditions in wildtype, Fancc<sup>-</sup>, Sod1<sup>-</sup> and Fancc<sup>-</sup>Sod1<sup>-</sup> cultures by 3.1, 1.81, 1.78 and 1.90 fold, respectively. When Fancc<sup>-</sup>Sod1<sup>-</sup> HPCs are grown in the presence of 0.001 mM TIRON, the average cell number at day 9 (3.61 x 10<sup>5</sup> ± 0.4) is no
longer statistically different from the average cell number of wildtype HPC grown under basal conditions (3.75 x 10^5 ± 0.3) (p=0.9). An unexpected observation was made when HPC were grown in the presence of 0.001 mM TIRON. We found that growth in TIRON stimulated differentiation of HPC cultures into a larger, highly granular and slightly adherent population as assessed by FACS analysis in all genotypes except for Fancec^-Sod1^- cultures. The significance of this observation is unknown however, it suggests that the rescue of HPC growth in the presence of TIRON is not attributable strictly to superoxide scavenging, since wildtype cells have significantly increased growth rates as well. It does suggest however, that HPC from Fancec^- Sod1^- mice are unable to respond normally to growth factors.

Similarly, a dose response of MnTMPyP, a SOD mimetic done on day 9 post-isolation, revealed that 5 μM MnTMPyP maximally increased growth of cultured HPC from all genotypes, n=5 for each genotype, each done in duplicate (Fig. 19a). MnTMPyP increases HPC growth over basal no drug conditions in wildtype, Fancec^-, Sod1^- and Fancec^-Sod1^- cultures by 1.1, 1.32, 1.31 and 1.69 fold, respectively. The average number of HPC from Fancec^-Sod1^- cultures counted on day 9 in basal conditions are statistically different from wildtype HPC cell numbers under the same conditions (p=0.02). However, when Fancec^-Sod1^- HPCs are grown in the presence of 5 μM MnTMPyP, the average cell number at day 9 (3.0 x 10^5 ± 0.3) is no longer statistically different from the average cell number of wildtype HPC grown under basal conditions (3.87 x 10^5 ± 0.5) (p=0.52). HPC from wildtype and Fancec^-Sod1^- mice were subsequently cultured in the presence of 5 μM MnTMPyP and counted to determine the rate of growth over 13 days (Fig. 19b). Interestingly, MnTMPyP increased growth of wildtype HPC over wildtype cells grown in basal conditions, however this was not a statistically significant difference. The resultant growth of HPC from Fancec^-Sod1^- mice is only partially rescued over 13 days in culture. Unlike the TIRON supplemented cultures, we did not find additional, differentiated cell populations in HPC cultures grown in the presence of MnTMPyP. Taken
together, these observations suggest that the specific scavenging of superoxide anion, either with TIRON or MnTMPyP, results in a partial correction of HPC growth which may, in part, be due to ROS.

**Inability to generate cell lines from Fancc<sup>−/−</sup>Sod1<sup>−/−</sup> mice.**

Since it was very difficult to obtain or grow sufficient numbers of primary BM-derived progenitors from Fancc<sup>−/−</sup>Sod1<sup>−/−</sup> mice, and also because we were interested in examining the biochemical events causing death in Fancc<sup>−/−</sup>Sod1<sup>−/−</sup> cells, we attempted to generate cell lines from Fancc<sup>−/−</sup>Sod1<sup>−/−</sup> mice. Mast cell lines were initially chosen for study because large numbers of these cells can be readily grown from primary BM cultures. Thus, while wildtype controls differentiated successfully into large numbers of homogenous mast cells over the 6 weeks in culture, Fancc<sup>−/−</sup>Sod1<sup>−/−</sup> BM cells failed to proliferate and after approximately 3 weeks in culture, the resulting cell population was highly heterogenous, with a combination of small cells and large, very granular cells (Fig. 20). The average number of mast cells recovered from wildtype cultures after 5 weeks in vitro was 4.5 x 10<sup>7</sup> c/ml (represents a 225-fold increase from the original cell number plated), while the average number of cells from Fancc<sup>−/−</sup>Sod1<sup>−/−</sup> cultures was 9.3 x 10<sup>5</sup> c/ml, a 5-fold increase. This result suggests that Fancc<sup>−/−</sup>Sod1<sup>−/−</sup> BM cells cannot properly expand and differentiate in vitro, and likely in vivo as well. This result was supported by our previous study which showed that TIRON induced differentiation of HPC in all cultures except for Fancc<sup>−/−</sup>Sod1<sup>−/−</sup> cultures.

Murine embryonic fibroblasts (MEFs) were chosen as a good alternative for the generation of a Fancc<sup>−/−</sup>Sod1<sup>−/−</sup> cell line. As these cells are not hematopoietic in origin, we hypothesized that the proliferation defect observed in BM-derived cells might not be manifested in fibroblasts. Thus, embryos were harvested from pregnant females on day 14 of a timed pregnancy and after processing all of embryos, they were genotyped. Wildtype, Fancc<sup>−/−</sup>, Sod1<sup>−/−</sup> and Fancc<sup>−/−</sup>Sod1<sup>−/−</sup> MEFs were retained and left to grow until confluency at which point they were to be transformed.
Intriguingly, MEFs from \textit{Fancc''Sodl''} embryos ceased proliferating once they were plated and could never be grown to confluency. Thus, out of a total of three \textit{Fancc''Sodl''} embryos, none grew to confluency, while all the wildtype and \textit{Fancc''} cells became confluent within a week and were successfully transformed. Furthermore, 3 of 5 \textit{Sodl''} MEFs were capable of growing to confluency, however they were slower to grow out. MEFs were also grown, post-embryo plating in 5% O$_2$ conditions, however this did not rescue growth sufficiently to allow for confluent cultures. These results were interesting because they suggested that the defect observed in \textit{Fancc''Sodl''} hematopoietic cells also extends to cells of completely different origin.

Since it became clear that most cells from \textit{Fancc''Sodl''} mice could not survive ex vivo for any significant amount of time, we attempted to create primary BM derived-macrophage (BMDM) cell lines by infection of BM cells immediately upon marrow flushing using a J2 myc/raf virus, (known to infect monocytes specifically). We hypothesized that if we could infect the cells immediately as they were flushed from the marrow, we might be able to minimize the amount of ROS-mediated toxicity from 20% oxygen. While 3 of 5 \textit{Fancc''Sodl''} BM cultures were successfully infected with the virus, the monocytes did not proliferate sufficiently to be selected as lines. Qualitative observations indicated that even after viral infection followed by two weeks of monocyte differentiation and macrophage outgrowth, the resultant cells from \textit{Fancc''Sodl''} cultures grew significantly slower and were never as confluent as wildtype controls. These results indicate that the 20% O$_2$ culture conditions are likely too toxic for cells from \textit{Fancc''Sodl''} mice and studies need to be done either in short term experiments or in vivo, where the concentration of oxygen is less than 5% O$_2$.

\textbf{3.4 DISCUSSION}

It is our hypothesis that superoxide anions are produced in human FA cells at a rate that overwhelms the capacity of the cell to detoxify them and this imbalance leads to ROS-mediated damage and progenitor cell death. This hypothesis is supported by the observations described in
this chapter using Fancc<sup>−/−</sup>Sod1<sup>−/−</sup> mice as a model. The goals of this study were a) to use a functional assay to measure the existence and frequency of early hematopoietic progenitors (CAFC) from the marrow of Fancc<sup>−/−</sup>Sod1<sup>−/−</sup> mice, b) to determine if the CAFC were undergoing excess ROS-mediated apoptosis (or whether they were simply not responding to growth factor expansion in vitro), and c) to quantify and attempt to 'rescue' the growth of HPC from liquid cultures in order to delineate the causes of decreased growth.

Hematopoiesis is a hierarchial process involving multiple lineages and differentiation states, thus it was important to evaluate the specific population of cells within Fancc<sup>−/−</sup>Sod1<sup>−/−</sup> mice that show deregulated growth. We used the CAFC assay system to evaluate the earliest progenitor subset from the marrow of wildtype, Fancc<sup>−/−</sup>, Sod1<sup>−/−</sup> and Fancc<sup>−/−</sup>Sod1<sup>−/−</sup> mice. Our CAFC data indicate that early progenitor cells do exist in the marrow of Fancc<sup>−/−</sup>Sod1<sup>−/−</sup> mice, albeit at lower frequencies then in wildtype cells and that there is an increase in apoptosis in this population of cells, likely due to ROS toxicity. This was demonstrated in two ways, first CAFC frequency can be partially rescued from Fancc<sup>−/−</sup>Sod1<sup>−/−</sup> cultures in the presence of 5% O<sub>2</sub>. This observation highlights the importance of measuring CAFC frequencies at all time points, since we observed a partial rescue of CAFC frequency at days 3-10 in 5% O<sub>2</sub> from Fancc<sup>−/−</sup>Sod1<sup>−/−</sup> cultures that would not have been evident if only day 28 LTC-IC were measured. Furthermore, qualitative observations of the CAFC colonies from Fancc<sup>−/−</sup>Sod1<sup>−/−</sup> cultures indicate that cellular morphology is severely affected, perhaps reflective of apoptotic membrane changes. Wildtype CAFC colonies are always large, whether these are early, day 7 CAFC or later, day 28 CAFC, and declining CAFC frequency in wildtype cultures is due to a decrease in the number of colonies, and not to the decreased size of the colonies. Therefore, there appear to be two factors at play in decreased Fancc<sup>−/−</sup>Sod1<sup>−/−</sup> CAFC frequencies, first, the natural decline in colony number with time that is also observed in wildtype cells and second, the diminished size of the actual CAFC colony with time/well. We hypothesise that it is the accumulation of superoxide anion over time in vivo that is reducing the CAFC potential to proliferate as well as leading to increased.
apoptosis. The number of cells required within a colony to be classified as a proper CAFC was arbitrarily selected as 5. This small number was chosen owing to the small CAFC colonies that grew from Fancc$^{+/}$Sod1$^{+/}$ cultures in 20% O$_2$. While these might not technically constitute a CAFC in the classical sense (20 cells are usually considered the minimum number for a CAFC), we wanted to demonstrate that the cells do in fact exist in these cultures and are simply not able to expand normally.

While a dramatic repopulating defect has been reported for Fancc$^{−/−}$ mice compared to wildtype controls, Fancc$^{−/−}$ test cells were able to contribute equally to both the lymphoid and myeloid compartments, indicating that the defect lies within the pluripotent progenitor compartment of Fancc$^{−/−}$ mice. This in vivo data corroborates our in vitro CAFC results nicely and strongly suggests that pluripotent progenitors are in fact the cells defective in FA. Interestingly, these Fancc$^{−/−}$ mice with severe repopulating ability do not naturally develop BM aplasia, and the defect is only observed once the cells have been removed from the donor animal and then transplanted into the recipient. It is possible that the effect observed is due to ROS-mediated toxicity while the cells were processed in in vitro 20% O$_2$ conditions. To address this question, the BM samples should be maintained either in 5% O$_2$, or in the presence of an antioxidant for the duration of their time ex vivo.

We chose to 'correct' the ROS overload within Fancc$^{−/−}$Sod1$^{+/−}$ cells in three ways; by growth in hypoxia, through the addition of a superoxide scavenger (TIRON), or a SOD mimetic (MnTMPyP). These studies required a population of cells that were easier to isolate than CAFC and that could be recovered in sufficient enough numbers. Thus, we used column purified Lin$^-$ HPC to examine the effects of the above conditions on cell growth. In keeping with our previous reports of diminished committed progenitor growth and CAFC frequencies from Fancc$^{−/−}$Sod1$^{+/−}$ mice, we found a severe reduction in HPC growth that was accompanied by an increase in the number of apoptotic nuclei on day 13 of culture. It is clear from these progenitor growth studies that the removal of superoxide, through its specific scavenging (with TIRON or MnTMPyP),
diminishes the course of cell death in Fancε⁻Sod1⁻ cultures, just as the specific removal of superoxide anions from FA lymphocytes modulated the course of the human disease ⁵⁷. These studies provide strong evidence that superoxide anion is a mediator of HPC death in Fancε⁻-Sod1⁻ HPC cultures.

SOD has been given as a potential therapeutic to FA patients with the expectation that it would stimulate marrow growth. 4 FA patients were treated with a 2-week infusion of rh-SOD (25 mg/kg daily) to determine whether rh-SOD had any effect on HPC growth or on the abnormal cellular phenotype. Lymphocyte chromosomal aberrations induced by DEB were decreased during rh-SOD treatment in two patients and BM progenitors were increased in one patient ⁵⁷. The results were neither convincing enough, nor was BM outgrowth sustained long enough to provoke further study however, disease progression was modulated. There are many potential reasons why the effect of rh-SOD was short-lived in these clinical trials. Native enzymes such as SODs are poor pharmacological tools because there are many limitations to their use as therapeutics. For example, they often have high molecular weights and therefore cannot penetrate cells, limiting the dismutation of superoxide only to the extracellular spaces, and native enzymes are highly susceptible to proteolytic digestion ¹³³. The use of a SOD mimic with high stability and high SOD activity in FA clinical trials might produce a better outcome.

*In vitro* models of oxidative stress have been useful in predicting the use of SOD mimetics (specifically Mn metalloporphyrins, such as MnTMPyP) as antioxidants in specific *in vivo* models of human disease ¹³³. Metalloporphyrins have been shown to be protective in *in vitro* oxidative stress models, and at micromolar levels, protect cultured cells against the toxicity of superoxide generators ¹³³. Using MnTMPyP, we were able to partially rescue the growth of HPC from both Fancε⁻ and Fancε⁻-Sod1⁻ mice. The correction of HPC growth from Fancε⁻-Sod1⁻ cultures was not complete, probably due to the fact that superoxide anions are known to readily and sometimes spontaneously dismutate to hydrogen peroxide (H₂O₂) which ultimately leads to hydroxyl radical (OH⁻) ¹³⁴. Another important mechanism by which superoxide anion attenuates
disease is through the interaction with nitric oxide (NO) to produce peroxynitrite (ONOO')\textsuperscript{134}. These highly damaging species can lead to lipid peroxidation, protein nitrosylation, induce DNA strand damage which is a trigger for the induction of poly (ADP-ribose) polymerase (PARP), all processes that induce cell death\textsuperscript{135}. Thus, the removal of superoxide anion prevents the formation of cytotoxic OH\textsuperscript{*} and ONOO'. The toxicity observed in \textit{Fancc\textsuperscript{+}Sodl\textsuperscript{+}} HPC is likely due to the accumulation of damage from all of these species, and the specific scavenging of superoxide alone only rescues damage due to this species and not its breakdown products. In order to completely rescue growth, HPC cultures might need to be treated with a cocktail of antioxidants such as L-NMMA, for NO scavenging, uric acid, for ONOO' scavenging and catalases, for H\textsubscript{2}O\textsubscript{2} scavenging.

It is well accepted that low levels of oxidative stress promote cellular proliferation instead of causing cellular death\textsuperscript{134}. This effect occurs through the myriad of signaling roles that exist for ROS in cells. The role for ROS in proliferation was suggested in our HPC cultures grown in the presence of TIRON and MnTMPyP. HPC cultures from wildtype, \textit{Fancc\textsuperscript{+}} and \textit{Sodl\textsuperscript{+}} mice had a distinct adherent population that was not evident in the \textit{Fancc\textsuperscript{+}Sodl\textsuperscript{+}} cultures and growth was fully restored in \textit{Fancc\textsuperscript{+}} and \textit{Sodl\textsuperscript{+}} HPC cultures. While we did not formally characterize this population (our results are preliminary and strictly qualitative), this differentiated population arose from the initial HPC cells plated. Thus, the resultant marrow hypocellularity and peripheral blood bicytopenia described in \textit{Fancc\textsuperscript{+}Sodl\textsuperscript{+}} mice could be due to two related mechanisms, increased apoptosis in the early progenitor compartments as well as decreased proliferative capacity from surviving progenitors.
CHAPTER FOUR - FancA<sup>−/−</sup> SodI<sup>−/−</sup> mice have a less severe phenotype compared to Fance<sup>−/−</sup>SodI<sup>−/−</sup> Mice

4.1 INTRODUCTION

While a role for the FA complex in the nucleus is not disputed, it is becoming increasingly clear that individual FA proteins have important functions that extend beyond the formation of the FA nuclear complex. A significant amount of FANCA, FANCG and FANCC proteins localize to the cytoplasm and the plasma membrane, where they independently interact with various other molecules<sup>16</sup>. While information is accumulating regarding these interacting proteins, little is known about the regulatory mechanisms of FA proteins, their interactions in the cytoplasm, or the stimuli required for nuclear translocation of the complex.

The FANCA gene encodes a protein which contains a bipartite nuclear localization signal at its extreme N-terminus, and a partial leucine zipper<sup>29,136</sup>. FANCA is localized to both the cytoplasm and to the nucleus and a FANCA mutant with a deleted NLS fails to correct MMC sensitivity, and also fails to bind to FANCC and FANCG in the cytoplasm<sup>41</sup>. This FANCA mutant however can still be phosphorylated. These results suggest that the phosphorylation of FANCA is necessary but not sufficient for its proper cellular function, and that the nuclear localization of FANCA involves several functional domains<sup>41</sup>. The mouse cDNA (Fanca) encodes a protein that shares 65% amino acid sequence identity with human FANCA, has a ubiquitous pattern of expression in embryonic and adult mouse tissue and its expression in human FA-A lymphoblast cells completely complements the sensitivity to MMC<sup>137</sup>.

Like FANCC, FANCA is known to interact with various non-FA proteins, including; the nuclear scaffold protein human α-spectrin II<sup>138</sup>; the cytoplasmic protein SNX5, required for intracellular receptor trafficking between organelles<sup>139</sup>; and BRG1, a component of the human SWI/SNF complex known to be involved in the remodelling of chromatin structure<sup>66</sup>.
Furthermore, bioinformatic analysis of the coding region of FANCA has suggested that the protein may have intrinsic peroxidase function. A region of the FANCA protein, encoded by exons 6 to 13 were reported to show limited similarity (4.2%) to the active domain of a superfamily of fungal, plant and bacterial heme (FPBH) peroxidases. Within this peroxidase domain, there are six active site amino acid residues that are of major functional importance and of these, three are conserved between the human FANCA and the FPBH peroxidase motif. Despite the conservation of critical residues, no biochemical function or peroxidase activity has been identified for FANCA, although this result is complicated by the fact that there is no known substrate for this putative peroxidase, making evaluation of enzymatic activity impossible.

Given the role of the FANCC protein in the regulation of both P-450 and GSTP1, known redox regulated proteins, we were interested in examining the possibility that FANCA may also have a role in regulating the cellular oxidant state. We described in Chapters 2 and 3 that Fanne" Sod1" mice have spontaneous BM defects both in vitro and in vivo that exist at the level of the earliest hematopoietic progenitors. To examine whether ROS-mediated FA pathogenesis observed in Fanne" Sod1" mice is a Fanne-specific effect or a more general FA defect, we generated FancA" Sod1" mice and compared the BM phenotype of this strain to the previous Fanne" Sod1" strain developed in our laboratory. We hypothesized that if the primary defect of the FA disease is to protect the cell from ROS-mediated toxicity, and if FANCA also has a role in this process, then Fanca-null mice bred with Sod1-deficient mice should result in a similar BM failure phenotype.
4.2 MATERIALS AND METHODS

Generation of FancA⁺Sod1⁺ mice and histological analysis

FancA⁺/⁻ mice (n=7, backcrossed to C57Bl/6) were bred with Sod1⁺/⁻ mice (n=5, backcrossed to C57Bl/6) until mice that were heterozygous at both loci were obtained. Locus-specific PCR was used to genotype mice. Brother-sister matings of FancA⁺/⁻ Sod1⁺/⁻ mice were carried out to produce litters having FancA⁺Sod1⁺ mice. Knockout mice and littermate controls at 8 weeks of age were used in all experiments. Viral antibody-free mice were housed in the Faculty of Medicine barrier unit according to protocols approved by the Animal Care Committee at the University of Calgary. For light microscopy, tissue samples were fixed in 30% formalin solution and embedded in paraffin and bone sections were first de-calcified before processing. For paraffin-embedded sections; hematoxylin and eosin (H&E) and Masson's trichrome were used.

BM Cell Preparation and Clonogenic Assays for committed hematopoietic progenitor cells

Mice were sacrificed at 8 weeks of age by CO₂ asphyxiation. Total BM cells were collected by flushing femurs with cold αMEM containing 5% FCS. Cell viability, > 90% in all samples, was determined by trypan blue exclusion. Whole BM cells were plated in 1.1 ml of 1% methylcellulose media supplemented with 10% FCS, 2 mM L-glutamine, 10⁻⁴ M 2-mercaptoethanol, 1% bovine serum albumin, 10 μg/ml bovine pancreatic insulin, 200 μg/ml human transferrin, 3 units/ml recombinant human erythropoietin, 10 ng/ml recombinant mouse IL-3, 10 ng/ml recombinant human interleukin-6 and 50 ng/ml recombinant mouse stem cell factor (SCF) (StemCell Technologies, Vancouver, BC). Cells were dispensed using a blunt-ended needle and cultured at a density of 8.5 x 10³ cells per 35 mm dish (each sample done in duplicate). Dishes were incubated for 10 days at 37 °C, 5 % CO₂ in air, ≥ 95 % humidity.
Colonies (> 20 cells) were counted on a gridded stage using an inverted light microscope and scored morphologically into CFU-GEMM, CFU-GM/G/M or BFU-E colonies.

4.3 RESULTS

Histological analysis of FancA''Sodl'' mice.

Necropsy and histological analysis of FancA''Sodl'' mice revealed modest abnormalities of the liver. On inspection, livers of FancA''Sodl'' mice (n=3) did not have the dramatic pale and yellow reticular surface pattern of the Fancc''Sodl'' mice described in Chapter 2. Liver sections were examined by light microscopy, with a typical sample shown in Fig. 21. Liver sections from FancA''Sodl''', FancA'', Sodl''', and FancA''Sodl'' mice were stained with Masson’s Trichrome. The distinct division between relatively healthy periportal hepatocytes and severely affected centrilobular hepatocytes observed from Fancc''Sodl'' mice was not as obvious in FancA''Sodl'' mice. Hepatocytes from wildtype, FancA'' and Sodl'' were fairly homogenous with few cytoplasmic vacuoles present. However, liver sections from FancA''Sodl'' mice did reveal some zone 3 hepatocyte change consisting of multiple cytoplasmic vacuoles (consistent with microvesicular steatosis), that did not displace the nuclei. No inflammatory cell infiltrates were present in the liver, and trichrome stain did not reveal evidence of increased collagen deposition. Oil red O staining (n=2) confirmed the presence of microvesicular steatosis in hepatocytes of FancA''Sodl'' mice while wildtype controls revealed modest amounts of oil red O-positive droplets distributed in a non-zonal pattern (data not shown due to the low quality of these frozen sections).

BM cellularity and body weights are slightly decreased in FancA''Sodl'' mice.

No developmental defects or gross skeletal abnormalities were detected in FancA''Sodl'' mice. Body weights of FancA'' and Sodl'' mice were not statistically different from
FancA^{+/+}Sod1^{+/+} controls (Fig. 22), however both male and female FancA^{−/−}Sod1^{−/−} mice, had statistically decreased body weights compared to wildtype controls at 8 weeks of age; n=3-4 animals per genotype (p=0.05, 0.008 for male and female respectively). Liver and spleen weights were not increased in any of the mutants as compared to FancA^{+/+}Sod1^{+/+} controls. We also assessed marrow cellularity from FancA^{+/+}Sod1^{+/+}, FancA^{−/−}, Sod1^{−/−} and FancA^{−/−}Sod1^{−/−} mice (Fig. 23) and found that the average cell number / femur was slightly decreased (although not significantly) in FancA^{−/−} and Sod1^{−/−} mice compared to wildtype controls. The average cell number / femur in FancA^{−/−}Sod1^{−/−} mice was further decreased (3.7 x 10^7 ± 0.21) compared to wildtype controls (4.9 x 10^7 ± 0.35), however, this difference was did not reach statistical significance (p=0.06).

Colony Forming Assays reveal defective growth from FancA^{−/−}Sod1^{−/−} committed progenitors.

We were primarily interested in the status of committed progenitor growth from FancA^{−/−}Sod1^{−/−} mice and how it compared to the results we obtained from FancC^{−/−}Sod1^{−/−} mice (see chapter 2). Thus, we plated whole BM into methylcellulose and enumerated the in vitro clonogenic potential of committed myeloid (CFU-GM) and lymphoid (CFU-pre-B) progenitors from FancA^{+/+}Sod1^{+/+}, FancA^{−/−}, Sod1^{−/−} and FancA^{−/−}Sod1^{−/−} mice. Fig. 24 represents the average number of progenitors/femur ± SEM from myeloid (a) and pre-B (b) methylcellulose assays for n=3-4 animals per genotype, with each experiment done in duplicate. This data shows that the total number of myeloid and pre-B progenitors/femur from FancA^{−/−}Sod1^{−/−} mice (p=0.01 and 0.009, respectively) was severely reduced when compared to FancA^{+/+}Sod1^{+/+} controls, where the number of myeloid and pre-B progenitors/femur from FancC^{−/−}Sod1^{−/−} mice was approximately 15-fold lower than from FancA^{+/+}Sod1^{+/+} controls. The number of colonies obtained from Sod1^{−/−} and FancC^{−/−} marrows was also significantly reduced (p=0.009 and p=0.04, respectively) in only the pre-B assays when compared to FancA^{+/+}Sod1^{+/+} controls. There were no significant differences in Sod1^{−/−} and FancC^{−/−} colony formation in the myeloid assays compared to wildtype controls.
We also evaluated our methylcellulose results from $FancA^{+} Sod1^{+}$ cultures to determine the frequency of the different cell types arising from a single myeloid progenitor. Thus, the myeloid colonies described in Fig. 24a were scored by cell morphology into CFU-GEMM, CFU-GM/G/M and BFU-E groups and Fig. 25 represents the frequency of progenitors/10^5 BM cells. We found that colonies enumerated from $FancA^{+} Sod1^{+}$ samples were mostly erythroid in origin, 57.4 ± 20.44% (p=0.12) with very few CFU-GM/G/M, 8.78 ± 8.0% and CFU-GEMM, 0.46 ± 0.21% colonies being present (p=0.002; p=0.003, respectively). $Sod1^{+}$, $Fanc^{+}$, and $Fanc^{+/+} Sod1^{+/+}$ progenitors, on the other hand, all gave rise to the different cell types at similar frequencies.

4.4 DISCUSSION

The purpose of this study was to determine whether the primary defect in FA is due to a general ROS-deregulation defect or if this role is specific to FANCC. Our results indicate that elevated levels of superoxide anion superimposed onto a $FancA^{+}$ background result in a FA model that is not as severe as the $Fanc^{+} Sod1^{+}$ mouse described in our earlier studies, however $FancA^{+} Sod1^{+}$ mice do demonstrate a significant reduction in clonogenic growth potential of committed progenitors compared to wildtype, $FancA^{+}$ and $Sod1^{+}$ controls.

The significance of the liver pathology observed from $Fanc^{+} Sod1^{+}$ mice is unclear since human FA patients have not been reported to have liver pathology (unless anabolic steroids have been administered). However, given that CYT P-450 enzymes are largely expressed, constitutively and inducibly, in the liver and that a role for Fanc was identified in the regulation of RED, our observation of a liver defect was of particular interest and indirectly supported the hypothesis that the FANCC gene product has a role in the regulation of RED and P-450 mediated-ROS production. While some abnormal liver pathology is evident in $FancA^{+} Sod1^{+}$ mice, the extent of the defect is not nearly as dramatic as previously observed for the $Fanc^{+}$.
Sod1<sup>−/−</sup> strain. The difference is likely due to the distinct role for FANCC in RED regulation. No similar role for FANCA has been observed<sup>53</sup> and the effect seen in FancA<sup>−/−</sup>Sod1<sup>−/−</sup> mice may be reflective of purely Sod1<sup>−/−</sup> effects. Sod1<sup>−/−</sup> controls did reveal small amounts of hepatic damage, however, the fact that this phenotype was not greatly exacerbated in the presence of a FancA-null background suggests that FancA may not have as prominent a role in the regulation of the CYT P-450 system in hepatocytes. It is important to note however, that the Fance<sup>−/−</sup>Sod1<sup>−/−</sup> strain used in Chapters 2 and 3 was of a mixed background and it is thus possible that the phenotype was reflective of a mutation within a modifier locus or due to some peculiar sensitivity of the Balb/c genetic background. However against this notion, when we backcrossed the Fance<sup>−/−</sup>Sod1<sup>−/−</sup> strain to C57Bl/6, (N=5), we found that the liver phenotype persisted as did the BM hypocellularity (n=2-3 mice).

The most telling of results obtained from Fance<sup>−/−</sup>Sod1<sup>−/−</sup> mice was the near non-existent clonogenic potential of committed progenitors in methylcellulose. FancA<sup>−/−</sup>Sod1<sup>−/−</sup> mice also showed a significant reduction in committed progenitor frequencies compared to wildtype controls, while both Sod1<sup>−/−</sup> and FancA<sup>−/−</sup> controls revealed a 2-3 fold decrease in progenitor frequency compared to wildtype mice, a result also seen from the Fance<sup>−/−</sup>Sod1<sup>−/−</sup> CFA data in Chapter 2. We can compare the extent of the hematopoietic defect between these strains by assessing the fold decrease in committed progenitors from each double knockout animal to its respective wildtype control. This analysis indicates that the fold decrease of committed progenitor growth from FancA<sup>−/−</sup>Sod1<sup>−/−</sup> mice is 16.6x, and 15.7x for pre-B and myeloid assays respectively, and in Fance<sup>−/−</sup>Sod1<sup>−/−</sup> mice is 66.1x, and 22.7x, for pre-B and myeloid assays. This comparison suggests that the hematopoietic defect is more severe in Fance<sup>−/−</sup>Sod1<sup>−/−</sup> mice compared to FancA<sup>−/−</sup>Sod1<sup>−/−</sup> mice. This difference in phenotypic severity is reminiscent of the clinical heterogeneity observed in FA patients. It is now well established that FA-C patients tend to have a significantly more severe form of the disease, with the onset of hematological
malignancy and BM aplasia occurring at a much younger age compared to other FA groups \textsuperscript{26}, and this may be reflected in our double knockout strains.

The role(s) for each of the FA proteins within the cytoplasm is diverse and it is possible that they do not significantly overlap in function until they are collectively called into the nucleus as a functional complex. Our results suggest that the Fancc protein has a more prominent, or a more direct role, in ROS regulation then the Fanca protein. However, the results are also in keeping with the idea that the FA defect may in fact be a general oxidant stress disorder since specific hematopoietic failure was observed in both knockout strains (over the single mutant controls). While FANCA may not be directly responsible for the regulation of proteins that generate superoxide anion, it may have a more indirect effect in the regulation of pro-oxidant states within cells. For example, FANCA has been shown to bind to components of the human SWI/SNF complex \textsuperscript{66}. It is possible that the FANCA protein is partially responsible for opening the chromatin at specific sites for the expression of genes needed to combat pro-oxidant environments. A more detailed analysis of gene expression profiles from HPC cultures of \textit{Fanca}^+/+\textit{Sod1}^- mice may reveal such a mechanism.
CHAPTER FIVE - A Novel Role for Nitric Oxide in Mediating Cytokine-Induced Growth Inhibition of Fancc-deficient Bone Marrow Cells.

5.1 INTRODUCTION

While the FA genes are ubiquitously expressed in humans and in mice, there is a specific hematopoietic defect within FA individuals that leads to progressive BM failure. In keeping with this observation, a specific role for FANCC in the survival and/or proliferation of hematopoietic progenitor cells (HPC) has been suggested \(^90\). As a potential mechanism to account for the marrow defect in FA, it has been reported that Fancc-/- HPC demonstrate increased hypersensitivity to the growth-inhibitory effects of three unrelated pro-inflammatory cytokines: IFNγ, TNFα, and MIP1α \(^70,76\). Consistent with this result, obtained using murine cells, HPCs from FANCC-deficient individuals have been shown to upregulate fas and interferon response factor 1 (IRF-1) gene expression at significantly lower doses of IFNγ than required for control cells \(^93\). The apoptotic responses were mediated via the caspase 8-dependent activation of caspase 3 \(^94\). Paradoxically however, FANCC cells appear to be hypersensitive to the effects of IFNγ. The activation of STAT1 in response to IFNγ in EBV-transformed FANCC lymphoblast cell lines can be suppressed \(^91\), depending on the nature of the mutation responsible for the loss of FANCC activity \(^95\).

We hypothesized that a common mechanism may be responsible for the inhibition of Fancc-/- hematopoietic colony formation in response to IFNγ, TNFα and MIP1α. Several lines of evidence pointed towards inducible nitric oxide synthase (iNOS)-derived nitric oxide (NO) as a possible candidate for mediating the inhibitory effects of these cytokines. NO, enzymatically generated from L-arginine by one of three NOS isoforms, is a free radical that is also able to react with oxygen to yield other reactive species that range from very stable anions to unstable peroxides \(^140\). NO is involved in a wide variety of biological processes, for example, NO made by
eNOS and nNOS have been implicated in the innate immune response, in tumor killing, control of vascular tone, and in chemotaxis. NO produced by iNOS in response to factors such as IFNγ, TNFα and Fas-L is seen during infection, inflammation, autoimmunity and apoptosis. Importantly, NO has also been shown to suppress human hematopoiesis in vitro. Both IFNγ and TNFα, potent inhibitors of hematopoiesis, are known to be capable of inducing iNOS expression and nitric oxide (NO) production in a variety of different cell types. MIP1α, another suppressor of hematopoiesis, has also been shown to induce NO generation by human peripheral blood mononuclear cells. Furthermore, NO not only amplifies MIP1α responses in lymphocytes, but also increases MIP1 mRNA levels, facilitating the recruitment of polymononuclear leukocytes and macrophages.

While much has been published regarding the sensitivity of FA cells to ROS, no information exists in the literature regarding either NO production from FANCC-deficient cells, or the responses of these cells to reactive nitrogen species. We performed experiments to test the hypothesis that an abnormal production of, and/or response to NO might account for the inhibitory effects of proinflammatory factors, IFNγ, MIP1α, and TNFα on Fance−/− BM cells. We observed the following: a) cytokine-dependent inhibition of hematopoietic progenitor growth from murine Fance−/− BM cells was prevented by an iNOS inhibitor; b) Fance−/− hematopoietic progenitors were hypersensitive to NO generating agents; and c) both iNOS gene expression, and NO production were elevated in primary Fance−/− macrophages following exposure to IFNγ and IFNγ/LPS.
5.2 MATERIALS AND METHODS

Clonogenic Assays for committed hematopoietic progenitor cells

Whole BM cells were plated in 1.1 ml of 1% methylcellulose media supplemented with 10% FCS, 2 mM L-glutamine, 10^4 M 2-mercaptoethanol, 1% bovine serum albumin, 10 μg/ml bovine pancreatic insulin, 200 μg/ml human transferrin, 3 units/ml recombinant human erythropoietin, 10 ng/ml recombinant mouse IL-3, 10 ng/ml recombinant human interleukin-6 and 50 ng/ml recombinant mouse stem cell factor (SCF) (StemCell Technologies, Vancouver, BC). Cells were dispensed using a blunt-ended needle and cultured at a density of 8.5 x 10^3 cells per 35 mm dish (each sample done in duplicate). Dishes were incubated for 10 days at 37 °C, 5% CO_2 in air, ≥ 95% humidity. Colonies (> 20 cells) were counted on a gridded stage using an inverted light microscope and scored morphologically into CFU-GEMM, CFU-GM/G/M or BFU-E colonies.

Peritoneal Macrophage Isolation

8 week old mice (N=7 on C57/B16 background) were injected with 1 ml of 3% thioglycollate. On day 5, the mice were sacrificed by CO_2 asphyxiation. A small incision was made on the body of the mouse which exposed the peritoneal lining and the skin was gently pulled away such that the peritoneal lining was kept intact. 10 ml of DMEM + 10% FCS was injected into the peritoneum and the media was collected using a 18 gauge needle. The cell suspensions were centrifuged at 1200 rpm for 5 min, resuspended at 1.5 x 10^6 cells / 2 mls media and grown in a 6 well dish in a humidified chamber in 5% CO_2 in air. Five hours later the adherent peritoneal macrophages were washed twice with warm PBS to remove the contaminating suspension cells and fresh DMEM + 10% FCS was added. The next day, IFNγ (10 ng/ml) with or without LPS (100 ng/ml) was added to the macrophage cultures. The supernatants were collected for nitrite ELISA tests and lysates were made from the cells.
**Column Purified Hematopoietic Progenitor (HPC) and BM-Derived Macrophage (BMDM) Isolations**

Whole BM cells were collected by flushing in αMEM + 5% FCS both femurs from 2 mice per genotype and pooling the samples. Cell viability, > 90% in all samples, was determined by trypan blue exclusion. HPC isolations were performed as previously described (in Chapter 3). For BMDM cultures, BM samples were centrifuged at 1200 rpm and resuspended at a density of $10^7$ cells/ml in a 10 cm$^2$ dish in DMEM + 10% FCS + 5% CSF-1 conditioned (cell-free) media. The next day all suspension cells were removed into a sterile 50 ml Falcon tube and the adherent (stromal) cells were discarded. The cells were centrifuged and resuspended in twice the original volume of fresh DMEM + 10% FCS + 5% CSF-1. Cells were plated at a density of $8.5 \times 10^6$ cells / well of a 6-well tissue culture dish and were allowed to grow in a humidified chamber in 5% CO$_2$ in air for 8-10 days or until the culture became adherent and confluent. Fresh media was added every third day.

**Immunoblotting and densitometry**

Macrophage cells were lysed in Phosphorylation Solubilization Buffer (PSB) (50 mM HEPES, 100 mM NaF, 10 mM Na$_4$P$_2$O$_7$, 2 mM Na$_3$VO$_4$, 2 mM EDTA, 2 mM NaMoO$_4$, 1% Triton X freshly added, pH 7.35) in the presence of the following additional protease inhibitors Leupeptin (1:1000), Aprotinin (1:1000), PMSF (1:1000) (Roche Diagnostics, Mannheim, Germany). Whole cell lysates were centrifuged for 5 min at 12,000 rpm to remove cellular debris and supernatants were collected into a fresh tube and stored at -20°C. Protein concentration was determined by Bradford-method-based assay. Lysate volume corresponding to 40 and 80 μg of total protein (iNOS and Stat1 blots respectively) was diluted 6:1 with Laemmli sample buffer and the samples were boiled for 5 min prior to electrophoresis. Total cell lysates were separated by SDS-PAGE at 150 V and transferred to PVDF membranes by electroblotting using a semi-dry
transfer method at 25 V for 45 min at RT in a solution containing semi-dry transfer buffer (192 mM glycine, 25 mM Tris 10% SDS and 20% methanol). Filters were blocked for 1 hour at RT in TBST (10 mM Tris (pH 8.0), 150 mM NaCl and 0.05% Tween-20) containing 5% BSA. Filters were incubated overnight at 4°C in TBST with 1% BSA with one of the following antibodies; anti-iNOS (1/1000), (Upstate Biotechnology, Lake Placid, NY): anti-Stat1 (1/1000), anti-P-Stat11 (1/1000), (Cell Signaling Technology, Beverly, MA) or anti-α-tubulin (1/500) (Sigma, St. Louis MI). After three TBST washes, filters were incubated for 1 h at RT with a horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Labs Inc., West Grove, Pennsylvania). Proteins were detected by chemiluminescence (Amersham, Arlington Heights, IL) using Flour-S-Max Imager equipped with densitometry software (Bio-Rad Laboratories Ltd. Mississauga ONT).

**Nitrite ELISA**

Supernatants were collected from the previously mentioned stimulated cell cultures and stored at -20°C. 10 μl of 30% (w/v) ZnSO₄ was added to a fresh eppendorf tube containing 250 μl of each supernatant sample, vortexed and incubated at RT for 15 min. The samples were centrifuged at 4000 rpm for 5 min to collect the debris and the clean supernatant was transferred to a fresh eppendorf tube containing 0.5 g cadmium beads. The samples were nutated in the presence of the beads overnight at RT. The next day the samples were transferred to a clean tube, the cadmium beads were removed and the supernatants were centrifuged at 10000 rpm for 5 min. 100 μl of nitrite standards and 100 μl of each sample were loaded in duplicate onto a 96 well ImmunoSorp ELISA plate (NUNC, VWR Inc.). 50 μl of Color Reagent 1 was added to each well and the samples were briefly mixed. 50 μl of Color Reagent 2 was added to each well and the whole plate was incubated at RT for 15 min for the samples to develop. Colour reagents and nitrite standards were provided by Oxford Biomedical Research (Oxford, MI). Absorbance was
measured at 540 nm in a Multiskan Ascent Microtiter Plate reader (Dynex Labsystems, Chantilly, Virginia). Data was collected as uM concentrations of nitrite based on a standard curve of nitrite done on each plate and was normalized to total protein.

**Flow cytometry**

1 x 10⁶ cells were resuspended in 500 µL PBS + 2% FCS (FACS buffer) and blocked on ice with 1 µg of anti-FcyRIIb (2.4G2, Pharmingen, Mississauga, ON) for 30 min. The cells were washed once in FACS buffer and then stained with one of the following antibodies for 1 hour at 4°C; 0.5 µg anti-CD11b-FITC, 0.5 µg anti-CD14-FITC or 0.5 µg anti-CD119-FITC (Pharmingen, Mississauga, ON). Cells were washed 3x with FACS Buffer and resuspended in 500 µL of buffer before analysis on a FACSort (Becton Dickinson, Mountain View, CA) flow cytometer equipped with CellQuest software (Becton Dickinson). Peritoneal macrophages were immunophenotyped for the following cell surface markers; CD11b, CD14, and CD119 (IFNγR). There was no difference in the % staining of any of these receptors between Fancc⁻/⁻ and wildtype macrophages (n=3).

**Chemicals**

Diethylenetriamine Nitric Oxide Adduct (DETA/NO) was purchased from Sigma-RBI (St. Louis, Missouri). S-nitroso-N-acetyl-D,L-penicillamine (SNAP), and N⁶-Monomethyl-L-arginine, (L-NMMA), were purchased from Calbiochem (San Diego, CA). rm IFN-γ, rm TNF-α and rm MIP-1α were purchased from R&D Systems Inc. (Minneapolis, MN). All chemicals were diluted in α MEM.
5.3 RESULTS

Cytokine-inhibited colony growth of \textit{Fanc}^c BM cells is rescued with L-NMMA.

BM cells from wildtype and \textit{Fanc}^c mice were plated into methylcellulose cultures in the presence of increasing doses of IFN\gamma. Consistent with previous reports \textsuperscript{70,76}, Fig. 26a demonstrates that \textit{Fanc}^c BM cells exhibited a dose-dependent inhibition of colony number in response to IFN\gamma (compared to wildtype controls) that was maximal at 1 ng/ml (p = 0.003), (n=4 animals per group). BM cells were plated in the presence of 1 ng/ml IFN\gamma and increasing concentrations of the iNOS inhibitor, \textsuperscript{G}-monomethyl-L-arginine, (L-NMMA). As shown in Fig. 26b, at 0.1, 0.25 and 0.5 mM L-NMMA there was complete rescue of \textit{Fanc}^c colony formation in the methylcellulose cultures (n=3). The average \textit{Fanc}^c colony numbers at these L-NMMA doses are not significantly different from those generated by the wildtype controls (p = 0.75, 0.85 and 0.82 respectively); in contrast, they are significantly different from \textit{Fanc}^c colony numbers when cells were grown in the presence of 1 ng/ml IFN\gamma in the absence of L-NMMA (p = 0.018, 0.025, 0.003). These results suggested that the inhibition of \textit{Fanc}^c colony formation by IFN\gamma was a consequence of NO generation.

Given this result, and the capacity of two unrelated cytokines to also induce NO production by hematopoietic cells, we examined whether inhibition of colony formation by TNF\alpha and MIP1\alpha was similarly reversible by L-NMMA. BM cells were plated in methylcellulose in the presence of either 0.5 ng/ml TNF\alpha, or 1 ng/ml MIP1\alpha, with or without L-NMMA, n=3 (Fig. 27a and b respectively). Fig. 27a shows that the growth of \textit{Fanc}^c progenitors when treated with TNF\alpha alone was significantly suppressed as compared to wildtype TNF\alpha-treated cultures (p =0.007). As seen in the case of the IFN\gamma-treated cultures, TNF\alpha-mediated inhibition of \textit{Fanc}^c colony growth was reversed in the presence of 0.25 mM L-NMMA (p = 0.36). Growth of TNF\alpha treated \textit{Fanc}^c cultures was significantly different from \textit{Fanc}^c cultures grown in the presence
of TNFα plus L-NMMA (p = 0.01). Fig. 27b shows that Fance− progenitors treated with MIP1α were significantly inhibited as compared to MIP1α-treated wildtype cultures (p = 0.003; n=4 animals per group). In contrast, growth of Fance− BM cells in the presence of MIP1α plus 0.25 mM L-NMMA was no longer significantly different from that of wildtype cells maintained under the same conditions (p = 0.21); also, the average colony number was not significantly different from Fance− MIP1α-treated cultures (p = 0.29). Increasing L-NMMA to 0.5 mM restored Fance− progenitor growth to wildtype levels under the same conditions (p = 0.41); and different from no L-NMMA (p = 0.02). As rescue of MIP1α treated progenitors occurred at a somewhat higher dose of L-NMMA, it suggested that at the concentration tested, MIP1α was generating higher levels of NO than the other two cytokines. Together, these results suggested that NO generation might be a common mechanism through which these three structurally-unrelated polypeptides bring about inhibition of hematopoietic progenitor cell growth.

**Fance− BM cells are hypersensitive to NO donors.**

Since Fance− BM progenitor cells show increased sensitivity (compared to controls) to the above cytokines and growth was reversed by L-NMMA exposure, we hypothesized that Fance− BM cells might also be hypersensitive to NO. To test this possibility, colony formation was measured in the presence of two mechanistically-distinct NO donors. BM cells from wildtype and Fance− mice were plated in increasing concentrations of S-nitroso-N-acetyl-D,L-penicillamine (SNAP), an NO donor, with colony number displayed as percent of maximal colony number (n=4 animals per group) in Fig. 28a. Although both wildtype and Fance− progenitors exhibited a dose-dependent inhibition of colony number in the presence of SNAP, Fance− progenitors generated fewer colonies at 0.06 and 0.25 μM SNAP as compared to wildtype controls (p = 0.04 and 0.05, respectively). The reduction in colony number was due to a similar reduction of growth from erythroid as well as GM/G/M progenitors in Fance− cultures,
while wildtype mice appeared to show a greater inhibitory effect on erythroid colonies as compared to GM/G/M, as previously observed for wildtype mice \(^{148}\).

Since SNAP produces large amounts of NO over a wide concentration range, and can also generate/donate additional ROS and sulfhydryls \(^{140,149}\), growth inhibition was difficult to attribute solely to NO \(^{149}\). Therefore, wildtype and Fancc" BM cells were plated in the presence of increasing doses of Diethylene triamine nitric oxide adduct (DETA/NO), a member of the NONOate class of NO donors with a half-life of approximately 20 hr in cell culture and with minimal potential for the generation of additional reactive species \(^{149}\). Fig. 28 depicts data from four separate experiments in which we observed a strong reduction in Fancc" BM colony formation at the lowest dose of 5 \(\mu\)M DETA/NO (\(p = 0.004\)) that was further reduced at the highest dose tested, 100 \(\mu\)M DETA/NO (\(p = 0.0009\)). As seen in Fig. 28, the effect of DETA/NO on wildtype colony formation was minimal at all doses of this agent. These results indicate that committed hematopoietic progenitors of Fancc" mice are highly sensitive to the growth-inhibitory effects of NO donors.

Apoptosis of IFN\(\gamma\)-treated HPC from Fancc" mice is inhibited by L-NMMA.

To determine whether IFN\(\gamma\)-mediated NO production inhibited the growth of more primitive hematopoietic progenitor populations (HPC), we cultured column purified HPC in 10 ng/ml IFN\(\gamma\), in the presence and absence of 0.5 mM L-NMMA. After 3 days in culture, cell counts were used to ascertain the effects of these conditions on HPC expansion (Fig. 29a), \(n=4\), each count done in duplicate. As expected, IFN\(\gamma\) inhibited the growth of both wildtype, 82% and Fancc", 58% HPC as compared to the untreated controls (\(p = 0.04\)). However, when cultured in the presence of IFN\(\gamma\) plus the addition of 0.5 mM L-NMMA, cell HPC growth was restored, being 109% for wildtype, and 114% for Fancc" compared to untreated control populations. To investigate the potential contribution of apoptosis to the inhibitory effect of IFN\(\gamma\), day 6 HPC were assayed for apoptotic nuclei using a flow cytometry-based TUNEL assay (Fig. 29b), \(n=4\).
These experiments revealed that the diminished proliferation of IFN\(\gamma\)-treated Fanc\(^c\) HPC was accompanied by a trend towards increased levels of apoptotic cells in the cultures. This effect was blocked by the addition of 0.5 mM L-NMMA to the IFN\(\gamma\)-containing cultures of both wildtype and Fanc\(^c\) HPC which demonstrated few if any apoptotic nuclei (Fig. 29b).

Fanc\(^c\) macrophages have elevated expression of iNOS.

Given the increased sensitivity of Fanc-deficient cells to NO-generating cytokines, and the ability of L-NMMA to blunt the effects of these, we hypothesized that altered regulation of iNOS might be a feature of these cells. Since progenitor cells that give rise to colonies in the methylcellulose experiments are difficult to purify in sufficient numbers to carry out signal transduction analyses, an alternate BM-derived primary cell source was selected to study the response of the iNOS gene. We first investigated the response of thioglycollate-elicited primary peritoneal macrophages to the combined stimulus of IFN\(\gamma\) plus bacterial lipopolysaccharide (LPS) and measured the expression of the iNOS protein. Fig. 30a (top panel) is a representative time-course immunoblot showing iNOS expression in peritoneal macrophages obtained from wildtype and Fanc\(^c\) mice following stimulation with 10 ng/ml IFN\(\gamma\) and 100 ng/ml LPS. It is evident that induction of iNOS protein occurs more rapidly in the Fanc-deficient cells, and reaches a higher level at the 12 hr time point than that of controls. This is again shown in the densitometry Fig. 30b which represents the average of five independent experiments, with the data expressed as iNOS expression normalized for loading using \(\alpha\)-tubulin. The increase in iNOS is significantly higher in Fanc\(^c\) macrophages as compared to wildtype controls at 8, and 12 hours post-stimulation (\(p = 0.02, 0.04\) respectively). This suggests that Fanc-deficient thioglycollate-elicited peritoneal macrophages show an altered regulation of iNOS protein expression following the potent inductive stimulus of IFN\(\gamma\) and LPS.

The expression of iNOS in peritoneal macrophages from wildtype and Fanc\(^c\) mice stimulated only with IFN\(\gamma\) alone did not consistently demonstrate increased iNOS expression in
the Fanecc" cells, showing this in only three of five independent experiments. The intraperitoneal injection of thioglycollate broth, however, constitutes a pro-inflammatory stimulus that could affect the baseline state of the macrophages, potentially leading to animal-to-animal variation in the responses to a weaker stimulus (for example, IFNγ alone). We therefore also assessed IFNγ-induction of iNOS in BM-derived macrophages (BMDM), cultured from total marrow cells for 7 days, in the absence of any pro-inflammatory stimulus prior to cytokine exposure. As seen in the immunoblot shown in Fig. 31a (upper panel), Fanecc" BMDM stimulated with 10 ng/ml IFNγ expressed higher levels of iNOS than controls. Densitometry analysis in Fig. 31b shows that iNOS expression was maximal in IFNγ-treated Fanecc" BMDM at 8 hours post-stimulation, while the greatest difference over control BMDM was at 5 hours (p = 0.04). This result indicated that Fanecc-deficient bone marrow derived monocytic cells were able to generate higher levels of iNOS post-IFNγ stimulation than control cells, suggesting a possible explanation for the increased sensitivity of these cells to the growth-inhibitory effects of this cytokine.

**Fanecc" macrophages have increased nitrite production.**

To determine whether the increased levels of iNOS seen in the Fanecc-deficient macrophages translated to increased production of NO, we measured levels of nitrite in the supernatants of stimulated macrophages. As shown in Fig. 32, we found a significant increase in nitrite production from Fanecc" macrophages when these were stimulated with IFNγ plus LPS. This difference was statistically distinct from that of wildtype samples at the 8 hrs post-stimulation point (p = 0.04). Fanecc" macrophages stimulated with IFNγ alone revealed an increase in nitrite production compared to wildtype samples at 5 and 8 hours, however, this increase did not reach statistical significance. Thus, there was evidence of a correlation between the levels of iNOS generated and *in vitro* NO production by the macrophage populations.
Phosphorylated Stat1 is increased in Fancc\textsuperscript{−/−} macrophages stimulated with IFN\gamma

Several transcription factors, including Stat1, are known to regulate expression of the iNOS gene in response to IFN\gamma stimulation\textsuperscript{140}. Given our results which show elevated iNOS expression in Fancc\textsuperscript{−/−} cells, we were interested in determining the tyrosine phosphorylation status of Stat1 in response to IFN\gamma. Thus, peritoneal macrophages from wildtype and Fancc\textsuperscript{−/−} mice were stimulated with 10 ng/ml IFN\gamma and phoso-Stat1 (P-Stat1) levels assessed over time by immunoblotting. Fig. 33\textsuperscript{a} is a representative experiment showing P-Stat1 levels in wildtype and Fancc\textsuperscript{−/−} peritoneal macrophages following stimulation with IFN\gamma (top panel) and normalized for loading using a Stat1 antibody (lower panel). Densitometry data, shown in Fig. 33\textsuperscript{b} represents four independent experiments with P-Stat1 expression being normalized for loading using anti-Stat1. This data shows that Fancc\textsuperscript{−/−} macrophages generate higher levels of P-Stat1 at 15 min post-stimulation (p = 0.04), as compared to wildtype controls. The possibility of increased cell surface expression of IFN\gamma receptor in Fanc-deficient cells was excluded by flow cytometry using anti-CD119 antibody staining. As Stat1 can be a positive regulator of iNOS expression\textsuperscript{150}, our results are in keeping with the increased levels of iNOS observed in IFN\gamma-stimulated Fanc-deficient BM cells. Another transcription factor, Hif1α, was recently shown to induce iNOS expression in response to IFN\gamma\textsuperscript{151}. In preliminary experiments, we examined Hif1α protein levels in peritoneal macrophages (n=2) following treatment with 10 ng/ml IFN\gamma for 14 hrs, in ambient oxygen. We observed an ~2-fold increase in the expression of Hif1α in Fancc\textsuperscript{−/−} macrophages over that of wildtype controls (Fig. 34). This transcription factor may act in concert with Stat1 to mediate the observed effects of IFN\gamma in Fanc-deficient cells.
5.4 DISCUSSION

It has been proposed that IFNγ and the two other pro-inflammatory cytokines we evaluated, TNF and MIP1α, whether released constitutively, or as a result of intercurrent illnesses, might play a role in the progressive failure of hematopoiesis seen in human FA. Although all murine FA models generated to date lack spontaneous marrow aplasia, increased sensitivity of Fancc<sup>-/-</sup> BM cells to these three cytokines has been demonstrated by both strains. Given that NO is suppressive to normal hematopoiesis, and the fact that all three cytokines are capable of inducing iNOS, it was important to evaluate the effects of the broad-spectrum NOS inhibitor, L-NMMA, on cytokine-inhibited Fancc<sup>-/-</sup> colony formation. Our data support the hypothesis that cytokine inhibited Fancc<sup>-/-</sup> progenitor growth in vitro is mediated primarily through NO generation. The effects of L-NMMA on IFNγ-mediated inhibition of hematopoietic cells was not confined to committed progenitors assayed in the methylcellulose CFAs, since rescue of more primitive HPC in suspension cultures was also achieved with L-NMMA.

The finding that NO donors were inhibitory at lower concentrations to Fancc<sup>-/-</sup> committed progenitor cells in methylcellulose cultures was a novel finding. This suggested that murine Fancc-deficient cells were more sensitive to the toxic effects of this radical or its derivatives. In keeping with this, we found that the increased number of TUNEL-positive cells in IFNγ-treated HPC cultures was returned to normal levels by the presence of L-NMMA. The apparent sensitivity of Fancc-deficient BM progenitors to NO is of considerable interest, and is similar to the well documented sensitivity of FA cells to oxygen. We were unable to find information about the role of reactive nitrogen species in the pathogenesis of FA, despite evidence that NO is an inhibitor of hematopoiesis.

A considerable amount of experimental evidence indicates that NO has a role in DNA damage, necrosis, and apoptosis. NO is a stable free radical that has been detected in many cell types and yields other radicals that range from very stable anions to unstable peroxides.
NO and its derivatives (N\textsubscript{2}O\textsubscript{3}, NO\textsuperscript{2-} or NO\textsuperscript{+}) are known to be genotoxic, causing DNA damage both directly and indirectly. Of particular interest to FA, NO readily reacts with superoxide, which may be elevated in FA cells \textsuperscript{51}, leading to the formation of the highly reactive entity, peroxynitrite (ONOO\textsuperscript{-}) \textsuperscript{141}. ONOO\textsuperscript{-} is capable of causing single and double-strand DNA breaks, oxidative lesions (such as 8-oxoG), induction of PARP, Fe\textsuperscript{2+} release, GSH depletion, and cell death \textsuperscript{152-154}. NO may also potentially cause damage indirectly by altering the activity of repair molecules, for example, O\textsuperscript{6}-methylguanine-methyltransferase, and Fpg, a bacterial protein responsible for removal of 8-oxoguanine residues from DNA \textsuperscript{155,156}. Many of the above mentioned lesions have the potential to induce chromosomal aberrations and other mutations that might predispose FA individuals to acute myeloid leukemia.

To gain some insight into the cell signal transduction events that might provide a mechanistic explanation for the increased sensitivity of BM-derived cells to IFN\gamma, a population of primary cells was required that could be obtained in sufficient numbers to permit biochemical analyses. For this reason, peritoneal and BM-derived macrophages were selected for study. iNOS and NO studies are readily performed in macrophages where the stimuli required for iNOS responses have been well characterized \textsuperscript{140}. Furthermore macrophages are appropriate for study in FA, given the possibility that cytokines and NO derived from these cells conceivably play a pathogenic role in FA. Strong stimulation of iNOS expression and subsequent NO production from murine macrophages can be achieved with IFN\gamma and LPS co-stimulation. Employing this stimulus, we found that Fancc\textsuperscript{-/-} peritoneal macrophages generated elevated levels of iNOS expression and NO production as compared to controls. When only IFN\gamma was used to stimulate Fancc\textsuperscript{-/-} peritoneal macrophages, elevated iNOS expression was observed in only three of five independent experiments. This variability may have been due to the fact that the recruitment of macrophages and neutrophils into the peritoneal cavity in response to thioglycollate broth results in the partial activation of these cells, in part owing to chemokine and cytokine production by
resident peritoneal macrophages \(^{157}\). For this reason, we were interested in assessing the iNOS response to IFN\(\gamma\) in naïve cells of the monocyte-macrophage lineage, thus BMDM were obtained for study. In keeping with the results observed when peritoneal macrophages were challenged with the potent IFN\(\gamma\) and LPS combination, BMDM from \(F anx^{-}\) mice also generated higher levels of iNOS in response to IFN\(\gamma\) as compared to controls. These results provide a plausible explanation for the increased sensitivity of \(F anx\)-deficient murine hematopoietic progenitors to IFN\(\gamma\), namely, that increased levels of iNOS, and NO, in these cells leads to growth inhibition that is greater than that of \(F anx\)-proficient cells. In this regard, it would be very interesting to establish whether \(F anx^{-}\)BM cells exhibit resistance to the growth-inhibiting effects of the cytokines tested herein.

Cells of the monocyte-macrophage lineage are highly sensitive to a wide variety of stimuli that lead to the induction of iNOS and NO production \(^{140}\). In addition, iNOS expression has been detected in CD34\(^{+}\) progenitor cells following exposure to IFN\(\gamma\), and NO in this system was shown to exhibit inhibitory effects on proliferation \(^{142}\). Interestingly, the constitutive expression of iNOS was described in BM macrophages of patients with myelodysplastic syndromes (MDS) as well as in idiopathic aplastic anemia \(^{158,159}\). Many contributory factors may be involved in promoting marrow aplasia in humans, while at the same time leaving other tissues uninvolved. These potentially include, a) the uniqueness of the marrow cell populations, many of which possess the ability to generate high levels of myelosuppressing NO, as well as NO-inducing cytokines in response to a wide variety of stimuli, b) the sinusoidal nature of the marrow vascular system, which may favor the accumulation of NO, chemokines, and cytokines c) and the myelosuppressive potential of NO. The regulation of the iNOS promoter is complex, and regulation of expression occurs at several different levels \(^{140}\). A large number of transcription factor consensus binding-site elements are present within the murine iNOS promoter, including sites for NF-\(\kappa\)B, STAT1 and HIF1\(\alpha\) \(^{140}\). Since we found that iNOS expression was higher in
IFNγ-stimulated Fancc-deficient cells, it was important to begin to define signal transduction pathways that might account for this differential effect. Along these lines, we have found that two transcription factors, P-Stat1 and possibly Hi1α, known positive regulators of iNOS gene expression following IFNγ stimulation, were expressed at higher levels in IFNγ-treated Fancc-deficient macrophages than in control cells. The novel finding that Hi1α levels may be altered in Fancc-deficient cells treated with IFNγ suggested a possible contributory mechanism for the increased levels of iNOS seen in Fancc-deficient cells. P-Stat1 levels were significantly increased in Fancc-deficient cells post-IFNγ stimulation for 15 minutes, a finding that contrasts with the reduced levels of IFNγ-induced P-Stat1 that have been seen in some human FA mutant cell lines. There are several potential reasons for this difference. First, we determined the P-Stat1 response to IFNγ in primary murine macrophages rather than EBV-transformed human lymphoblasts; secondly, the murine cells used here were null for the Fancc protein, as compared to human cells which generally harbor point mutations of FANCC. Thirdly, there exists the possibility that our observations are a characteristic of the predominantly C57B/6 (N=7) genetic background of our Fancc-deficient mice. Indeed, since all of our findings have been generated through the use of Fancc-deficient primary mouse cells, it would be of considerable interest to determine whether dysregulation of iNOS and NO production and/or the increased sensitivity NO generating compounds are characteristics that are also shared by human FA hematopoietic cells.
CHAPTER SIX - DISCUSSION

6.1 Results Summary

The primary objective of this thesis was to study the role of increased reactive oxygen and nitrogen species on hematopoiesis in Fancc - deficient mice in vivo. The overall conclusion from this work is that elevated levels of either superoxide anion or nitric oxide lead to aberrant hematopoiesis in Fancc'' cells both in vivo and in vitro that is not evident in the wildtype or single mutant controls. Since the Fancc protein may be responsible for the regulation of redox potential within the cell through its interactions with RED and GSTP1\textsuperscript{59,61}, then a lack of Fancc would be predicted to lead to elevated levels of ROS. However, FA-null mice do not develop 'spontaneous' BM failure unless they have been challenged with MMC in vivo. Due to intrinsic differences between murine and human cells, the levels of ROS that might play a role in marrow aplasia may not be present in Fancc-null mice unless environmental, or genetic factors are introduced to create oxidative stress. Thus, we hypothesized that if elevated ROS/RNS were an important pathogenic mechanism in FA, then one would expect that a Fancc'' mouse with an intrinsic pro-oxidant state would resemble human FA.

In Chapter 2 we observed that a lack of Sod1, superimposed onto a Fancc - null background results in a hematopoietic phenotype with some similarities to human FA, namely, decreased BM cellularity, reduced peripheral blood RBC and leukocytes, and a complete lack of committed progenitor growth in vitro. Our model is different from the human FA disease in that Fancc''Sod1'' mice develop microvesicular steatosis, and do not develop thrombocytopenia or malignancy with age. It is likely that Fancc'' mice have additional in vivo compensatory mechanisms for dealing with ROS since FA null mice do not spontaneously develop aplasia. It is possible that in humans with FA, a polymorphism or the loss of one allele of a redox or antioxidant gene would result in a pro-oxidant state that might influence disease progression. FA
patients and cell lines have not been systematically evaluated for heterozygosity of either other FA genes, or additional candidate genes involved in the regulation of oxidant state. Furthermore, there is the possibility that antioxidant proteins may be damaged, via nitrosylation for example, resulting in diminished activity, and hence a pro-oxidant state in vivo. Factors such as these would help to further explain the heterogeneity differences described for FA in combination with genotype-phenotype correlations that are already known.

Since hematopoiesis is hierarchical in nature, we were interested in identifying the specific population of cells that was defective in Fancc<sup>−/−</sup>Sod1<sup>−/−</sup> mice and that eventually resulted in diminished peripheral blood counts, BM hypocellularity and reduced committed progenitor numbers. Therefore, in Chapter 3 we investigated whether Fancc is required for normal early progenitor growth in vitro and if the presence of increased intrinsic superoxide anion further affects growth in this compartment. We found that early hematopoietic progenitors do exist in the marrow of Fancc<sup>−/−</sup>Sod1<sup>−/−</sup> mice and, the specific scavenging of superoxide anion partially modulated the course of cell death in both CAFC and Lin<sup>−</sup> HPC cultures, suggesting that superoxide-mediated toxicity was at least partially responsible for both the apoptotic phenotype and the lack of proliferation observed. We were not able to describe all of the reactive species that were elevated in Fancc<sup>−/−</sup>Sod1<sup>−/−</sup> progenitor cells, however, we hypothesize that the dismutation of superoxide anion into H<sub>2</sub>O<sub>2</sub>, OH<sup>−</sup> or ONOO<sup>−</sup> (through interactions with NO) all contribute to lack of progenitor growth.

The major goal in the creation of a FancA<sup>−/−</sup>Sod1<sup>−/−</sup> mouse strain was to determine whether the hematopoietic defect observed in Fancc<sup>−/−</sup>Sod1<sup>−/−</sup> mice was specific to the Fancc gene alone, or if the FA disease is due to a more general ROS-mediated toxicity defect. In this preliminary study, we observed that FancA<sup>−/−</sup>Sod1<sup>−/−</sup> mice have a reduction of committed progenitor growth in methylcellulose cultures, although the defect was not as dramatic as that observed for Fancc<sup>−/−</sup>Sod1<sup>−/−</sup> mice. Furthermore, hepatic steatosis did not develop in FancA<sup>−/−</sup>Sod1<sup>−/−</sup> mice, and was
presumably due to the function of the Fancc protein in the direct regulation of RED, known to be highly expressed and inducible in the liver.

In Chapter 5 we identified a novel mechanism for the observation that FA BM cells are hypersensitive to the effects of inhibitory cytokines in vitro. We were able to show that the growth of BM progenitors can be fully restored to wildtype levels when cytokine-treated cultures were grown in the presence of a NOS inhibitor, L-NMMA. We were also able to show that FA BM progenitors were hypersensitive to NO donors in vitro. These results raise some interesting clues about the source of internal cross-linkers in human FA patients and implicate a new pathway to the pro-oxidant states already described for this disease.

6.2 A Role for FA Proteins in Redox Regulation.

Many intriguing questions remain in the field of FA research. Perhaps the most pressing of these has to do with the primary defect in this disease and resolution of the two major hypotheses that exist. The first suggests that the FA proteins are involved in the repair or recognition of specific types of DNA damage, and the lack of an FA protein results in an abnormal repair ‘surveillance’ system that is manifested primarily by chromosomal aberrations. The observation that the FA proteins co-localize with BRCA1 at nuclear foci in response to damage induction or that DNA repair proteins co-exist in a large surveillance complex that scans the genome for errors is an intriguing and likely concept. If in fact the FA proteins are part of a large repair machine, they conceivably have a distinct role in the repair of a very specific type of DNA lesion, such as the homologous recombination repair pathway, involved in the correction of cross-links.

While several studies have clearly shown a role for the FA protein complex, direct or indirect, in the repair of damaged DNA, the ‘damaging’ source remains unidentified. DNA cross-link formation does not occur spontaneously in vivo, and if a small subset of cross-links did
appear spontaneously, the reason(s) why the BM cells are preferentially affected cannot be explained by this model. It is also clear that the FA proteins have distinct roles outside of the nuclear complex and that these roles are important in the proper function of the cell. The idea that the FA proteins have both nuclear and cytoplasmic functions that are not mutually exclusive is analogous to other chromosomal instability disorders with cancer predisposition such as ataxia telangiectasia where the mutant protein, ATM has a role in both DNA repair and ROS detoxification. Given the interactions described for FANCC and FANCG with cytochrome P-450 enzymes and FANCC in the regulation of GSTP1, it seems likely that these FA proteins have an indirect role in maintaining cellular oxidant states. Thus a mutation in one of these proteins necessarily leads to elevated ROS levels and an upset in the balance of detoxifying antioxidants.

These studies raise the possibility that FA proteins may have a more general role in the regulation of structurally similar proteins that are required for the protection against oxidative stress. In keeping with this hypothesis, and in light of our results in chapter 5 which implicate nitric oxide synthase (NOS) and its product NO in the pathogenesis of FA, it is important to note that the NOS enzyme has similar functional domains as the cytochrome P-450 reductases. The N-terminal region of NOS contains a heme oxidase domain and the C-terminus contains a flavin reductase domain, both of which are strictly conserved between the two molecules. Based on this similarity and our observation that FA BM cells are hypersensitive to NO donor drugs in vitro, it would be worth investigating a possible interaction between FANCC or FANCG and NOS. Perhaps the improper regulation of this enzyme within a subset of monocytes in the BM environment would lead to slightly elevated levels of NO species. It is known that superoxide anion is a by-product of P-450 reduction and that P-450 and NOS can exist in similar cells. Thus, the abnormal regulation of P-450 by either mutant FANCC or FANCG proteins could lead to elevated levels of superoxide anion, known to rapidly react with NO to produce the very potent oxidizing by-product peroxynitrite (ONOO⁻). The result is a pro-oxidant state that develops
within the cell as a result of ROS and/or RNS build up, that in turn overwhelms the antioxidant systems of the cell leading to cellular damage.

6.3 A Role for NO in FA BM Aplasia

It is important to remember that the BM is the most consistently and severely affected tissue in FA. Aplasia is the major cause of morbidity in FA patients and research should be focused on the reasons why this organ is so sensitive to a lack of FA protein function. Explanations for the inhibitory effects of IFNγ on FANCC HPC cannot rely primarily on the observation that STAT1 has decreased phosphorylation \(^9\) since it is known that IFNγ does not always relay its signaling messages through STAT1 \(^34\). Furthermore, FA HPC are also hypersensitive to the killing effects of TNFα and MIP1α, which do not signal through STAT1 \(^34\). A more general defect must exist within FA HPC or within the BM microenvironment in order to explain the sensitivity of FA BM cells to induced cell death from unrelated cytokines. We have proposed in the second half of this thesis that NO, either from activated macrophages or from natural cellular processes such as Ca\(^{2+}\) signaling, may have a direct role in the selective death of FA HPC.

The particular sensitivity of FA cells to DNA interstrand cross-linkers and the fact that this sensitivity exists primarily within the BM microenvironment \textit{in vivo}, suggests that damage from an endogenous cross-linker that either exists within, or is produced from, cells in the BM of FA patients, leads to eventual BM aplasia. We believe that NO, either alone or in combination with other ROS, may lead to the formation of endogenous cross-linkers initiating the cellular phenotype observed for FA. NO is a good candidate for this role since all mammalian cells can produce low levels of NO and are also influenced by this species \(^140\). NO is known to cause mutagenesis leading to cell death, protein and lipid damage and defective cellular signaling. In fact, the majority of the cellular phenotypes described for FA to date can be explained by NO-
mediated effects (Fig 35). NO can lead to DNA damage either directly or through one of its breakdown products, N₂O₃ or ONOO'. For example, a high level of 8-hydroxy-2-deoxyguanine (8-OHdG) bases have been detected in the DNA of FA cells. 8-OHdG is a damaged DNA base considered to be a sensitive marker for oxidative DNA damage and can arise as a result of direct ROS damage as well as from ONOO' induced damage. Thus ONOO', the product of superoxide anion and NO, and a potent oxidizing agent, can lead to a specific type of DNA damage that is commonly found in FA cells. A study that examined the levels of 8-OHdG in FA cells with and without L-NMMA treatment would help to determine whether endogenous NO levels were increased in FA patients.

Perhaps more importantly, NO exposure can also lead to the formation of single-strand breaks, DNA intra- and interstrand cross-links and DNA-protein cross-links. The direct attack of N₂O₃ and (to a lesser extent) ONOO' on DNA can lead to DNA deamination with the end result being the formation of abasic sites and the formation of single strand breaks after endonuclease cleavage. While the exact mechanism for cross-link formation from NO treatment is not well understood, it is has been observed in vitro where cross-link formation corresponds to approximately 6% of the guanine deamination product xanthine. It is thought that the FA proteins have a role in the repair of double strand breaks (DSB) because increased HR activity has been described in FA-null nuclear extracts. A genetic study was done in E.coli to assess the genes that were required for the repair of NO-induced damage: AP-endonuclease-deficient strains and DSB repair-deficient strains were very sensitive to killing by NO, suggesting that recombinational repair must be in place in order to survive NO exposure.

NO is known to regulate the enzyme activity of various proteins (guanylate cyclases, cytochrome P-450 mixed function oxidases, thiols, catalases) by its interaction with Fe-S and heme centers. Given the observations that FANCC and FANCG regulate P-450 enzymes and studies which have identified SOD deficiencies in FA cells without the identification of SOD mutations, it would be interesting to assess peroxynitrite-induced protein damage in FA cells.
Using a marker of peroxynitrite-specific protein damage, 3-nitrotyrosine (3-nTyr), antioxidant proteins in FA cells could be studied for increased levels of 3-nTyr. Interestingly, both ONOO⁻ and lipid peroxidation have been indirectly implicated in FA before, with the result that PHGPₓ, an enzyme responsible for destroying lipid hydroperoxides and malondialdehyde, a product of lipid peroxide degredation, were found to be elevated in SV40-transformed FA fibroblasts. Along these lines, the specific nitrosylation of SOD and its resultant inactivation in FA patients might lead to a severe phenotype that was very similar to the Fancë⁺Sod₁⁻ mouse that was described within this thesis. The existence of modifier genes within complex genetic disorders has been documented and can contribute to the clinical heterogeneity seen in patients with mutations in the same genes.

NO-induced 3-nTyr formation has been shown to effect signaling molecules in cytokine stimulated macrophages. The endogenous production of NO has been shown to inhibit signalling pathways induced by IFNγ, by nitrating tyrosine residues in STAT1, thereby preventing phosphorylation. Similarly, endogenous NO is capable of modifying proteins containing cysteine residues by S-nitrosylation of the thiol group of that particular cysteine. Caspases can be targeted and inactivated by NO via this S-nitrosylation process and upon activation of the Fas apoptotic pathway, the caspase becomes denitrosylated and activated. NO can regulate CD95 expression on both normal and tumor cells, which can lead to apoptosis through the activation of upstream FLICE caspases. Furthermore, FA HPC have been shown to have higher levels of fas receptor (CD95) and active caspase 3 upon stimulation with low doses of IFNγ resulting in elevated apoptosis in FA cells. Many transcription factors such as NFKB are known to be regulated by the levels of reactive species within the cell and degregulated levels of ROS in FA cells have been shown to effect NFKB pathways. Constitutive expression of NFKB from untreated FA SV40-transformed fibroblasts occurs through the formation of ROS since the addition of antioxidants and CYT P-450 inhibitors both reversed this effect. This pathway
provides an interesting feedback mechanism since NO is also known to stimulate iNOS expression through NFκB. NO is produced by all mammalian cells at low levels in response to many stimuli and is required for many normal cellular functions. Recently, NO has been shown to be both necessary and sufficient for the onset of fertilization and is thought to be an important aspect of Ca²⁺ signaling. Furthermore, activated macrophages and endothelial cells can induce large amounts of NO through the specific activation of iNOS. The BM microenvironment consists of many different cytokines required for the proper differentiation and turn-over of particular hematopoietic lineages. These stimuli may initiate signaling cascades that FA cells are particularly sensitive to, such as the IFNγ cascade. Similarly, ROS are known to be involved in growth factor signaling cascades and an upset in this balance may lead to stunted and abnormal differentiation or cellular death.

The wide range of cellular phenotypes observed in FA patients and mice indicates that the FA proteins are involved in a myriad of cellular processes. All such processes, while they appear to act independently, likely work in concert to maintain cellular homeostasis, be it DNA damage repair, redox regulation of proteins, or some combination of the two. Although the primary defect in FA remains elusive, deficiencies in pathways such as apoptosis, repair of DNA mutations, cell cycle function or control of ROS/RNS toxicity have been described in FA cells from both human and mouse. FA research has clearly shown, particularly through the study of FANCC, that the pathogenesis of FA is highly complex with several different proteins having independent roles within different compartments of the cell, that do not necessarily have a common function. Cloning of the genes involved in FA and the subsequent creation of mouse models was an enormous leap forward for FA researchers. Current work should focus on the specific sensitivity and pathology of the BM compartment, on the better understanding of the cytoplasmic roles of the FA proteins, on the specific type of DNA damage that FA cells are
required to correct and if this is a direct or indirect role for the FA complex. Only through the understanding of all aspects of this disease will appropriate therapies become available.
BIBLIOGRAPHY


90


44. Buchwald M, Moustacchi E. Is Fanconi anemia caused by a defect in the processing of DNA damage? Mutat Res. 1998;408:75-90


48. Papadopoulo D, Guillouf C, Mohrenweiser H, Moustacchi E. Hypomutability in Fanconi anemia cells is associated with increased deletion frequency at the HPRT locus. Proc Natl Acad Sci USA. 1990;87:8383-8387


141. Selleri C., Maciejewski JP. Nitric oxide and cell survival: megakaryocytes say "NO". J Lab Clin Med. 2001;137:225-230


156. Wink D.A., Laval J. The Fpg protein, a DNA repair enzyme, is inhibited by the biomediator nitric oxide in vitro and in vivo. Carcinogenesis. 1994;15:2125-2129


Table I. Clinical and Cellular Phenotypes in Fanconi Anemia

<table>
<thead>
<tr>
<th>Clinical Phenotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Growth retardation, short stature</td>
</tr>
<tr>
<td>• Hypogonadism and reduced fertility</td>
</tr>
<tr>
<td>• Skeletal malformations (absent or abnormal thumbs and radii)</td>
</tr>
<tr>
<td>• Microcephaly</td>
</tr>
<tr>
<td>• Skin hyperpigmentation / cafe au lait spots</td>
</tr>
<tr>
<td>• Congenital heart disease</td>
</tr>
<tr>
<td>• Hearing loss</td>
</tr>
<tr>
<td>• Elevated serum α-fetoprotein</td>
</tr>
</tbody>
</table>

Disease Onset

- Bone Marrow Failure; Aplastic Anemia
- Cancer Predisposition; Acute Myeloid Leukemia and / or Squamous Cell Carcinomas, Solid Tumors

<table>
<thead>
<tr>
<th>Cellular Phenotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Spontaneous chromosomal instability (chromatid breaks and interchanges, radial formation)</td>
</tr>
<tr>
<td>• Spontaneous arrest and delay at the G₂ phase of the cell cycle (4n post-replication)</td>
</tr>
<tr>
<td>• Hypersensitivity to DNA inter-strand cross-linking agents such as mitomycin C, diepoxybutane, cisplatin, and cyclophosphamide.</td>
</tr>
<tr>
<td>• Hypersensitivity to elevated oxygen concentrations (35% O₂)</td>
</tr>
</tbody>
</table>


<table>
<thead>
<tr>
<th>Complementation Group</th>
<th>No. of Pathogenic Mutations</th>
<th>Chromosomal Location</th>
<th>Exons</th>
<th>% Identity to Mouse&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Protein Product</th>
<th>Evolutionary Conservation</th>
<th>Knockout Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>FANCA</td>
<td>&gt; 100</td>
<td>16q24.3</td>
<td>43</td>
<td>65%</td>
<td>145 kDa</td>
<td>Fish&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Reduced Fertility</td>
</tr>
<tr>
<td>FANCB&lt;sup&gt;a&lt;/sup&gt;</td>
<td>?</td>
<td>?</td>
<td>?</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>FANCC</td>
<td>12</td>
<td>9q22.3</td>
<td>14</td>
<td>63%</td>
<td>56 kDa</td>
<td>Fish&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Reduced Fertility</td>
</tr>
<tr>
<td>FANCD1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>?</td>
<td>?</td>
<td>?</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>FANCD2</td>
<td>5</td>
<td>3p25.3</td>
<td>44</td>
<td>NA</td>
<td>145 kDa</td>
<td>Worm/Fly</td>
<td>Reduced Fertility /Ionizing Radiation</td>
</tr>
<tr>
<td>FANCE</td>
<td>4</td>
<td>6p21.3</td>
<td>10</td>
<td>60%</td>
<td>53 kDa</td>
<td>Fish&lt;sup&gt;b&lt;/sup&gt;</td>
<td>NA</td>
</tr>
<tr>
<td>FANCF</td>
<td>6</td>
<td>11p15</td>
<td>1</td>
<td>NA</td>
<td>37 kDa</td>
<td>Fish&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Reduced Fertility</td>
</tr>
<tr>
<td>FANCG</td>
<td>18</td>
<td>9p13</td>
<td>14</td>
<td>83%</td>
<td>68 kDa</td>
<td>XRCC9</td>
<td>Reduced Fertility</td>
</tr>
</tbody>
</table>

NA, data not available
<sup>a</sup> Remains to be identified
<sup>b</sup> BLAST searches for homologous regions in the FA proteins reveals no significant similarity in non-vertebrate, yeast or bacteria. Significant BLAST hits were identified in fish; *Tetraodon nigroviridis* (FANCA, FANCE, FANCF), *Takifugu rubripes* (FANCC) and zebrafish *Danio rerio* (FANCG).
<sup>c</sup> values represents the % amino acid sequence identity between the mouse cDNA and the human sequence
Figure 1. Mutations Identified in the Fanconi Anemia Genes
The exon structure of each cloned FA gene is shown as well as the approximate location for each mutation. Mutations surrounded with a box are the most common for that particular gene. The greatest number of pathogenic mutations have been identified in FANCA, and many of these are deletions due to the large number of Alu repeats within the gene.
Figure 2. The current knowledge of protein interactions and potential function of the FA proteins.
Table III. Current Mouse Models for Fanconi Anemia

<table>
<thead>
<tr>
<th>Model</th>
<th>Genetic Strategy</th>
<th>Similarities to human phenotype</th>
<th>Differences from human phenotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fanca -/-</strong></td>
<td>Fanca exons 4-7 replaced with lacZ-neo</td>
<td>reduced fertility, cross-linker sensitivity</td>
<td>no congenital malformations, hematological abnormalities or tumor formation</td>
<td>[Cheng, 2000]</td>
</tr>
<tr>
<td><strong>Fancc -/-</strong></td>
<td>Fancc exons 8 or 9 replaced with neo</td>
<td>Reduced fertility, cross-linker and cytokine sensitivity, repopulating ability decreased</td>
<td>no congenital malformations, hematological abnormalities or tumor formation</td>
<td>[Chen, 1996] [Whitney, 1996] [Haneline, 1998] [Haneline, 1999]</td>
</tr>
<tr>
<td><strong>Fancg -/-</strong></td>
<td>Fancg exons 2-9 replaced with neo</td>
<td>reduced fertility, cross-linker and ionizing radiation sensitivity, no Fancd2-L protein isoform</td>
<td>no congenital malformations, hematological abnormalities or tumor formation</td>
<td>[Yang, 2001]</td>
</tr>
<tr>
<td><strong>Fancd2 -/-</strong></td>
<td>Fancd2 exons replaced with neo</td>
<td>Reduced fertility, cross-linker and ionizing radiation sensitivity</td>
<td>no congenital malformations, hematological abnormalities or tumor formation</td>
<td>[Houghtaling, 2001]</td>
</tr>
<tr>
<td><strong>FANCC transgenic</strong></td>
<td>human FANCC ubiquitously overexpressed</td>
<td>HPC protected against fas apoptosis</td>
<td>N/A</td>
<td>[Wang, 1998]</td>
</tr>
<tr>
<td><strong>FNT</strong></td>
<td>Breeding of (hu)TNFα Tg to Fancc-/- mice</td>
<td>BM colony growth is decreased</td>
<td>marrow cellularity and peripheral blood counts are normal</td>
<td>[Otsuki, 1999]</td>
</tr>
<tr>
<td><strong>Fanca -/- Fancg -/- double ko</strong></td>
<td>Breeding of the single knockout animals</td>
<td>Phenotype same as single knockouts</td>
<td>Phenotype same as single knockouts</td>
<td>[van de Vrugt, 2000]</td>
</tr>
</tbody>
</table>

*results were presented at conference proceedings and have not been published since, information is limited as a result.
Figure 3. The major sources of reactive species found within the mammalian cell. Breakdown products from oxygen and L-arginine result in reactive oxygen and nitrogen species, respectively.
Fig. 4 Body Weights from 8 wk old Fancc<sup>−/−</sup>Sod1<sup>−/−</sup> mice.
Anaesthetized male (dark bars) and female (gray bars) mice were weighed and the average weight (in grams) ± SEM is represented above. There is a slight decrease in body weight in Fancc<sup>−/−</sup>Sod1<sup>−/−</sup> mice, however, this difference is not significant (n=5-6).
Fig. 5 Histological examination reveals zonal hepatic microvesicular steatosis in Fancc-/-Sod1-/- mice.
Histology of Fancc-/-Sod1-/- and control livers (magnification 400x). Sections of Fancc-/- (a, e); Sod1-/- (b, f); Fancc+/Sod1+- (c, g) and Fancc-/-Sod1-/- (d, h) livers were stained with Masson's trichrome (a-d) and oil red-O (e-h). While Fancc-/-, Sod1-/- and Fancc+/Sod1+- show normal morphology, Fancc-/-Sod1-/- hepatocytes demonstrate a zone 3 abnormality characterized by abundant cytoplasmic vacuolation. Oil red-O staining demonstrates prominent microvesicular zone 3 lipid accumulation without nuclear displacement in Fancc-/-Sod1-/- mice. Small amounts of lipid droplets are present in Sod1-/- mice.
Fig. 6 Electron Microscopy of hepatocytes from Fanc-/-Sod1-/- mice reveals no increase in organelle damage.
Hepatocytes from Fancc+/Sod1+/- (a and c) and Fancc-/Sod1-/- (b and d) mice. a) a normal multi-nucleated centrilobular hepatocyte (5400x) and c) a higher magnification showing normal organelles (11,750x). b) microvesicular steatosis in a centrilobular Fancc-/-Sod1-/- hepatocyte (5400x) and d) (11,750x).
Fig. 7 Primary hepatocytes from *Fancc"Sod1"* mice have increased superoxide levels. The level of superoxide is reflected by the RLU value and was measured using luminol dependent chemiluminescence. Each value represents the mean ± SEM for 5 mice per group, with each sample in triplicate. *, p < 0.05; **, p < 0.001 by the Student's *t* test. *Sod1"* mice have a significant increase (p=0.01) in the RLU value compared to wildtype mice. *Fancc"Sod1"* mice had a synergistic increase in RLU values of 1.62 (p=0.0008) compared to wildtype mice.
Fig. 8 Liver-specific expression of Mn Sod and HO-1 is increased in Fancc"Sod1" mice. Autoradiographs showing total liver lysates immunoblotted with antibodies against Mn Sod and HO-1, and normalized for loading with anti-tubulin Ab. HO-1 and Mn Sod protein expression in A) control, B) Fancc"Sod1"/" and C) Fancc"Sod1"/" livers indicates an increase in protein expression in D) Fancc"Sod1" samples.
### Table IV  Peripheral Blood Values from 8-10 wk old mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>n</th>
<th>RBC (10^6/l)</th>
<th>WBC (10^3/l)</th>
<th>HGB (g/l)</th>
<th>PLT (10^4/l)</th>
<th>MCV (fl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fancc&lt;sup&gt;+&lt;/sup&gt; Sod1&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>15</td>
<td>10.02 ± 0.27</td>
<td>3.69 ± 1.09</td>
<td>163.4 ± 4.47</td>
<td>605.3 ± 47.2</td>
<td>52.8 ± 0.69</td>
</tr>
<tr>
<td>Fancc&lt;sup&gt;-/-&lt;/sup&gt; Sod1&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>18</td>
<td>9.66 ± 0.27</td>
<td>6.59 ± 0.86</td>
<td>161.9 ± 4.50</td>
<td>708 ± 49.3</td>
<td>52.3 ± 0.62</td>
</tr>
<tr>
<td>Fancc&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>9</td>
<td>9.68 ± 0.20</td>
<td>1.55 ± 0.23</td>
<td>158.6 ± 4.05</td>
<td>435 ± 26.9</td>
<td>53.5 ± 0.42</td>
</tr>
<tr>
<td>Sod1&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>10</td>
<td>8.72 ± 0.42</td>
<td>1.94 ± 0.56</td>
<td>147 ± 3.96</td>
<td>745.5 ± 49.1</td>
<td>54.1 ± 1.0</td>
</tr>
<tr>
<td>Fancc&lt;sup&gt;+&lt;/sup&gt; Sod1&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>9</td>
<td>7.9 ± 0.36</td>
<td>0.96 ± 0.20</td>
<td>136 ± 4.52</td>
<td>719 ± 93.7</td>
<td>58.5 ± 0.68</td>
</tr>
</tbody>
</table>

Values represent average ± SEM for the indicated number of animals per group.

*, p < 0.05; **, p < 0.0005

CBCs were quantified using a Sysmex 9500 automated blood analyser.
Fig. 9 Hypocellularity and increased fat accumulation in FancC\(^{+/+}\)Sod1\(^{++}\) bone marrow.
Metaphyseal sections of leg bones from FancC\(^{+/+}\)Sod1\(^{++}\), FancC\(^{-/-}\), Sod1\(^{-/-}\) and FancC\(^{-/-}\) Sod1\(^{-/-}\) mice (magnification 400x). Marrow fat content (clear areas) is increased in FancC\(^{-/-}\) Sod1\(^{-/-}\) mice compared to FancC\(^{+/+}\)Sod1\(^{++}\) controls. FancC\(^{-/-}\) controls revealed only rare fat cells, while Sod1\(^{-/-}\) mice did show some increase in fat spaces.
<table>
<thead>
<tr>
<th>Genotype</th>
<th>n</th>
<th>Average cellularity/femur (10^7)</th>
<th>PGP1</th>
<th>B220</th>
<th>Ly6G</th>
<th>CD11b</th>
<th>CD14</th>
<th>PMN</th>
<th>Term-119</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fancc&lt;sup&gt;-/-&lt;/sup&gt; Sod1&lt;sup&gt;+/+&lt;/sup&gt;</td>
<td>6</td>
<td>3.98 ± 0.62</td>
<td>2.89</td>
<td>0.86</td>
<td>1.28</td>
<td>1.33</td>
<td>0.05</td>
<td>1.21</td>
<td>1.36</td>
</tr>
<tr>
<td>Fancc&lt;sup&gt;-/-&lt;/sup&gt; Sod1&lt;sup&gt;+/+&lt;/sup&gt;</td>
<td>7</td>
<td>4.08 ± 0.57</td>
<td>3.14</td>
<td>1.21</td>
<td>1.48</td>
<td>1.42</td>
<td>0.14</td>
<td>1.46</td>
<td>1.30</td>
</tr>
<tr>
<td>Fancc&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>6</td>
<td>3.41 ± 0.33</td>
<td>2.21</td>
<td>0.77</td>
<td>0.87</td>
<td>0.77</td>
<td>0.04</td>
<td>0.73</td>
<td>1.2</td>
</tr>
<tr>
<td>Sod1&lt;sup&gt;+/+&lt;/sup&gt;</td>
<td>5</td>
<td>3.53 ± 0.57</td>
<td>2.7</td>
<td>0.88</td>
<td>1.23</td>
<td>1.2</td>
<td>0.06</td>
<td>1.1</td>
<td>1.05</td>
</tr>
<tr>
<td>Fancc&lt;sup&gt;-/-&lt;/sup&gt; Sod1&lt;sup&gt;+/+&lt;/sup&gt;</td>
<td>6</td>
<td>2.33 ± 0.19</td>
<td>1.68</td>
<td>0.56</td>
<td>0.79</td>
<td>0.73</td>
<td>0.01</td>
<td>0.85</td>
<td>0.72</td>
</tr>
</tbody>
</table>

Average BM cellularity /femur was measured using trypan blue staining on a hemocytometer of BM aspirates and is represented as the average ± SEM. Fancc<sup>-/-</sup> Sod1<sup>+/+</sup> cellularity is decreased by approximately 52 % compared to Fancc<sup>+/+</sup> Sod1<sup>+/+</sup> (p=0.08). Absolute numbers of cell values represent the average % staining x average cellularity for 5 mice per group and is consistently reduced by a minimum of 40 % compared to Fancc<sup>-/-</sup> Sod1<sup>+/+</sup> controls. Cellularity is decreased in Sod1<sup>+/+</sup> and Fancc<sup>-/-</sup> mice as well.
Fig. 10 FACS analysis of Lin⁺ cells for progenitor markers.
Whole BM samples from Fancc⁺/⁻ Sod1⁺/⁻ and Fancc⁻/⁻ Sod1⁻/⁻ mice were immunophenotyped by FACS analysis to determine the number of Lin⁺ BM cells also carrying Sca₁, ckit or CD34 markers on their cell surface. There was approximately a 40% decrease in the absolute number of Lin⁺ cells with any of the above markers from Fancc⁺/⁻ Sod1⁺/⁻ marrows. Data is from two mice per genotype.
Table VI. PI/Annexin FACS Analysis from total BM samples

<table>
<thead>
<tr>
<th>Genotype</th>
<th>n</th>
<th>Annexin+ PI-</th>
<th>Annexin+ PI+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fancc+/- Sod1+/-</td>
<td>5</td>
<td>25.49 ± 4.59</td>
<td>15.52 ± 3.08</td>
</tr>
<tr>
<td>Fancc-/- Sod1-/-</td>
<td>5</td>
<td>28.38 ± 3.83</td>
<td>16.44 ± 6.12</td>
</tr>
</tbody>
</table>

FACS Staining was used to quantitate the number of apoptotic cells from total BM samples of 8-9 wk old mice. Annexin+/PI- values represent cells initiating the apoptotic process and Annexin+/PI+ values represent cells that have already undergone apoptosis and death.
Fig. 11 Colony Forming Assays reveal abnormal progenitor growth from Fancc''Sod1'' mice.

a) Colony forming assays reveal decreased numbers of progenitors in Fancc''Sod1'' marrows. Myeloid (dark bars) and Pre-B (hatched bars) CFUs were measured for Sod1'', Fancc''Sod1''/+, Fancc''/Sod1''/+, Fancc''/Sod1''+/- and Fancc''Sod1''-/- mice. The decrease in the number of progenitors/femur is highly significant when Fancc''Sod1'' mice are compared to Fancc''/+Sod1''+/- controls. Values represent the average number of progenitors/femur of 6 mice per group ± SEM. * = p<0.05, ** = p<0.001.

b) Fancc''Sod1'' progenitors fail to generate normal ratios of CFU-GEMM, CFU-GM/G/M and BFU-E. Myeloid colonies from a) were assessed morphologically to determine the cell types contributing to the colonies. CFU-GEMM (dark bars), CFU-GM/G/M (light gray bars) and BFU-E (hatched bars) colonies were scored by eye and the values represent average percent of cell type ± SEM (n = 6-8 mice per group). Ratios of CFU-GM/G/M, and CFU-GEMM were significantly different from Fancc''/+Sod1''+/- controls (p=0.002; and p=0.003, respectively).
Table VII. Reagents used to try to rescue colony growth of methylcellulose colonies from Fancc<sup>+</sup>Sod1<sup>+</sup> mice.

<table>
<thead>
<tr>
<th>REAGENTS</th>
<th>CELLULAR EFFECT</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cell Density</strong></td>
<td>• 2x</td>
</tr>
<tr>
<td></td>
<td>• 5x</td>
</tr>
<tr>
<td></td>
<td>• 10x</td>
</tr>
<tr>
<td><strong>Hypoxia</strong></td>
<td>• 5 % O₂</td>
</tr>
<tr>
<td><strong>Pan-Caspase Inhibitor</strong></td>
<td>• ZVAD-fmk (10, 50 µg)</td>
</tr>
<tr>
<td><strong>Antioxidants/Drugs</strong></td>
<td>• Ammonium pyrrolidinedithiocarbamate (APDC) (0.1, 1, 10 µM)</td>
</tr>
<tr>
<td></td>
<td>• 4,5-Dihydroxy-1,3-benzen Disulfonic Acid (TIRON) (0.05, 0.5 mM, 5 µM)</td>
</tr>
<tr>
<td></td>
<td>• Superoxide Dismutase (SOD) (100 ng, 1, 10, 50 µg)</td>
</tr>
<tr>
<td></td>
<td>• EUK-8, Dihydrate (5, 7, 10, 25 µM)</td>
</tr>
<tr>
<td></td>
<td>• [Mn(III)tetrakis (1-methyl-4-pyridyl)porphyrin pentachloride] (MnTMPyP) (1, 5, 20, 50 µM)</td>
</tr>
<tr>
<td></td>
<td>• prevents induction of NO synthetase by inhibiting NOS mRNA translation</td>
</tr>
<tr>
<td></td>
<td>• superoxide anion scavenger</td>
</tr>
<tr>
<td></td>
<td>• scavenger of superoxide anion</td>
</tr>
<tr>
<td></td>
<td>• synthetic complex with SOD and catalase activities</td>
</tr>
<tr>
<td></td>
<td>• SOD mimetic</td>
</tr>
<tr>
<td><strong>Growth Factors</strong></td>
<td>• SCF (50, 100, 200 ng)</td>
</tr>
</tbody>
</table>

Each experiment was repeated three times with each sample done in duplicate. All tests used littermate Fancc<sup>+</sup>Sod1<sup>+</sup> or Fancc<sup>+</sup>Sod1<sup>+</sup> mice as controls.
Fig. 12 FACS of Lin⁻ cells from Fance⁻/⁻Sod1⁻/⁻ marrows after column selection. Whole BM samples from Fance⁺/⁺Sod1⁺/⁺ and Fance⁻/⁻Sod1⁻/⁻ mice were depleted of Lin⁺ cells by negative selection and the resultant column purified progenitors were immunophenotyped by FACS analysis. Both the percent staining and the absolute number of Lin⁻ cells with Sca, ckit and/or CD34 markers from Fance⁻/⁻Sod1⁻/⁻ marrows was similar to Fance⁺/⁺Sod1⁺/⁺ controls. Data is from 3 mice per genotype.
Fig. 13 Colony Forming Assays from aged 6-8 month old mice.
Colony forming assays were done from aged cohorts of animals and revealed that the decreased number of progenitors from Fancc'' Sodl'' marrows persisted with age. Myeloid (dark bars) and pre-B (hatched bars) CFUs were measured for Sodl'', Fancc'', Fancc''/Sodl''/+, Fancc''/Sodl''/−, Fancc''/Sodl''+/−, and Fancc''/Sodl''−/− mice. The decrease in the number of progenitors/femur is highly significant when Fancc'' Sodl''−/− mice are compared to Fancc'' Sodl''+/− controls. Values represent the average number of progenitors/femur of 5 mice per group ± SEM. * = p<0.05, ** = p<0.001.
Table VIII. Frequency of CAFC / $10^5$ BM cells in hypoxic (5 \%) and normoxic (20 \%) conditions

<table>
<thead>
<tr>
<th>Genotype</th>
<th>No. wells Tested</th>
<th>% O$_2$</th>
<th>Day 3</th>
<th>Day 7</th>
<th>Day 14</th>
<th>Day 21</th>
<th>Day 28</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fancc&quot;Sod1&quot;</td>
<td>32</td>
<td>20 %</td>
<td>6.9 ± 1.1</td>
<td>28.6 ± 3.2</td>
<td>16.8 ± 2.6</td>
<td>8.7 ± 0.9</td>
<td>5.8 ± 0.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5 %</td>
<td>7.1 ± 0.9</td>
<td>22.1 ± 2.0</td>
<td>17.1 ± 2.8</td>
<td>10.6 ± 1.4</td>
<td>6.9 ± 1.2</td>
</tr>
<tr>
<td>Fancc&quot;</td>
<td>28</td>
<td>20 %</td>
<td>4.8 ± 0.8</td>
<td>13.9 ± 1.6</td>
<td>10.5 ± 0.8</td>
<td>5.6 ± 1.5</td>
<td>4.9 ± 0.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5 %</td>
<td>5.3 ± 0.9</td>
<td>16.1 ± 1.8</td>
<td>14.4 ± 1.8</td>
<td>7.8 ± 1.3</td>
<td>5.5 ± 0.8</td>
</tr>
<tr>
<td>Sod1&quot;</td>
<td>28</td>
<td>20 %</td>
<td>3.9 ± 0.7</td>
<td>9.0 ± 1.0</td>
<td>6.5 ± 1.1</td>
<td>3.8 ± 1.2</td>
<td>1.9 ± 0.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5 %</td>
<td>6.8 ± 1.5</td>
<td>10.9 ± 1.2</td>
<td>8.4 ± 1.8</td>
<td>6.4 ± 1.8</td>
<td>3.1 ± 0.7</td>
</tr>
<tr>
<td>Fancc&quot;Sod1&quot;</td>
<td>24</td>
<td>20 %</td>
<td>1.9 ± 0.4$^*$</td>
<td>2.2 ± 0.3$^*$†</td>
<td>0.68 ± 0.1$^<em>$†</em></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5 %</td>
<td>3.3 ± 0.4$^*$</td>
<td>6.3 ± 0.7</td>
<td>4.3 ± 0.7$^*$</td>
<td>0.75 ± 0.2$^<em>$†</em></td>
<td>0.49 ± 0.1$^*$†</td>
</tr>
</tbody>
</table>

$^*$ represents a significant difference between Fancc" Sod1" and wildtype
† represents a significant difference between Fancc" Sod1" and Fancc" Sod1"
$^*$ represents a significant difference between Fancc" Sod1" and Sod1", $p<0.05$, ratio of the log differences compared to Fancc" Sod1" samples within the same oxygen concentration. The statistical software is based on Poisson statistics and was provided with the L-calc program used to analyze CAFC frequency.
Fig. 14 a) Representative CAFC colonies at day 7 in 20% oxygen. Long-term BM cultures from all genotypes were grown in 20% oxygen conditions and measured for CAFC frequency. CAFC colonies appear as non-refractive colonies in the same plane as the stroma (see circles for examples). In wildtype and Fance<sup>+/+</sup> cultures, the CAFC are extensive, while both Sod1<sup>−/−</sup> and Fance<sup>−/+</sup>Sod1<sup>−/−</sup> cultures have smaller CAFC colonies.
Fig. 14 b) Representative CAFC colonies at day 7 in 5% oxygen. CAFC colonies from wildtype and \textit{Fance}^{-/-} cultures grown in 5% oxygen conditions appear as large as when cultured in 20% oxygen. \textit{Sod1}^{-/-} CAFC colonies are larger in 5% oxygen, while \textit{Fance}^{+/+}\textit{Sod1}^{-/-} CAFC colonies are significantly larger and are found with increasing frequency in culture as well.
Fig. 15 Morphological changes of CAFC colonies from *Fance<sup>-/-</sup>*-*Sod1<sup>−/−</sup>* cultures. a) and b) represent day 7 and day 10 colonies respectively from *Fance<sup>+/+</sup>*-*Sod1<sup>+/+</sup>* mice in 20% oxygen. These colonies had the same morphology when grown in hypoxic conditions. c) and d) represent day 7 and day 10 colonies grown in 20% oxygen from *Fance<sup>-/-</sup>*-*Sod1<sup>−/−</sup>* mice. e) and f) represent day 7 and day 10 CAFC from the same *Fance<sup>-/-</sup>*-*Sod1<sup>−/−</sup>* mice grown in 5% oxygen.
Fig. 16 HPC growth in liquid media reveals abnormal proliferation and increased apoptosis.

a) HPC were grown in liquid media supplemented with SCF, IL-3 and IL-6 for 13 days post column isolation and proliferation was determined of triplicate cultures. Data is represented as the average ± SEM of triplicate cultures from n=5 mice per genotype. b) FACS analysis of dUTP-positive nuclei from day 13 HPC grown in vitro. FACS data is from 10,000 events. Data represents the average percent staining ± SEM for n=3 per genotype. * p 0.05.
Fig. 17 Hypoxia partially rescues HPC growth from *Fancc*<sup>/−</sup>*Sod1*<sup>/−</sup> mice.

HPC cultures were grown in liquid media in 20% (dark bars) and 5% (gray bars) oxygen conditions and proliferation was measured on day 9 of culture. *Fancc*<sup>/−</sup>*Sod1*<sup>/−</sup> HPC numbers could be partially rescued to wildtype levels when grown in hypoxic conditions. Data is represented as the average ± SEM of duplicate cultures from n=3-4 mice per genotype. * p 0.05
Fig. 18 TIRON partially rescues HPC growth from *Fancc<sup>−/−</sup>*Sod1<sup>−/−</sup> mice.

HPC cultures were grown in liquid media in the presence of increasing concentrations of TIRON, a cell permeable superoxide scavenger. Cell number was determined on day 9 of culture. Data is represented as the average ± SEM of duplicate cultures from n=3-4 mice per genotype.

* p  0.05
Fig. 19 MnTMPyP dose response and partial rescue of HPC proliferation in vitro.

a) HPC cultures were grown in liquid media in the presence of increasing concentrations of MnTMPyP, a SOD mimetic. Cell number was determined on day 9 of culture. 5μM was chosen as the concentration which resulted in optimal growth for each genotype.

b) HPC were subsequently cultured in the presence of 5 μM MnTMPyP and cell number was determined as above for 13 days post-isolation. Data is represented as the average ± SEM of duplicate cultures from n=3-4 mice per genotype. * p 0.05
Fig. 20 Primary Mast Cell cultures from Fancc	extsuperscript{−/−} Sod1	extsuperscript{−/−} marrows do not grow in vitro.
Whole BM samples from wildtype and Fancc	extsuperscript{−/−} Sod1	extsuperscript{−/−} mice were cultured for 5 weeks in liquid media in the presence of SCF and IL-3 and a representative picture of a typical culture is shown above. Wildtype cultures consisted of a homogenous population of mast cells, while the Fancc	extsuperscript{−/−} Sod1	extsuperscript{−/−} cultures contained a highly heterogenous cell population with many dead cells. Arrows represent an example of a single mast cell.
**Fig. 21 Histological examination of FancA⁺⁺Sod1⁺⁺ mice livers.**  
Sections of FancA⁺⁺Sod1⁺⁺, FancA⁻⁻, Sod1⁻⁻ and FancA⁺⁺Sod1⁻⁻ livers were stained with Masson's trichrome (magnification 400x).  
FancA⁻⁻ and FancA⁺⁺Sod1⁻⁻ sections reveal normal liver morphology and some Sod1⁻⁻ sections have very mild pathology.  
FancA⁻⁻ Sod1⁻⁻ hepatocytes demonstrate a moderate zone 3 abnormality characterized by cytoplasmic vacuolation.  
Oil red-O staining revealed mild microvesicular zone 3 lipid accumulation in FancA⁺⁺Sod1⁻⁻ mice and a small amount of lipid droplets are present in Sod1⁻⁻ mice, however the quality of the photographs was poor.
Fig. 22 Body weights are significantly decreased in $FancA^{+/+}Sod1^{+/+}$ mice. Anaesthetized male (gray bars) and female (dark bars) mice were weighed and the average weight (in grams) ± SEM is represented above. There is a significant decrease in body weight in $FancA^{+/+}Sod1^{-/-}$ mice. $n=3-4$ mice per genotype. * $p < 0.05$. 
Fig. 23 Marrow cellularity from $FancA^{-/-}Sod1^{-/-}$ mice is decreased. Femurs from wildtype, $FancA^{+/+}$, $Sod1^{+/+}$ and $FancA^{-/-}Sod1^{-/-}$ mice were flushed and the cellularity per femur measured. $FancA^{-/-}Sod1^{-/-}$ mice had a statistically significant decrease in femur cellularity compared to wildtype controls. Data is represented as average femur cellularity ± SEM of n=3-4 mice per genotype. * p 0.05.
Fig. 24 Colony Forming Assays reveal abnormal progenitor growth in *FancA<sup>+</sup>Sod1<sup>+</sup>* mice. Colony forming assays reveal decreased numbers of progenitors in *FancA<sup>+</sup>Sod1<sup>-</sup>* marrows. a) Myeloid and b) Pre-B CFUs were measured for *Sod1<sup>+</sup>, FancA<sup>+</sup>, FancA<sup>+</sup>Sod1<sup>+</sup>, and *FancA<sup>-</sup>Sod1<sup>-</sup>* mice. Both myeloid and pre-B CFU are decreased in *FancA<sup>-</sup>Sod1<sup>-</sup>* mice compared to wildtype controls, and the number of pre-B progenitors/femur is significantly decreased from *Sod1<sup>+</sup>, FancA<sup>-</sup>, and FancA<sup>-</sup>Sod1<sup>-</sup>* mice compared to *FancA<sup>+</sup>Sod1<sup>+</sup>* controls. Values represent the average number of progenitors/femur of 3-4 mice per group ± SEM. * = p<0.05, ** = p<0.001.
Fig. 25 Frequency of BM progenitors from $FancA^{+/+}Sod1^{+/+}$ mice is abnormal. $FancA^{-/-}Sod1^{-/-}$ progenitors fail to generate normal ratios of CFU-GEMM, CFU-GM/G/M and BFU-E. Myeloid colonies from Fig. 24a) were assessed morphologically to determine the cell types contributing to the colonies. CFU-GEMM (dark bars), CFU-GM/G/M (light gray bars) and BFU-E (hatched bars) colonies were scored by eye and the values represent the average percent of cell type ± SEM (n = 3-4 mice per group). The ratios of CFU-GM/G/M, and CFU-GEMM were significantly different from $FancA^{+/+}Sod1^{+/+}$ controls. * p 0.05
Fig. 26 Inhibition of Fancc<sup>−/−</sup> colony formation by IFNγ is reversed by L-NMMA.

a) Colony formation by Fancc<sup>−/−</sup> and littermate control BM progenitor cells plated in methylcellulose in the presence of increasing concentrations of IFNγ. There is a dose-dependent inhibition of colony formation from Fancc<sup>−/−</sup> progenitors.

b) Colony formation by BM cells from wildtype and Fancc<sup>−/−</sup> mice grown in the presence of 1 ng/ml IFNγ and with increasing concentrations of L-NMMA. L-NMMA completely rescues colony growth. Data points represent the average number of colonies counted per methylcellulose plate; n = 3 mice. * P < 0.05; ** P < 0.005.
Fig. 27 Inhibited colony formation by TNFα and MIP1α is also reversed by L-NMMA.

a) In the presence of 0.5 ng/ml TNFα, there was a reduction in colony formation by Fancc−/− BM cells as compared to littermate controls which was reversed by addition of 0.25 mM L-NMMA.  b) Reduction in Fancc−/− BM cell colony number in the presence of 1 ng/ml MIP1 was partially reversed by the addition of 0.25 mM L-NMMA and completely reversed with 0.5 mM L-NMMA.  Data points represent the average number of colonies counted; n = 4 mice.  * P < 0.05; ** P < 0.005.
Fig. 28 \textit{Fancc}\textsuperscript{−/−} BM progenitors show increased sensitivity to NO-generating drugs.
a) Progenitor cell growth in the presence of increasing concentrations of the NO donor, SNAP. b) Growth of progenitor cells in the presence of increasing concentrations of DETA/NO. Percentage maximal colony formation was determined by dividing the number of colonies scored at a given concentration of NO donor by the number of colonies scored in the absence of the NO donor; \( n = 4 \) mice per group. * \( P \leq 0.05 \), ** \( P < 0.005 \).
Fig. 29 Inhibition of Fancc^-/- HPC growth by IFNγ was reversed by L-NMMA.

a) Fancc^-/- and wildtype HPC grown in the presence of 10 ng/ml IFNγ, with and without the addition of 0.5 mM L-NMMA. Bars indicate percent of control (no cytokine added) for each of the two genotypes. 
b) Flow cytometry TUNEL analysis showing percentage of HPC cell nuclei that were dUTP-positive staining in untreated, IFNγ-treated (10 ng/ml), and IFNγ plus 0.5 mM L-NMMA. IFNγ plus 0.5 mM L-NMMA decreased dUTP-positive numbers to a level similar to that of the untreated group. Flow data was based on 10,000 events. n = 4 mice for each group. * P < 0.05.
Fig. 30 Elevated iNOS expression in stimulated Fancc⁻/⁻ peritoneal macrophages.
Peritoneal macrophages were stimulated for 0 - 12 hours with IFNγ 10 ng/ml +/- LPS 100 ng/ml, and whole cell lysates assayed for iNOS expression by immunoblotting. a) is a representative filter showing iNOS protein expression in Fancc⁻/⁻ and control peritoneal macrophages following IFNγ plus LPS stimulation (top panel), with α-tubulin (bottom panel) as the loading control. b) Densitometric representation of five independent experiments showing a significant difference in iNOS expression between Fancc⁻/⁻ and wildtype peritoneal macrophages at 8 and 12 hours post-stimulation. * P < 0.05.
Fig. 31 Elevated iNOS expression in Fancc\(^{-}\) BM-derived macrophages stimulated with IFN\(\gamma\).

BM-derived macrophages were stimulated for 0 - 12 hours with IFN\(\gamma\) 10 ng/ml and whole cell lysates were assayed for iNOS expression by immunoblotting

a) is a representative blot showing iNOS expression in BMDM following IFN\(\gamma\) stimulation (top panel), with \(\alpha\)-tubulin (bottom panel) as the loading control.  
b) Densitometric representation of four independent experiments showing a significant increase in iNOS expression within Fancc\(^{-}\) BMDM at 5 hours post-stimulation, with expression of iNOS reaching a maximum at 8 hours. \(\ast P < 0.05\).
Fig. 32 Increased NO production by \textit{Fancc}^\ْ \textit{macrophages}.

Supernatants from the peritoneal macrophages used in iNOS expression studies (Fig. 30) were harvested and NO levels (as nitrite) were quantitated by ELISA. There was a significant increase in nitrite levels in the supernatants of \textit{Fancc}^\ْ cells (at 8 hrs) when macrophages were stimulated with IFN\γ plus LPS as well as when the cells were stimulated with IFN\γ alone (although the latter did not reach significance). * $P < 0.05$. 
Fig. 33 Stat1 phosphorylation is augmented in IFNγ-stimulated Fancc<sup>−/−</sup> macrophages.
Peritoneal macrophages from wildtype and Fancc<sup>−/−</sup> mice were stimulated with IFNγ and cell lysates were collected at various time points for Stat1 immunoblotting analysis. a) is a representative filter showing P-Stat1 (top panel), and total Stat1 (bottom panel). b) is densitometry analysis derived from four independent experiments that demonstrates a significant increase in P-Stat1 signal within Fancc<sup>−/−</sup> macrophages at 15 min post-stimulation with IFNγ, *P < 0.05.
Fig. 34 Hif1α expression is increased in IFNγ stimulated Fancc-/- macrophages.
Peritoneal macrophages from wildtype and Fancc-/- mice were grown in 20% oxygen in the absence or the presence of IFNγ and Hif1α protein levels were measured in the nuclear lysates. Data is represented as the average of two experiments and shows an increase in Hif1α protein from Fancc-/- macrophage lysates both constitutively and a further increase in the presence of IFNγ.
NO• and O₂•; Converging pathways to FA?

Activated macrophages, P450 Reductase, NADPH Oxidase, METC, GF or Ca²⁺ signaling

Effects phosphatase levels

Defective Signaling

Lipid peroxidation

O₂• + NO•

ONOO•

N₂O₃

Base deamination, abasic sites

Cell cycle arrest

Caspase Activation

Protein damage: nitrosylation, inactivation

Protein -3-nTyr

Single, double strand breaks, DNA cross-links

Chromosomal rearrangements, translocations

DNA base damage; mutagenic

Damage to repair proteins Fapy, MGMT

IFNγ, TNFα, IL-1β, Hif1α, STAT1

Induce MIP1α expression
Can regulate NFκB and Hsp levels
Can induce fas expression on cells
NO-induced damage is repaired by homologous recombination

APOPTOSIS or

Predispose to cancer, AML