Clonal Selection:

The Leukemic Process in Fanconi Anemia

by

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CERTIFICATE OF APPROVAL

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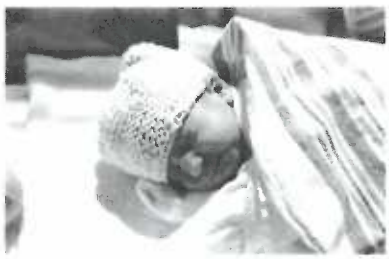
Martin Gustav Mateus Lensch (1917-1983)

paxtecum, paxvobiscum ☙ *MWL*

** note: my sonny-boy, Dante August Francis Fiorillo Lensch was born February 7, 2002



Abstract: ***(499 words)***

This thesis seeks to define the malignant process in leukemia, though understanding the myriad events that govern hematopoietic development represents a daunting task. Myelodysplastic syndrome (MDS) and acute myelogenous leukemia (AML), while common clinical entities, are among the diseases of blood whose pathogenic origins remain obscure. However, as has been the case with many common human diseases, insights regarding that which is frequent have been gained by studying that which is rare. In order to develop greater understanding of MDS and AML pathogenesis, the autosomal recessive, cancer-predisposition syndrome Fanconi anemia (FA) has been studied.

FA is a complex, genetic syndrome that presents mainly in childhood with an incidence of approximately 1 in 200,000. The condition demonstrates genetic heterogeneity and eight complementation groups have been described, each group corresponding to a distinct gene. FA is pleiotropic and skeletal, integumentary, nervous, renal, and cardiac defects may be present in each patient. Wide variability between affected individuals is not uncommon though one aspect is nearly invariant: the eventual development of bone marrow failure (BMF). BMF is the leading cause of death in FA and likely results from hematopoietic stem (HSC) and progenitor cell (HPC) hypersensitivity to environmental apoptotic cues such as cytokines, alkylating agents, and radiation. Those individuals that do not die from events incident to bone marrow failure,

exhibit an extraordinarily elevated risk for the onset of MDS, AML, and solid tumors. Due to the relationship between Fanconi anemia protein dysfunction and leukemia, hematological malignancy in FA was investigated as a "model system" for understanding the leukemic process.

MDS/AML in FA regularly demonstrates complex, non-random cytogenetics, an often rapid clinical course, and intractability to therapy. These observations parallel those frequently noted in non-FA patients with therapy-related or secondary disease. Based upon the similarities between FA-related and non-FA, secondary MDS/AML, a model for a common adaptive evolution in each condition has been proposed and is presented herein. This model draws from the observation that leukemic clones in both conditions may be resistant to apoptotic stimuli such as the inhibitory cytokine interferon-gamma (IFNγ). By investigating pathways that regulate elements of hematopoietic tissue homeostasis (pathways upon which FA proteins impinge) key leukemogenic events were sought.

In illustrating a study of leukemogenesis in Fanconi anemia and its relationship to leukemia in certain non-FA contexts, this thesis hopes to make three points: (1) that genetic events can be defined, resulting from selection, that are key to the initiation of MDS/AML in FA and that such events contribute to changes in cellular sensitivity to apoptotic stimuli during disease progression, (2) that defects of the FA protein pathway not only result in leukemia in hereditary

FA, but may also exacerbate the presentation and progression of leukemia in non-hereditary (acquired) FA patients, thereby lending credible evidence to the theory that FA-related and non-FA related MDS/AML share important features, and (3) that molecular signatures of cellular insensitivity to normal homeostatic mechanisms may indicate events required for the progression of malignancy, not only in Fanconi anemia but in leukemia in general.

Abbreviations used (in their order of appearance in the text):

AML = acute myeloid leukemia
MDS = myelodysplastic syndrome
FA = Fanconi anemia
BMF = bone marrow failure
HSC = hematopoietic stem cell
HPC = hematopoietic progenitor cell
IFN = interferon
BMT = bone marrow transplantation
WHO = World Health Organization
FAB = French American British
CD = cluster of differentiation
SCF = stem cell factor
KDR = kinase domain receptor
VEGF = vascular endothelial growth factor
CML = chronic myelogenous leukemia
ABL = Ableson tyrosine kinase
BCR = breakpoint cluster region
CBF = core binding factor
ETO = eight-twenty-one
APL = acute promyelocytic leukemia
PML = promyelocytic leukemia
RAR = retinoic acid receptor
TSG = tumor suppressor gene
LOH = loss of heterozygosity
Rb = retinoblastoma
TGF = transforming growth factor
IRF = interferon response factor
PCR = polymerase chain reaction
Mb = megabase
NQO1 = NAD(P)H:quinone oxioreductase
FANCA = FA complementation group gene A
FANCC = FA " " gene C
FANCD2 = FA " " gene D2
FANCE = FA " " gene E
FANCF = FA " " gene F
FANCG = FA " " gene G
FA-B = FA complementation group B
FA-D1 = FA complementation group D1
DNA = deoxyribonucleic acid
MMC = mitomycin C
DEB = diepoxybutane
PNH = paroxysmal nocturnal hemoglobinuria
SCN = severe congenital neutropenia
GPI = glucosylphosphatidylinositol
PIG-A = phosphatidylinositol glycan class A
FAS = a member of the TNF-receptor superfamily
CN = cyclic neutropenia
ELA = elastase
G-CSFR = granulocyte colony-stimulating factor receptor
G-CSF = granulocyte colony-stimulating factor
CFU = colony forming unit
RNA = ribonucleic acid
PKR = dsRNA dependent protein kinase
PHSC = pluripotential hematopoietic stem cell
MHPC = multipotential hematopoietic progenitor cell
LSC = lymphoid stem cell
eryth = erythrocyte
eos = eosinophil
gran = granulocyte
mono = monocyte
mac = macrophage
meg = megakaryocyte
plat = platelet
baso = basophil
CFU-GM = colony forming unit granulocyte/macrophage
BFU-E = burst forming unit erythroid
LDBMC = low density blood mononuclear cell
SIE = STEEL factor, interleukin 3, erythropoietin
IL = interluekin
EPO = erythropoietin
CFU-GEMM = colony forming unit granulocyte, erythrocyte, monocyte, macrophage
FCS = fetal calf serum
DEPC = diethyl pyrocarbonate
MOPS = 3-(N-morpholino)propanesulfonic acid
cDNA = complementary DNA
MAS = microarray analysis suite
cRNA = complementary RNA
PM = perfect match
MM = mismatch
AD = average difference
SF = scaling factor
GADPH = glyceraldehydes-3-phosphate dehydrogenase
FATC = Fanconi anemia transcriptome consortium
SEM = standard error of the mean
RA = refractory anemia
SD = standard deviation
UoC-M1 = University of Chicago M1 leukemia
FCS = fetal calf serum
ATCC = American Type Culture Collection
DMEM = Dulbecco's minimal essential media
BSA = bovine serum albumin
Ig = immunoglobulin
IRES = internal ribosomal entry site
PBS = phosphate buffered saline

MPZ = myelin protein zero
dNTP = deoxynucleotide triphospate
EtOH = ethyl alcohol
CEPH = The Centre d'Etude du Polymorphisme Humain
TPO = thrombopoietin
QRT-PCR = quantitative reverse transcription polymerase chain reaction
HeLa = Henrietta Lacks
EBV = Epstein-Barr virus
NPM = nucleophosmin
BRCA1 = breast cancer 1
STAT = signal transducer and activator of transcription
TOPO = topoisomerase
Ub = ubiquitin
VEGF = vascular endothelial growth factor
NOD = non-obese diabetic
SCID = severe combined immunodeficiency
FLT-1 = fms-like tyrosine kinase 1
KDR = kinase domain receptor
DTT = 1,4-Dithio-DL-threitol
CUL = cullin
HIF = hypoxia inducible factor
VHL = von Hippel-Lindau
Hy = hygromycin
tk = thymidine kinsae
HAT = hypoxanthine, aminopterin, thymidine
PHA-P = phytohemagglutinin-P
DMSO = dimethyl sulfoxide
PEG = polyethylene glycol
ELISA = enzyme linked immunosorbent assay
VPF = vascular permeability factor
PBML = peripheral blood mononuclear leukocyte
CHO = Chinese hamster ovary
HMC = human mast cell leukemia
JAK = Janus kinase
HGPRT = hypoxanthine guanine phosphoribosyltransferase
HSV = herpes simplex virus
PKC = protein kinase C
PTK = protein tyrosine kinase
MAP = mitogen activated protein
MAPK = MAP kinase
BCL = B-cell lymphoma
HPH = HIF prolyl hydroxylase
MCP = multicatalytic protease
OMIM = Online Mendelian Inheritance in Man
SRC = Rous sarcoma
FISH = fluorescent *in-situ* hybridization

Introduction

Fanconi anemia (FA) is an autosomal recessive disorder associated with a high risk of bone marrow failure and myeloid leukemia. The genes for six of the eight to eleven FA complementation groups have now been cloned. The cloning of these genes offers a unique opportunity to examine the molecular biological function of the FA proteins in pathways that may lead to a better understanding of myeloid leukemogenesis. The objective of the work presented herein was to define the processes by which leukemic clonal evolution occurs in patients with Fanconi anemia and to determine whether Fanconi protein dysfunction occurs in patients with sporadic acute leukemia.

Chapter One:

Background and Significance: Clonal evolution in Fanconi anemia

1-1: An historical introduction to leukemia and chemotherapy

Philipus Theophrastus Paracelcus (1493-1541) *Courtesy of the National Library of Medicine*

It is most likely that acute myelogenous leukemia (AML) has been a cause of human disease for centuries. In his *Elf Traktat*, published in the early 1500's, the Swiss physician Paracelsus (1493 – 1541) wrote of various types of consumption affecting the liver and spleen that produced "... *a thirst in the liver and kidneys, a change in the urine...*" along with "*spleen pricks*".[1] These disease manifestations were attributed to deficiencies of dew and rain and the recommended treatment was to orally consume a fluid elixir of powdered pearls.[1] Recognition of AML as a discrete, clinical entity came three hundred years later in 1827 by the French surgeon Alfred Velpeau though the first detailed, microscopic examinations of cases were described by Craigie and Bennett of

Alfred A. L. Velpeau (1795-1867) *Courtesy of the National Library of Medicine*

Edinburgh in 1845.[2,3] The term leukemia (rather, *Leukämie* or *Weissesblut* – literally: "white blood") was coined by the renowned father of pathology, Rudolf Virchow in his seminal work of 1847.[2] Therein, Virchow described a condition (also relying on the observations of earlier colleagues) of edema, bleeding, exhaustion, fever, and splenomegaly. At autopsy, he noted microscopically that the patient's peripheral blood reflected what he referred to as an inverted ratio of "mucous cells" (leukocytes) to red blood cells. Two years later, Virchow described the existence of two varieties of leukemia, one labeled as "lymphatic" and the other "splenic" based upon similarities of the defective cells in each form to those normally present in the lymph nodes and spleens of healthy individuals.[2] The recognition of chronic and acute forms of leukemia was made by Friedreich in 1857 and in 1870, Neumann reported that the splenic form was related to abnormalities of the bone marrow and introduced the term myelogenous leukemia.[2]

Rudolph L. K. Virchow (1821-1902) *Courtesy of the National Library of Medicine*

Various regimens for treating leukemia were attempted early on but it was not until World War I that the myelosuppressive effects of nitrogen mustards were noted (and rather nefariously at that). In World War II, after a mustard gas-filled cargo ship exploded in Bari Harbor, Italy the specific applicability of nitrogen mustards and similarly acting compounds (alkylating agents) to treating leukemia was discovered by noting their ability to induce hypocellularity of the

hematopoietic (blood forming) bone marrow space.[2] It was in this manner, beginning with the crude descriptions of Velpeau regarding blood, "*...the consistency and color (of) the yeast of red wine...*" that the recognition of leukemia as a distinct (and treatable) disease entity occurred.

Now, over 170 years later, the term acute myelogenous leukemia (AML) is still in use. While microscopes have improved phenomenally, affording one the ability to discern subtle, sub-cellular hallmarks of the leukemic blast (such as stained secretory granules or chromosomal translocations and deletions), it is also the age of the molecular biologist; allowing investigation into key pathogenic mechanisms in this group of diseases at their most fundamental level. However, despite nearly two centuries of progress, the diagnosis of AML is still largely made microscopically at the cellular level. While treatable, the major pharmacological compounds still in use for AML (anthracycline antibiotics and cytosine arabinoside) can contribute profound drug-related side effects and toxicity. Furthermore, certain anti-neoplastic agents, particularly alkylating agents and topoisomerase inhibitors, are themselves leukemogenic, leading, in some cases, to what are known as therapy-related or secondary leukemias (see section 1-6). Clearly, therapies making use of recombinant hematopoietic growth factors offer promise to many leukemic patients and bone marrow transplantation (BMT) offers additional hope for long-term remission and even cure to others. However, none of these therapies is toxic only to leukemic cells. Less toxic,

molecularly "targeted therapies" will evolve only when the stepwise progression of normal to neoplastic stem cells is fully characterized in molecular terms.

A good deal has been learned about the leukemic blast, it's cytogenetic defects, surface marker phenotype, and clonal growth potential. It is also widely accepted that leukemic blasts are part of a single clone of malignant cells derived from a mutant hematopoietic stem cell.[4] What genetic changes account for the conversion of a normal to a leukemia stem cell?

1-2: Leukemia has a molecular genetic basis

One of the hopes of modern biology is that by understanding the mechanisms by which leukemia arises and progresses, one might be able to develop rational, molecularly-targeted therapies to address the cancer process *specifically* while sparing surrounding tissues from the collateral damage associated with current chemotherapy. While obtaining such information has led to remarkable therapeutic efficacy in certain varieties of leukemia,[5,6] a definitive, molecular description (and thus a "magic bullet" treatment) has remained elusive in AML. If one thing has been learned, it is that a single event is not responsible for the onset and propagation of the disease. Rather, AML (like all cancers) is genetic (though rarely hereditary) in that it likely results from somatically acquired genetic anomalies that occur over time, each contributing in additive (or synergistic) manners to the disease process through alterations of normal cellular

proliferation, differentiation, and/or lifespan. Investigations into such genetic alterations and how they impair normal hematopoietic ontogeny have been built upon observations made in the field of clinical hematology.

1-3: Modern classification of leukemias

AML is a clonal disorder of the hematopoietic stem cell (HSC) in that malignant cells derive from a common progenitor.[7] Clinically, AML is classified according to the lineage of cells involved (myeloid), their degree of maturation (primitive), and their relative numbers in the marrow space (set at 20% by the World Health Organization (WHO)[8] and 30% by the French-American-British (FAB) Cooperative Group).[9] A system of classification has been proposed by the FAB that divides AML cases into subtypes and has been widely used for over 25 years.[9] Additional categorical distinctions are made according to factors such as cellular morphology, surface antigen presentation, and the presence of karyotypic abnormalities.

The morphology of healthy myeloid cells changes during their developmental progression from a common, hematopoietic stem cell (HSC). Normally, all peripheral blood myeloid cells are terminally differentiated and have individually recognizable precursors (in bone marrow) that define distinct stages of lineage maturation. As shown in figure 1-1, hematopoietic ontogeny progresses from the pluripotent hematopoietic stem cell (capable of producing all

blood lineages, both myeloid and lymphoid), to the multipotential hematopoietic progenitor cell (capable of producing all myeloid lineages), and culminates in seven, differentiated myeloid cell types at the distal end: erythrocytes (red cells), megakaryocytes (platelet-producing), neutrophils (granulocytes), monocytes (macrophage-producing), basophils, eosinophils, and mast cells. Roughly speaking, in the peripheral blood, white cells (leukocytes) represent but 0.1% of the cellular volume, the remainder being enucleate red blood cells. AML can occur in all myeloid cell types or be restricted to a sub-set of these depending at which point in ontogeny the leukemic blast has become independent of its normal developmental profile.[10-12] The clinical symptoms frequently noted in AML patients reflect insufficient cellular populations in differentiated lineages. Patients may present with fatigue (due to low red blood counts), excessive bleeding and bruisability (related to deficiencies of platelet-producing megakaryocytes), or infections (resulting from a paucity of functional neutrophils). While clinical symptoms reflect what might initially be thought of as cellular deficiencies, the paradox of leukemia is that patients have elevated white blood cell counts; the cells are simply incapable of fulfilling defined activities due to their lack of differentiation.

The poorly differentiated leukemic blast maintains a primitive morphology with a high nuclear to cytoplasmic ratio. While leukemic blasts may be difficult to discern from one another microscopically without stains (or even with simple preparations such as Wright's/Giemsa), it is often possible to group them into

specific myeloid lineages according to differential enzymatic activities detected by cytochemical stains or via surface antigen presentation.[13] Many leukemic blasts continue to present primitive hematopoietic surface antigens[12] such as CD34 (hematopoietic stem cell and vascular endothelial cell antigen),[13] CD117 (also known as c-KIT, the receptor for stem cell factor (SCF))[14], or KDR (kinase domain receptor; the vascular endothelial growth factor (VEGF) receptor 1).[15] Others experience limited differentiation and may be classified according to cytochemical reactivity in conjunction with later-stage myeloid antigens such as CD38 (which varies inversely with CD34 presentation during myeloid development) or lineage-specific antigens including CD41 (platelet glycoprotein IIb) and CD61 (platelet glycoprotein IIIa) (both restricted to megakaryocytic cells) or glycophorin A (erythroid cells), to name but a few of the many markers currently available.[13] As an additional diagnostic tool, analysis of chromosomal content and morphology represents a means of not only categorizing leukemic subtypes, but also of lending prognostic information to cases within a given subtype. All of these markers - lineage-specific proteins and cytogenetic morphology - have been utilized during the course of the investigations described below.

1-4: Dominantly-acting genetic events in leukemogenesis

Certain varieties of leukemia are (in part) identified by the presence of specific chromosomal rearrangements. The vast majority of cases of chronic

myelogenous leukemia (CML) contain the Philadelphia chromosome,[16] a translocation between chromosomes 9 and 22[17,18] that creates a novel, fusion gene by combining portions of the Abelson tyrosine kinase gene (*ABL*) from chromosome 9[19] and the breakpoint cluster region (*BCR*) on chromosome 22.[20] Characteristic chromosomal translocations are also found in some cases of AML.

Recurrent events include t(8;21) involving the amino-terminal portion of the DNA binding protein core-binding factor-alpha (CBFα; also known as AML1) and the carboxy-terminal portion of the eight twenty-one (ETO) transcription factor.[8,21,22] AML1/ETO is present in some cases of *de-novo* pediatric and adult AML but is seen in over 80% of cases of FAB type-M2 AML.[21] Acute promyelocytic leukemia (APL) cases frequently derive from t(15;17) that merges the promyelocytic leukemia gene (*PML*) on chromosome 15 and the retinoic acid receptor-alpha gene (*RARα*) on chromosome 17 (*PML/RARα*).[8,21,22] Each of these chromosomal fusions is a signature event for certain leukemic subtypes and are representative of specific varieties of transforming activity, *i.e.* dominantly-acting, gain-of-function (or oncogenic) events. This is because fusion genes create an activity (or alter the specificity of an existing process) not normally present within a cell.[22] However, not all transforming events in leukemogenesis are thought to be dominant.

1-5: Recessively-acting genetic events in leukemogenesis

Recessive (loss-of-function) abnormalities are also instrumental in the development of leukemia. Instead of being defined by the creation of novel fusion genes or overexpression of existing genes, these events derive from loss-of-function anomalies for regulators of cellular homeostasis. Such abnormalities are frequently indicated by structural (that is terminal or interstitial) or numerical (*i.e.* whole chromosome) losses of genetic material that result in a non-standard chromosomal content (aneuploidy) and are believed to indicate genomic regions encoding tumor suppressor genes (TSG).[23-25] Chromosomal deletions in leukemia are most often restricted to the malignant cells[26] in keeping with the clonal nature of AML. Recurring, non-random chromosomal anomalies have been characterized in many cases of primary AML (*i.e.* leukemia presenting as a first malignancy with no prior disease or exposure history) though they are much more prevalent in secondary AML (see section 1-6).[27] Frequent aberrations include monosomy 5 and 7, and deletions of 5q, 7q, and 20q.[28-31] That these same chromosomal anomalies are often seen in multiple, unrelated cases of AML (occasionally as the sole anomalies) has led to their recognition as likely causes and not effects of leukemogenesis. The presence of certain numerical/structural chromosomal anomalies often has prognostic significance and clones bearing 5q-, monosomy 7, and/or 9q- are among those with particularly poor outcomes.[27] The causes of cytogenetic instability in this form of AML is a subject of intense interest and work described herein proposes that somatic losses of genes mapping to each of the aforementioned commonly deleted chromosomes directly contributes to leukemic transformation.

Transformation by recessive genes is thought to come about by "loss of heterozygosity" (LOH) for key, negative-regulatory factors. The seminal observation for such a phenomenon was made by Alfred Knudson in 1971 when he statistically analyzed the disease retinoblastoma (Rb), an analysis that yielded persuasive data indicating that two events (*i.e.* two mutations, one in each allele of a single gene) were required for the transformation of retinal neurons.[32] Subsequent studies have demonstrated a variety of genetic mechanisms for how such losses could come about including: loss of both chromosomes, loss of one chromosome with point mutations or gene deletions on the other, loss of one chromosome with reduplication of the remaining (mutation bearing) chromosome, mitotic recombination, and/or gene conversion.[23] To this list of possible events has been added the phenomenon of epigenetic gene inactivation by alterations in the methylation status (and thus changes in transcriptional activity) of disease-causing genes.[33]

Many of the genes located in deleted regions, where gene function has been defined, encode proteins either directly or indirectly involved in transducing environmental cues for mitotic inhibition or apoptosis.[34,35] Consequently, a functional lack of both tumor suppressor alleles, through LOH, results in the subversion of modulatory growth control signals. Therefore, it follows that cells transformed by inactivation of such genes will be insensitive to one or more extracellular cues that control mitotic arrest or programmed cell death (as the

signaling pathway has been disrupted by LOH for a key signaling component). Examples of this have been previously demonstrated in tumor-derived tissue. For example, ablation of transforming growth factor-beta (TGFβ) induced cell cycle arrest can result from loss of function for either the $p21^{WAF1/Cip1}$ protein[34] or $p27^{KIP1}$.[35] In leukemia however, tumor-suppressor gene identification has proven to be a difficult job.

While a specific chromosomal translocation defines the genomic location of the transforming activity in CML (for example), AML presenting with monosomy 7, though bearing a cytogenetic anomaly that also suggests a genomic area in which to search for a candidate gene (or genes), presents a much more daunting task when one considers that thousands of genes, many with key growth-regulating activities, are encoded in the suggested genomic interval. Though tumor suppressor genes have been identified that are important in certain cases of AML,[36] fewer chromosomal deletion-associated leukemias have met with gene identification success. What successes there are have been limited in that the identified anomalies account for a small percentage of cases (see below in section 1-14 regarding *IRF-1*). Other large, chromosomal scale analyses have yielded even less despite intensive investigation into important sub-chromosomal regions such as 7q22.[37] While the frequency of specific chromosomal deletions lends much weight to the hypothesis that key genes are to be found within certain genomic intervals,[25] some findings perhaps speak to the contrary.

Recent work has suggested (rather intriguingly) that aneuploidy *itself* is directly transforming and not merely a situation that unmasks already present mutations in the residual allele.[38-40] That the result of aneuploidy is the creation of a specific chromosomal anomaly no doubt speaks to the presence of selective pressure which forces the retention of particular chromosomal abnormalities. In studies by Sweetser *et al*, using highly variable PCR-based markers to interrogate regions of genetic variability, 53 children with *de novo* AML were found to have very low frequencies of LOH in conjunction with common numerical/structural chromosomal abnormalities, suggesting that LOH for tumor suppressor genes may be of less importance in AML than solid tumor forms of cancer.[26] These results are mirrored by work from Shannon's group that describes a highly detailed analysis of interstitial deletions of chromosome 7q in AML patients.[37] Therein, the authors indicate that homozygous deletions (resulting in LOH) were not observed over a 2-Mb interval along chromosome 7q22 in individuals with either monosomy 7 or del(7q).[37] 7q22 is a chromosomal segment defined as a minimally-deleted region or deletion "hot-spot" by analysis of many patients with partial deletions of chromosome 7. Observations such as these have contributed to the notion that haploinsufficiency for certain genes may also promote malignancy.[41,42] Haploinsufficiency has been shown to play a key role in at least one form of leukemia, familial thrombocytopenia with propensity to develop AML (OMIM 601399), resulting from dosage disparity at the core binding factor 2A (*CBF2A*, also known as *AML1* and *RUNX1*) locus.[43] However, while

additional work regarding the effects of aneuploidy itself will no doubt continue, the search for tumor suppressors of particular importance to hematopoietic cells will go on as well, taking clues from genomic areas indicated by reoccurring chromosomal deletions. While not infrequent in primary leukemias, such complex cytogenetics are nearly universal in secondary leukemias *i.e.* those cases derivative of: an environmental exposure (such as benzene), previous therapy, antecedent myelodysplastic syndrome (MDS), or genetic predisposition (such as in the hereditary condition Fanconi anemia).

1-6: Secondary leukemia

Subsets of AML patients have a very high incidence of clonal chromosomal abnormalities including patients with secondary AML,[44,45] patients progressing to AML from MDS,[25,28,46] and those whose AML arises from a background of genetic predisposition, such as in the autosomal recessive syndrome Fanconi anemia (FA).[47-49]

Exposure to certain aromatic, organic compounds has frequently been associated with an increased risk of MDS and AML.[50-58] Among the best studied are those cases occurring subsequent to benzene exposure. Such cases demonstrate not only an elevated incidence of aneuploidy but of the specific, non-random chromosomal defects frequently noted in other forms of secondary AML (*e.g.* 5q-, monosomy 7, and trisomy 8).[59-64] The exact mechanisms for

benzene-related leukemogenesis are unclear though specific relationships between benzene (or its metabolic byproducts) and the onset of MDS/AML have been related to defects of topoisomerase II function,[65] RAS signaling,[66] and key metabolic pathways (such as NAD(P)H:quinone oxidoreductase or NQO1).[67]

In patients where AML results from treatment with alkylating agents for prior malignancies (such as cyclophosphamide; [2[bis-(2-chloroethyl)-amino] tetrahydro-2H1,3,2-oxazaphosphorine-2-oxide]), there is a particularly high incidence of deletions.[21,44,68,69] In a cohort of 257 secondary MDS/AML patients ascertained over a 26-year period at the University of Chicago, approximately 94% were found to have clonal chromosomal abnormalities.[70] Of these, the two most common were monosomy 7 (29%) and 5q deletion (20%). Together, abnormalities of chromosomes 5 and 7 were found in 71% of cases.[70] These anomalies are in contrast to therapy-related defects following treatment with topoisomerase inhibitors (such as Etoposide) where chromosomal translocations are the most frequent result.[71,72]

1-7: Myelodysplastic syndrome

Myelodysplastic syndrome (MDS or myelodysplasia) is a clonal, preleukemic condition.[46,73,74] Thought primarily to be a disease of the elderly, MDS has a median age of onset in excess of 65 years.[46] The rates if pediatric MDS are difficult to ascertain (estimated to be less than 3-9% of childhood

hematological malignancies), partially due to the rapidity with which juvenile patients often progress to frank AML and preferential diagnosis of other myeloproliferative syndromes in children.[75] MDS in children and adults is characterized by maturation arrest in myeloid lineages with or without an increased blast percentage. Cases with increased blasts by definition must bear less than 20% (WHO)[8] or 30% (FAB)[9] in the marrow space. As is the case in MDS as well as AML, the FAB has established a disease classification scheme that delineates five subsets of MDS based upon cellular morphology, marrow and peripheral blood blast percentages,[76] and more recently cytogenetics and surface antigen presentation.[77]

Instead of being a discrete disease entity, many consider MDS to be near the proximal end of a disease spectrum that includes AML as a distal manifestation. MDS may progress from aplastic anemia, a condition proposed to be a "pre-pre-leukemic" state and thus, perhaps at the most proximal end of the same disease spectrum.[78] 20 to 30% of MDS patients will progress to overt AML.[79] The majority of cases suffer morbidity and mortality related to MDS, events identical to those seen in AML including bleeding (thrombocytopenia), infection (neutropenia), and fatigue (anemia) with the latter being the most common complaint bringing individuals to seek medical attention.[46] Treatment of MDS is nearly identical to that undertaken in AML (including the use of hematopoietic growth factors) save for the relatively infrequent use of bone marrow transplantation due to the advanced age of the majority of patients. Also

similar to AML, chromosomal anomalies that are identical to those seen in AML are present in the majority of cases of MDS[28] where they also confer prognostic significance.[44,80-84]

In evaluating a large, multicenter population of 550 patients with MDS, 38-73% of cases had complex, non-random cytogenetics akin to those seen in AML. The most frequent were 5q-, monosomy 7, 7q-, trisomy 8, and 20q-.[46] In contrast, chromosomal translocations commonly seen in AML (such as the aforementioned t(8;21) and t(15;17)) are rarely noted in MDS.[46] Though the previously mentioned report by Sweetser *et al* concluded that LOH in conjunction with common chromosomal deletions was a rare event in AML,[26] work by Xie and colleagues has shown LOH to be a frequent occurrence in conjunction with these common deletions.[85] Furthermore, studies performed by Mori *et al* suggest that specific chromosomal anomalies (such as those mentioned above) can be noted to arise in myelodysplastic clones as they evolve to AML.[25] The observation of identical chromosomal anomalies suggest that key genetic events are common in the pathogenesis of both MDS and AML (lending credence to the hypothesis that they are truly different points along the same disease continuum). However, as is also the case with both AML and MDS, identification of key genes has, by and large, not yet occurred despite intensive investigation in many laboratories. Due in part to the difficulties related to identifying genes important to the onset and progression of MDS/AML, research into conditions of genetic predisposition to MDS/AML represents a robust strategy for addressing the question. Patients

with the cancer predisposition syndrome Fanconi anemia not only experience an incredibly elevated risk for the development of MDS/AML, but also of the type bearing complex, non-random cytogenetics that are identical to those seen in MDS/AML.

1-8: Leukemia in Fanconi anemia

Fanconi anemia (FA) is a rare, autosomal-recessive disorder consisting of skeletal abnormalities, *café au lait* spots, hypersensitivity to cross-linking agents,[86,87] bone marrow failure,[88] and a high frequency of myelodysplasia and acute myelogenous leukemia.[47,89] Acute myeloid leukemia is the most common neoplasm that develops in patients with FA, although the risk of cancer in general is increased.[47,90] The age-related risk of acute myeloid leukemia in FA is substantial and estimated to be 15,000 times greater than that of the general population.[91,92] Treatment in these patients is extraordinarily difficult, in part because of the inherent sensitivity of all FA cells to certain chemotherapeutic agents (a situation that also makes conditioning for BMT exceedingly difficult).

To date, eight FA complementation groups (each group corresponding to a unique gene) have been defined by somatic cell fusion studies and are represented as FA-A through FA-G including FA-D1 and FA-D2.[93,94] Recent unconfirmed and unpublished results from Joenje's laboratory suggest that three additional complementation groups (I, J, and K) may also exist.[95] Sixty to 65% of

all FA patients fall within complementation group FA-A. Six FA genes have been cloned. They are *FANCA* from chromosome 16q24.3,[96,97] *FANCC* from 9q22.3,[98] *FANCD2* from 3p25.3,[94] *FANCE* from 6p22-p21,[99] *FANCF* from 11p15,[100] and *FANCG* from 9p13.[101] The genes for FA-B and FA-D1 are currently unknown.

Though the Fanconi anemia gene products play obvious roles in the growth and differentiation of normal bone marrow cells (bone marrow is the only organ that consistently fails in children with FA) the exact function of the protein products of these genes is unknown though they are thought to ultimately impact cellular ability to recognize and/or repair DNA damage. This hypothesis derives from the observations that spontaneous chromosomal breakage is often present in biopsies from patients with FA.[102] Furthermore, cellular hypersensitivity to DNA damage can be demonstrated via *in vitro* cellular treatment with cross-linking agents such as mitomycin C (MMC) or diepoxybutane (DEB) which both produce a high percentage of chromosomal breaks and the formation of end to end chromosomal fusions termed "radials".[103-105] Such observations have led to FA being termed a condition of genetic instability, a situation that undoubtedly has some bearing on the profound incidence of leukemia in these patients.

Greater than 20% of FA patients will develop malignancy, primarily AML, while the remainder die from events related to bone marrow failure.[106] Alter has recently reported that FA patients that do not die of bone marrow failure have a 79% incidence of malignancy.[107]

For patients who develop AML, prognosis is poor. Complex cytogenetics including 5q-, monosomy 7, 7q-, 20q-, and trisomy 8; chromosomal anomalies identical to those seen in MDS/AML.[47,49,108-114] That identical chromosomal deletions are found in MDS, AML, and MDS/AML occurring in FA suggests a common mechanism that likely involves LOH for key tumor suppressor genes in each condition. Such an aneuploidy-producing mechanism either impacts merely the genetic integrity of certain chromosomes only (*i.e.* is a process that judiciously deletes genetic material from but a subset of chromosomes) or, alternatively, provides an escape valve for a cell under some selective pressure by deleting genes that encode proteins that serve as effector mechanisms for that pressure. While the former is not without precedent (*e.g.* the loss of material distal to chromosome-specific fragile sites) the latter scenario is more probable, particularly in light of the findings we describe herein on cells from children with Fanconi anemia.

1-9: Clonal adaptation in Fanconi anemia and other pre-leukemic conditions

While the LOH model has enormous scientific support it is incomplete. It presumes, but does not clarify, the important role of selective pressure in the process of clonal evolution. As eloquently stated by Painter (from work in bacterial genetics), "organisms with a relatively high mutation rate are favored during periods of selection".[115] We can infer that such a phenomenon also

applies to single cells with proliferative capacity (including HSC's) and may include aneuploidy as a manifestation of genetic instability,[116,117] (though Painter was specifically referring to the effects of mutator genes in prokaryotes).

Clonal selection is a fundamental tenet of biology in both prokaryotes (*e.g.* the evolution and proliferation of antibiotic resistant strains of bacteria) and eukaryotes (*e.g.* that which occurs in the evolution of multiple drug resistant forms of cancer). In early leukemogenesis, when a single, phenotypically normal stem cell inactivates or deletes its only remaining functional allele for a particular tumor suppressor gene, selective pressure is critical to permit the outgrowth of that mutant clone. If inactivation of such a mitotic inhibition pathway occurred in a normal stem cell, it would lead to a proliferative advantage over its neighbors. However, this would be the case only if the inhibitory signaling pathway of interest existed in a molecular context that was chronically or repetitively active. Only in this setting would the inactivating mutation enhance the replication and/or survival of the mutant clones. Examples of such a phenomenon are not without precedent in hematological malignancy and include selection/clonal evolution in the leukemia-predisposition conditions paroxysmal nocturnal hemoglobinuria (PNH) and severe congenital neutropenia (SCN).

Clonal Adaptation in PNH

PNH is an acquired disorder of complement-dependent destruction of peripheral blood cells (hemolysis; which leads to abundant heme in the blood stream [hemoglobinuria]), excessive clotting (thrombosis), and bone marrow failure.[118,119] The condition derives from defects in the glucosylphosphatidyl-inositol (GPI) anchor that tethers many extracellular proteins to the cell surface[120,121] including CD59 (a major inhibitor of reactive lysis), the loss of which is thought to be the major disease-promoting effect of GPI deficiency in the red cell.[122] Somatic mutations in the X-chromosome-linked *PIG-A* (phosphatidylinositol glycan class A) gene have been shown to result in the condition.[118] The acquired nature of the disease is supported by the observation that *Pig-A* knock-out mice have an embryonic lethal phenotype.[123] As the defect impacts both granulocytic and B-cell lineages,[118] and also that mutations are restricted to disease cells (the result of random X-inactivation and selection in affected females),[124] PNH is believed to be a clonal disorder of the HSC. Selection for the defective cells occurs over time and patients experience an increased risk for the onset of both aplastic anemia (early on) and leukemia (later in the disease course) in GPI-deficient cells.[118] The mechanism by which GPI-deficient cells are selected for is not completely understood though the presence of an autoreactive T-cell that targets normal GPI anchors has been proposed[119] in addition to the observation that non-mutant cells in PNH patients are more sensitive to FAS-mediated apoptosis.[125] The latter suggests that the mutant cells may be capable of resisting periods of selective pressure in the form of sporadic episodes of apoptosis in the marrow microenvironment.

Clonal adaptation in SCN

Severe congenital neutropenia (SCN, also known as Kostmann's syndrome) is characterized by regular infections and severe neutropenia and demonstrates granulocyte maturation arrest.[126] SCN is related to a curious condition known as cyclic neutropenia (CN or cyclic hematopoiesis) wherein production of several hematopoietic lineages fluctuates in a 21-day periodic cycle.[127] Each condition demonstrates an increased risk for MDS/AML (approximately 10%).[128] Dominant mutations in the serine protease gene neutrophil elastase (*ELA2*) on 19p13.3 cause both SCN and CN by contributing to tissue destruction via unrestricted proteolysis at sites of inflammation.[127,128] As such, the presence of the *ELA2* mutation is associated with excessive, apoptotic cell death.[128,129] SCN was initially believed to derive from mutations in the granulocyte colony-stimulating factor receptor gene (*G-CSFR*) as such defects were shown to cause hypersensitivity to the proliferation-inducing effects of G-CSF in mutant cells.[126] However, other studies demonstrated that *G-CSFR* mutations in SCN were acquired at a later stage of disease progression and were therefore, not initiating events.[130] More recently, it has been proposed that G-CSF therapy (commonly used to treat these conditions) might actually exacerbate the progression to MDS/AML by virtue of the robust myeloproliferative effects imparted by G-CSF in SCN patients.[131,132] Based upon these observations, a model has been proposed wherein early, ELA2-activating

mutations initiate the disease process and later, G-CSF hypersensitivity develops that leads to myeloproliferation by protecting against the apoptotic defect associated with mutant *ELA2*.[129] Such selective pressure models are certainly not limited to PNH and SCN.

Clonal Adaptation in Fanconi Anemia

Another selective force for such events could be an hematopoietic stem cell pool in which every member responded in an exaggerated manner to physiologically relevant levels of mitotic inhibitory factors. Hematopoietic stem and progenitor cells from children with Fanconi anemia (FA) and FA knockout mice are examples of this. Colony forming units (CFU) from bone marrow of children with the C complementation group (FA-C) and knockout mice with disruptions of the group C gene (*Fancc*) are hypersensitive to interferon gamma (IFNγ) and tumor necrosis factor alpha (TNFα).[133-135] Work performed by Pang *et al* in our laboratory has demonstrated that excessive, ground state activation of the double-stranded RNA-dependent protein kinase (PKR) mediates apoptotic hypersensitivity to IFNγ, TNFα, and double-stranded RNA (as well as these in combination) in murine embryonic fibroblasts and hematopoietic cells from *Fancc* knockout mice.[136] Furthermore, the exaggerated apoptotic responses of FA cells after exposure to these factors was completely suppressed when cells expressed a dominant-negative form of PKR.[136] We have proposed that such hypersensitivities are not only involved pathogenically in the development of

bone marrow failure in FA[134] but may also exert a unique selective-pressure that contributes to the evolution of malignant stem cells by forcing the emergence of cytokine resistant cells.[137]

In the sections that follow in this chapter, a model is presented for the evolution of neoplastic clones in children with Fanconi anemia. It is also argued that MDS in FA patients is a *de facto* model of secondary MDS in non-FA adults (Table 1-1). In view of the observations of Morley *et al* who studied busulfan (1,4-butanediol dimethanesulfonate) treated mice several years ago,[138] exposure of adults to alkylating agents may lead to chronic stem cell injury (perhaps akin to that seen in FA), thereby creating a similar selective force for clonal evolution in damaged stem cells. This would suggest that progenitors and stem cells from leukemogen-treated adults share certain functional abnormalities with those from FA bone marrow.

1-10: FA and the apoptotic phenotype

In an effort to better understand the possible FA gene functions, a murine model for FA-C has been established by targeted embryonic stem-cell disruption of the murine *Fancc* gene.[133] Progenitor cells from these knockout mice exhibit hypersensitivity to cross-linking agents similar to that found in Fanconi anemia patients.[133] Of more relevance to our model of selective pressure, cells from these mice are also hypersensitive to biological factors that ordinarily serve as

environmental cues for mitotic arrest or programmed cell death. Specifically, the mice demonstrate hypersensitivity to the inhibitory effects of IFNγ *in vitro* and *in vivo*.[133] Haneline has also reported hypersensitivity to IFNγ, TNFα, and the chemokine MIP1α.[135] More recently, Braun and his co-workers have demonstrated that the characteristic gonadal failure in *Fancc*$^{-/-}$ mice is completely reversed in *Fancc*$^{-/-}$ /*Bax*$^{-/-}$ double knockouts, confirming that a primary function of Fancc is to prevent apoptosis.[139]

Given these exaggerated apoptotic responses in human FA-C progenitor cells, the high incidence of bone marrow failure is not surprising.[133] A number of findings support this notion. First, strong evidence exists that intramedullary IFNγ release may be pathophysiologically involved in the development of acquired aplastic anemia.[134] Secondly, low levels of IFNγ prime the pathway for the "death domain" containing, pro-apoptotic receptor FAS in FA-C cells.[134] Finally, a high fraction of CD34^{+} cells from children with FA express FAS.[140]

1-11: FA and FAS

The FAS protein (CD95) is a member of the tumor necrosis factor (TNF) super-family.[141] Its intracellular portion contains a region known as the "death domain" and is required for activation of a signaling pathway (after FAS ligand (FAS-L) binding) that leads to caspase activation and apoptosis.[142,143] Freshly

isolated normal $CD34^+$ cells do not express *FAS* but *FAS* is induced by IFNγ stimulation.[134,144,145] However, compared to CD34+ cells from normal volunteers, a higher fraction of $CD34^+$ cells from children with FA express *FAS*.[140]

In work performed in our laboratory, the highly suppressive effects of IFNγ in FA progenitor cells was abrogated by the addition of neutralizing antibodies to FAS and the toxicity of IFNγ was augmented by the addition of an agonistic FAS antibody.[134] The term "FAS-priming" was used in this context to describe the state of heightened responsiveness to FAS ligation.[146] These studies confirm an important role of the *FAS* pathway in the apoptotic phenotype of FA progenitor cells, observations supported by findings that transgene overexpression of human *FANCC* cDNA protects murine progenitor cells from FAS-induced cell death.[147]

1-12: Cytogenetic abnormalities in Fanconi anemia hematopoietic cells

Cytogenetic abnormalities identical to those found in patients with secondary MDS/AML have been noted in bone marrow aspirates of FA patients including $5q^-$,[110,113,114] monosomy 7,[48,108,112-114] $7q^-$,[109,113] $20q^-$,[113] and trisomy 8.[111,113] Berger and Jonveaux reviewed 58 cases of bone marrow dysplasia in FA and found that approximately 35% bear abnormalities of chromosome 7.[113] Abnormalities of chromosome 1 were also quite prevalent in this cohort (43%). It is tempting to simply attribute clonal evolution in FA cells to a random

mutational process derivative of the widely accepted feature of chromosomal instability. However, the chromosomes involved are non-random, therefore, functional consequences of chromosomal alterations are fully expected.

The Fanconi anemia complementation group C gene or *FANCC* is ubiquitously expressed.[98] If clonal evolution occurred stochastically in all somatic cells in children with FA, then one would expect to see neoplastic disorders that were evenly distributed across different organ systems. This is not the case. While epithelial cancers do occur, the dominant clonal neoplastic disorders in children with FA are MDS and AML. One point of view, a view we espouse and support with experiments described herein, is that the cooperative factor in leukemogenesis is the selective pressure created in stem cells and progenitors by virtue of the FA mutation itself. These mutations (at least those found in children with the C complementation group) clearly result in exaggerated apoptotic responses via heightened responsiveness to FAS-mediated signaling.[134] Taken from the teleological view of stem cells at high risk of death, protective mutations would involve precisely the gene products that transduce the aberrant signal in the apoptotic pathway. This could be through gene conversion at the Fanconi locus itself (a phenomenon that occurs in nature, resulting in mosaicism)[148] or by inactivation of proteins that normally connect hematopoietic cells to a distal apoptotic response. The strongest evidence in support of this model in non-FA MDS/AML is found in work on human chromosome 5q in hematopoietic cells.

1-13: Chromosomal deletions and loss of function mutations

Chromosomal deletions are a common finding in hematopoietic cells from MDS and AML patients. Bone marrow cells of 40 to 90% of MDS/AML patients bear cytogenetic abnormalities.[28] In keeping with the Knudson model[32] it is widely presumed that the deleted material contains tumor suppressor loci and that the remaining alleles are at risk for inactivating mutations. In conditions with mutator phenotypes or chromosomal instability (such as Fanconi anemia) the machinery is in place for inactivation of the remaining locus.

1-14: 5q- and IRF-1 as models

If one were to design *inactivating* mutations that spare highly apoptotic Fanconi anemia stem cells and progenitors, one would clearly wish to inactivate genes encoding factors that transduce apoptotic signals. This is especially relevant in cells that have engaged one or more of the mitotic inhibitory factors to which they are hypersensitive (or perhaps, a downstream factor that the cell inappropriately activates even in the absence of extracellular cues). Loss of the long arm of chromosome 5 ($5q^-$) and monosomy 5 are less common cytogenetic findings in patients with Fanconi anemia though common in secondary MDS/AML. Interferon response factor-1 (*IRF-1*), encoded on chromosome 5q31.1,[149,150] is necessary for the transduction of an apoptotic response to IFNγ

in progenitor cells,[151] and functions as a tumor suppressor gene.[152] Ordinarily, *IRF-1* is inducible (by IFNγ, for example). In FA cells, it is expressed constitutively and low doses of IFNγ are capable of increasing its expression even further.[153] IRF-1 is itself a transcription factor that binds to an element in the promoters of interferon responsive genes and enhances their expression.[154] The overall biological effect of *IRF-1* expression in progenitor cells is growth inhibition and/or apoptosis.

In 1993 Willman and colleagues confirmed genomic loss of *IRF-1* via southern analysis and fluorescent *in-situ* hybridization (FISH) in a sub-population of blasts from patients with chromosome 5q- related MDS/AML.[149] All patients demonstrated reductions of *IRF-1* signals on southern blots probed with the *IRF-1* cDNA.[149] This reduction was in proportion to the number of undifferentiated blasts and 5q- clones in each specimen. Additionally, a sub-population of the individuals studied had a significant number of cells with no *IRF-1* signal at all by FISH. Since that time, others have found that *IRF-1* is not always lost and in some cases the locus is retained on 5q.[155,156] However, it is likely that *IRF-1* function can be lost in other ways.

Is *structural* loss of *IRF-1* the culprit in secondary MDS/AML? The work by Willman and colleagues indicates that this may play a role in some patients.[149] However, this is likely not the case in FA. *Functional* loss of *IRF-1* (or a similarly acting protein) is a more likely possibility. There are *IRF-1* related lesions that do

not depend upon gross deletion of 5q material though they potentially implicate abnormal function of *IRF-1*.[157,158] For example, Harada *et al* have demonstrated that transcriptional skipping of exons 2 and 3 of *IRF-1* can be found in hematopoietic cells from AML and MDS patients.[157] It is expected that other mechanisms (failure of transcriptional activation or accelerated rates of mRNA decay for example) will be discovered in MDS/AML cells that can account for reduced IRF-1 production or function in patients with no detectable abnormality of 5q or *IRF-1*. Such studies are currently being carried out in our laboratory.

Interdiction of *IRF-1* expression in interferon-treated normal progenitor cells not only abrogates the growth-inhibitory response, but also *converts* it to a *growth-enhancing* response.[151] Opportunities to test the notion that IFNγ responses might be reversed in colony forming units (CFU) from the marrow of patients with MDS have presented themselves. In some cases, progenitor cells with no known abnormalities of chromosome 5q have exhibited such "paradoxical", stimulatory growth curves (Figure 1-2). Whether IRF-1 activity is lost in these cells is as yet unknown.

1-15: Evolution of leukemic clones from bone marrow failure states

Functional inactivation of both loci for *IRF-1* predicts not only the loss of an apoptotic response in interferon-treated cells but also the conversion of IFNγ to a *de facto* growth factor.[151] The LOH demonstrated at the *IRF-1* locus serves

as an instructive model for the outgrowth of abnormal clones in MDS and AML and likely derives from the subversion of a mitotic inhibitory pathway that normally controls stem and progenitor cell replication. In the pro-apoptotic context of FA, a hematopoietic stem cell that experienced this type of deletion would have an enormous selective advantage for growth and production of daughter cells because: (a) it would *proliferate* in response to IFNγ exposure and (b) non-deleted cells exposed to IFNγ would undergo apoptosis (Figure 1-3).

1-16: Early (anti-apoptotic) vs. late (apoptotic) events in MDS cells

There has been much work published on the apoptotic activity of bone marrow cells in patients with MDS. Raza and colleagues have reported that a high fraction of bone marrow cells undergo programmed cell death (apoptosis) in patients with myelodysplasia.[159] Given that myelodysplastic syndromes are known to be disorders of ineffective hematopoiesis, these findings are not surprising. In fact, hematopoietic disorders other than myelodysplasia, including non-neoplastic diseases, can demonstrate a similar increase in apoptotic cells.[160,161] The work described within this thesis is focused on clonal stem cell evolution. In that regard, it must be emphasized that in fully evolved MDS marrow, cells in the *progenitor pool* are not undergoing excessive apoptosis.[162] Indeed, if primitive stem and progenitor cells were undergoing high rates of programmed cell death they would have no clonal advantage whatsoever. This thesis has focused *specifically* on the earliest genetic events that occur in stem

cells that later lead to MDS, those forces required to set the stage for the emergence of a dominant clone, and later-stage, proliferative processes utilized by selected clones to foster their outgrowth. This thesis will not address the process by which more differentiated dysplastic daughter cells of such clones die.

1-17: Conclusion; chapter one

Because of the shared links of alkylating agent exposure (secondary AML) and alkylating agent sensitivity (Fanconi anemia) as well as the overall incidence and type of unique cytogenetic abnormalities found in common between patients with secondary MDS/AML and individuals with Fanconi anemia, I hypothesize that the evolution of FA to MDS/AML is a paradigm of secondary MDS/AML in non-FA patients.[137] The evolution of myelodysplastic syndromes and acute myelogenous leukemia in FA patients serves as a model system for investigators studying the evolution of certain hematopoietic disorders. The model begs a set of critically important questions for investigators seeking to better understand early molecular steps in the evolution of leukemic clones. What genes are involved in MDS associated with monosomy 7 (and other common chromosomal abnormalities) and do their products influence function of either *IRF-1* or other genes that act in similar, tumor suppressing capacities in hematopoietic stem cells? What sorts of lesions do the "normal" alleles develop that result in their inactivation and are they heritable? Do individuals at risk for secondary AML

have a pro-apoptotic phenotype (as do FA patients) prior to clonal evolution and if so, what signaling pathways are involved? Can the risk of MDS/AML in FA patients be reduced with effective FA gene therapy? Finally, if gene therapy does not reduce the incidence of clonal evolution in Fanconi anemia, do the evolving leukemic clones occur in the corrected or uncorrected stem cells? With regard to this latter question, Gregory and colleagues have demonstrated in a single case of clonal evolution in FA, that despite mosaicism wherein the majority of hematopoietic stem cells from the patient were reverted (via mitotic recombination between alleles in a compound heterozygote to create one wild-type allele and one bearing both mutations in *cis*), clonal evolution still occurred.[163] Furthermore, the evolving clone was shown unambiguously to belong to the non-reverted HSC fraction.[163] Further analysis into the origins of clones bearing cytogenetic abnormalities in hematological malignancies associated with FA will no doubt shed more light on this point. The donation of hematopoietic and non-hematopoietic cells from children and adults with Fanconi anemia will continue to serve as key components in studies designed to answer these questions. Additional knockout mice with nullizygous mutations at other cognate FA complementation group loci are certain to be valuable resources. The answers to these and related questions will influence our comprehension of myeloid leukemogenesis, not only in patients with FA but those whose myeloid leukemia develops notwithstanding perfectly functional FA genes.

Figure 1-1

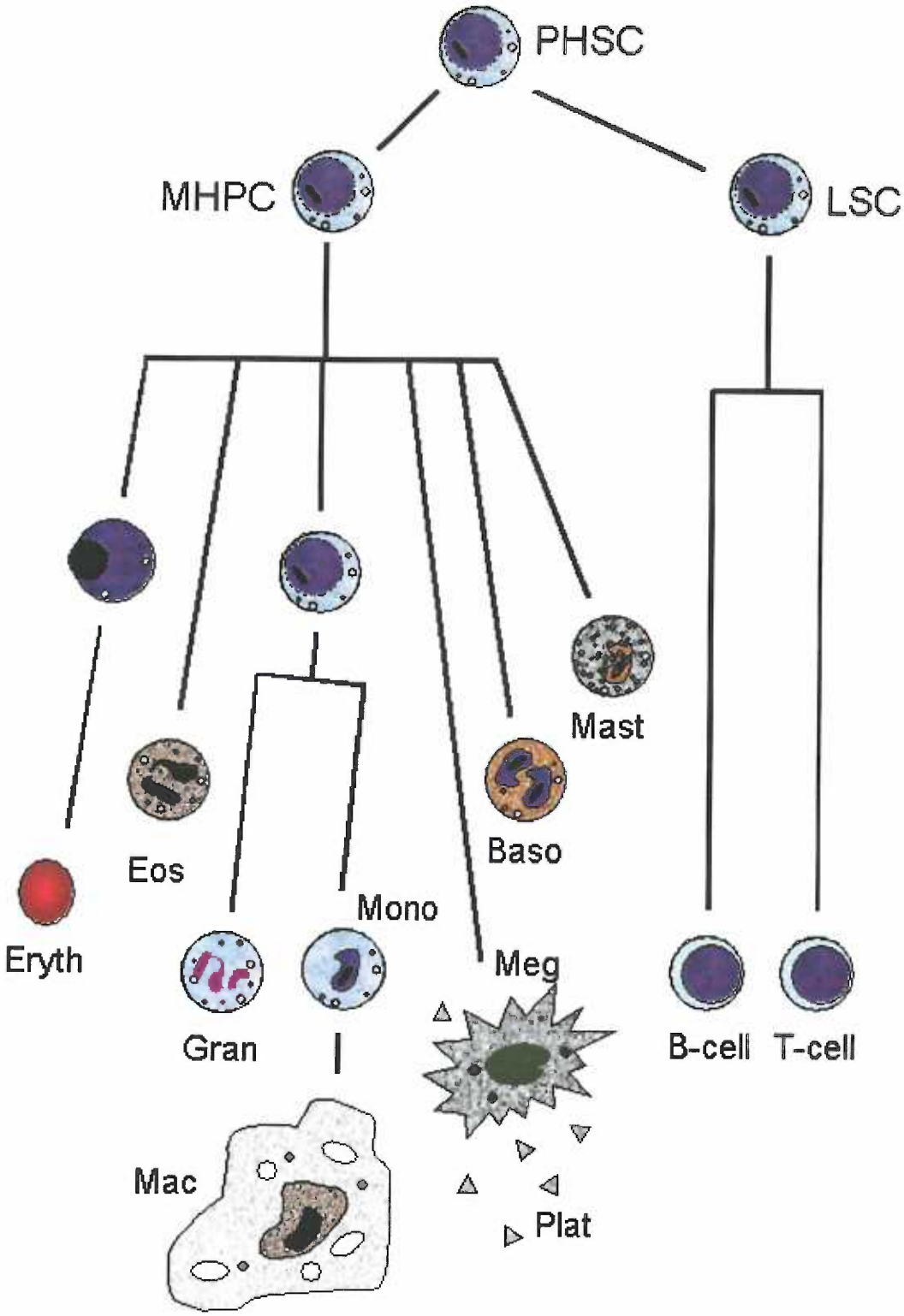

PHSC
MHPC
LSC
Mast
Eos
Baso
Eryth
Mono
Meg
Gran
B-cell
T-cell
Mac
Plat

Figure 1-1 Legend: Hematopoietic ontogeny

Blood cell production (hematopoiesis) derives from a common, pluripotential, hematopoietic stem cell (**PHSC**). The PHSC is capable of forming all lineages, both myeloid and lymphoid. Lymphoid cells (**B-cells**, **T-cells**, and other associated entities such as the plasma cell [not shown]) are derivative of the lymphoid stem cell (**LSC**). The forbearer of the myeloid lineage, the multipotential hematopoietic progenitor cell (**MHPC**) gives rise to the seven myeloid lineages: erythrocytes (**Eryth**), eosinophils (**Eos**), granulocytes (**Gran**), monocytes (**Mono**) (the progenitors of the macrophage [**Mac**]), megakaryocytes (**Meg**) which produce platelets (**Plat**), basophils (**Baso**), and mast cells (**Mast**).

Figure 1-2

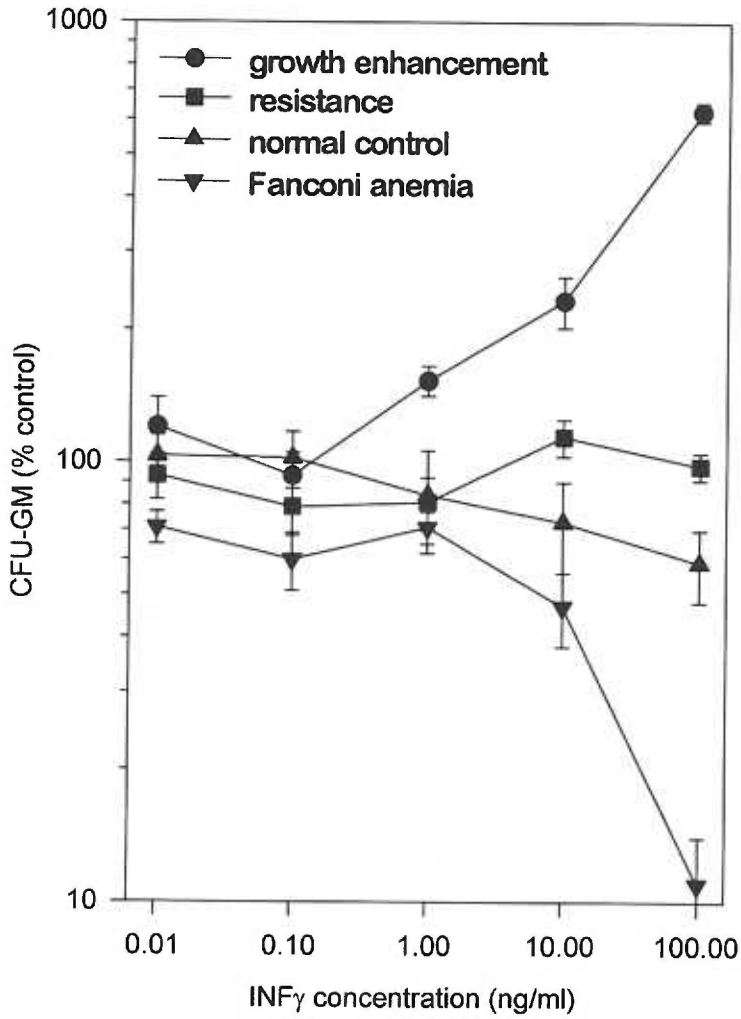

1000
100
10
CFU-GM (% control)
growth enhancement
resistance
normal control
Fanconi anemia
0.01
0.10
1.00
10.00
100.00
INFγ concentration (ng/ml)

Figure 1-2 Legend: Variable responses of CD34+ bone marrow cell exposure to IFNγ. Bone marrow derived CFU-GM progenitors from normal volunteers (-▲-) (n=9) exhibit a dose dependent inhibition in colony forming capacity with increasing concentrations of IFNγ. Progenitor cells from a child with Fanconi anemia (FA-A) (-▼-) exhibit IFNγ hypersensitivity. In contrast, certain populations of MDS/AML patients demonstrate insensitivity (-■-) (a 78 year-old male with secondary AML and monosomy 7) or growth stimulation (-●-) (a 72 year-old male with MDS and t(2;5)) over an identical range of IFNγ concentrations. All curves represent mean (± S.D.) CFU-GM growth expressed as percent control colony growth (no interferon) in methylcellulose cultures containing hSCF (50 ng/ml), IL-3 (10 ng/ml), and erythropoietin (2 U/ml). In the individual patients, means were calculated from colony counts in triplicate plates at each IFNγ dose. Colony-forming assays were performed by Keaney Rathbun in our laboratory.

Figure 1-3

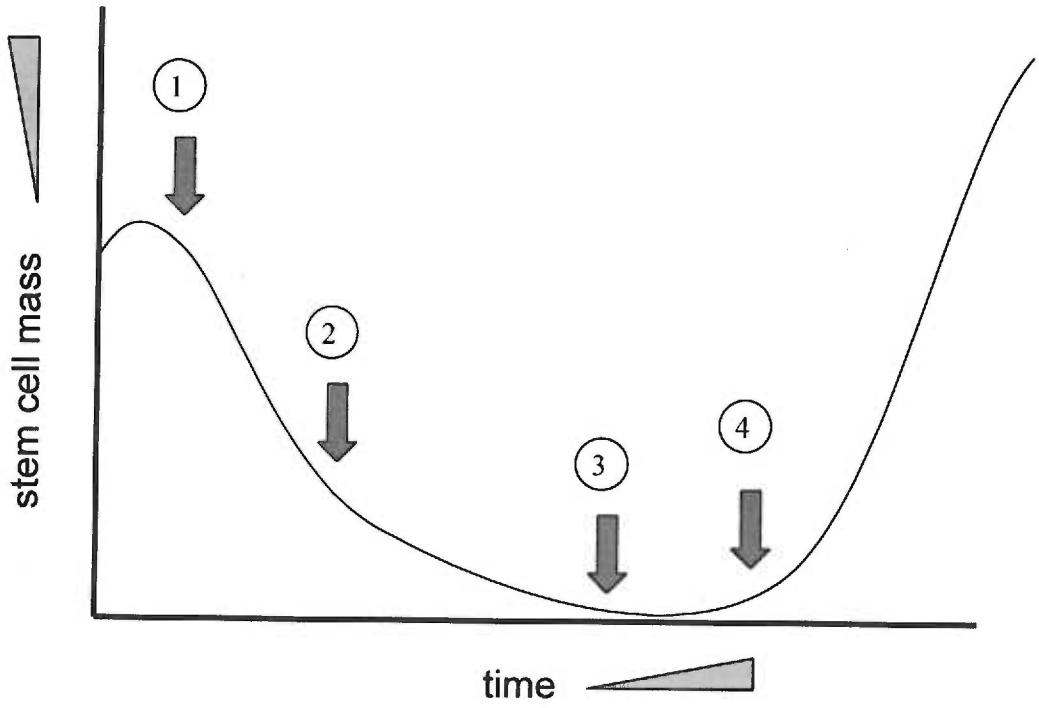


Figure 1-3 Legend: A schematic of stem cell mass over time in the marrow of an individual with Fanconi anemia (FA). (1) stem cell numbers decline in children with FA due to a high rate of apoptosis, (2) bone marrow failure results, (3) chromosomal instability leads to inactivating mutations of two alleles of a gene encoding a critical signal transducer of apoptosis, and (4) the mutant clone begins to expand.

Table 1-1: Similarities between MDS/AML in Fanconi anemia and secondary MDS/AML in non-FA patients

	FA-MDS/AML	2° MDS/AML
role of alkylators	cellular hypersensitivity *	high level exposure **
prevalence of cytogenetic defects	high	high
frequency of complex rearrangements	high	high
prevalence; deletions of chromosome 7	24% [113]	29% [70]

* the *sine qua non* of Fanconi anemia

** alkylating agents can induce chronic bone marrow failure [138]

Chapter Two:

Identifying the functional consequences and potential molecular causes of leukemia-initiating events in Fanconi anemia

2-1: Introduction

Hematopoietic cells, including committed bone marrow progenitors, from children and adults with Fanconi anemia (FA) are hypersensitive to apoptotic cues.[134-136,147,164-167] Chapter one described a model regarding clonal evolution in Fanconi anemia (FA) progressing to myelodysplastic syndrome (MDS) and acute myelogenous leukemia (AML). This model holds that the pro-apoptotic phenotype in FA hematopoietic stem and progenitor cells (HSC), deriving in part from known cellular hypersensitivity to inhibitory cytokines such as interferon-gamma (IFNγ), not only results in bone marrow failure (BMF)[134] but also represents a robust source of selective pressure for the emergence of apoptosis-resistant clones.[137] It went on to predict that malignant HSC's from FA patients should be resistant to the very factors that induce excessive apoptosis in the BMF phase of FA (see figure 1-3). Such resistant HSC's would necessarily have a pronounced competitive advantage over their apoptosis-prone counterparts.[137]

To evaluate this prediction, CD34+ bone marrow cells were obtained from a cohort of normal controls as well as FA patients that had either MDS, AML, or bone marrow failure without malignancy and exposed to a range of IFNγ doses in clonal methylcellulose assays. Colony forming unit assays (CFU-A) from six of

six FA patients without evidence of malignancy demonstrated statistically significant IFNγ-hypersensitivity in hematopoietic progenitor cells compared to normal controls (p = 0.005) while three of three FA patients with MDS/AML repeatedly demonstrated IFNγ-resistance that was not statistically significant from normals (p = 0.989). These data, described in detail below, represent a "proof-of-principle" for the model of adaptive clonal evolution described in chapter one of this thesis.

This cohort of FA patients also contained a multiplex pedigree wherein two siblings, both with FA (FA-A), were at different stages of disease progression. Specifically, one sibling had FA while the other had FA in addition to evidence of clonal evolution (1q trisomy) and MDS. Colony-forming unit-granulocyte/macrophage (CFU-GM) and burst-forming unit-erythroid (BFU-E) populations from the FA-MDS sibling were interferon-resistant while progenitors from the sibling without MDS were interferon-hypersensitive. Based upon the premise that these siblings represent distinct stages of disease progression on the same genetic and environmental background, we hypothesized that differences in bone marrow gene expression profiles between them would lead us to the characterization of malignancy-initiating genes in FA.

To test this prediction, gene expression microarray analysis (Affymetrix, Human Genome U95A Array)[168] was carried out using RNA isolated from low-density bone marrow cells from each of these individuals. In comparing

transcriptomes between siblings, it was noted that most genes were expressed at equivalent levels (> 97%). By conservatively masking data from all points held in common between siblings, a list of 327 differentially expressed genes was obtained. In this data set, 131 transcripts were found to be expressed ≥ two-fold in FA-MDS cells and 196 were expressed ≥ two-fold in non-MDS cells. These 327 differentially-expressed genes represent candidates for factors influencing the survival of FA hematopoietic cells. The details of these experiments and candidate genes are outlined below.

2-2: Materials and methods

LDBMC and CD34+ cell isolation - Bone marrow biopsies were obtained from patients and normal volunteers under IRB-approved protocols of informed consent. Heparinized bone marrow aspirates were obtained by iliac crest puncture in unsedated adult donors and in pediatric patients under general anesthesia. Low density bone marrow cells (LDBMC's) were isolated via density centrifugation on Ficoll-Paque of heparinized bone marrow mixed 1:1 with complete medium (RPMI-1640). Gradients were centrifuged at 1,200 X g for 20 minutes at 20°C. LDBMC's were rinsed three times in room temperature sterile PBS before either RNA extraction (see below) or purification of the CD34+ cell fraction via antibody-conjugated magnetic particles using a commercial kit per the manufacturer's instructions (Miltenyi Biotech, Auburn, CA).

Colony forming unit assays - $8x10^5$ CD34+ cells were resuspended in 1 ml of serum-free RPMI-1640, added to 3 ml of methylcellulose medium (Stemcell Technologies, Vancouver, B.C.), SIE (human stem cell factor [hSCF, 50 ng/ml], interleukin-3 [IL-3, 10 ng/ml], and erythropoietin [EPO, 2U ng/ml]), and mixed prior to seeding in triplicate in 35 mm polystyrene tissue culture dishes (1 ml each = $2x10^5$ CD34+ cells per dish). These cultures were incubated in a humidified, 5% CO_2-buffered atmosphere at 37°C. Colony forming unit assays were quantitated visually on a dissecting microscope at day-14 for CFU-GM, BFU-E, and CFU-GEMM and the results plotted with either standard deviations or standard error of the mean, as indicated.

Total RNA isolation – LDBMC's were obtained as indicated above and held in RPMI-1640, 10% heat-inactivated fetal calf serum (FCS), and the SIE cocktail at 37°C in a humidified, CO_2-buffered incubator for 18 hours prior to use. Total RNA was then extracted from LDBMC's using commercial kits (Qiashredder© and RNeasy©) per the manufacturer's instructions (Qiagen, Valencia, CA). Purified RNA was redissolved in diethyl-pyrocarbonate (DEPC) water (Qiagen, Valencia, CA) and evaluated spectrophotometrically (via 260nm/280nm λ UV light) in both DEPC-water (to determine the concentration and yield) and 10 mM Tric-HCl, pH 7.5 (to determine the purity). Total RNA was then reprecipitated for storage (-70°C) using 1/10 volume 3 M sodium acetate and 2.5 volumes of 100% ethanol. Prior to use, total RNA was pelleted at 12,000 x g for 15 minutes at 4°C, rinsed with 70% ethanol (in DEPC water) and recentrifuged as before. RNA pellets

were dried at room temperature for 5 minutes and then redissolved to approximately 1 μg/μl based upon the original spectrophotometric determination prior to storage at -70°C. Total RNA integrity was estimated by analysis of ribosomal RNA bands in formamide-containing agarose gel electrophoresis with 100 ng of total RNA in 1X MOPS buffer (0.2 M MOPS (3-(N-morpholino)propanesulfonic acid), 0.05 M sodium acetate, 0.01 M EDTA) relative to a 100 bp DNA size standard (Life Technologies, Gaithersburgh, MD), stained with ethidium bromide, and recorded photographically. Based upon the method outlined here, approximately 500 ng of total RNA per million LDBMC's was obtainable (on average) from children with FA. Amounts of total RNA sufficient for two independent labelings (including pre-labeling purity controls) were obtained from approximately 15 ml of a heparinized bone marrow aspirate.

cRNA synthesis and labeling - Protocols for the preparation of material for the Affymetrix microarray analysis have been previously reported.[168] By way of summary, total RNA was used as template for first strand cDNA which in turn served as the template for synthesis of double-stranded cDNA. Double-stranded cDNA was then used to synthesize labeled cRNA probes via incorporation of biotin-tagged ribonucleic acids in a cell-free, *in vitro* transcription (IVT) system (Affymetrix Inc., Santa Clara, CA). This biotin-tagged cRNAs were then fragmented to less than 200 bases and hybridized to the Affymetrix HU-95A microarray chip per the conditions of the microarray maufacturer.[168]

Hybridization was then performed with fluorophore-conjugated avidin and individual hybridization events detected by a scanning laser.

Data analysis - Image files (cell file) obtained for each microarray chip hybridization were uploaded to the OHSU Microarray Shared Resource Bioinformatics Suite at the PVAMC and analyzed by using proprietary software algorithms (Microarray Analysis Suite [MAS], v 4.0, Affymetrix Inc., Santa Clara, CA). The data was then restricted to a smaller set (data recoding) based upon signal comparisons within specimen replicates (*i.e.* intrasample comparisons using the same patient cRNA hybridized to two distinct chips) and also by pairwise comparisons between samples. This is performed to exclude data points reflecting non-reproducible calls (such as data points that were not present in each intraspecimen replicate) or those that were non-informative between siblings (such as genes not expressed in each individual).

A breakdown of the result of data recoding is as follows: of 12,625 genes, 70 were excluded as they did not agree within intraspecimen replicates, a further 7,579 were omitted as they were differentially expressed by less than a 2-fold difference between siblings, and 4,649 were removed as they were absent from each specimen. This resulted in a final list of 327 data points representing genes invariantly expressed in each sibling at greater than a 2-fold difference. In assigning the relative value of increased (I) or decreased (D), the specimen from the sibling with FA alone was set as the standard so that increased "calls" are

those genes expressed more highly in the sibling with FA/MDS and decreased calls denote reduced expression in this same individual. The data were entered into a spreadsheet pivot table along with the following additional information: chromosomal location, Entrez identifier, average difference for each intraspecimen replicate (4 total, 2 for each sibling), calls (I or D) for each pairwise comparison (R1xJ1, R1xJ2, R2xJ1, and R2xJ2), and finally, a brief description or name of the gene identified.

Results:

2-3: Proof-of-principle for adaptive evolution model

The model outlined in chapter one of this thesis described a role for selective pressure in the evolution of malignant clones in Fanconi anemia. In short, it stated that the finding of apoptotic hypersensitivity in FA bone marrow progenitors (deriving from events related to interferon-gamma (IFNγ) exposure)[133-136,165] represents an apoptotic hurdle that FA hematopoietic stem and progenitor cells (HSC/HPC) must overcome (via mutation) if they are to proliferate. The occurrence of such a mutation in an HSC/HPC would be functionally manifested as acquired resistance to IFNγ (an adaptive response of an HSC/HPC to a hyperapoptotic ground state).[137] This apoptotic resistance in turn would promote malignancy by permitting uncontrolled growth and opportunities attending this proliferative response to acquire compounding

genetic changes. As such, the acquisition of apoptotic resistance would be an early change in a precancerous clone. To test this theory, the IFNγ responsiveness of bone marrow progenitor cells was determined in three populations: individuals with FA plus evidence of MDS or AML, those with FA alone, and normal controls. As shown in figure 2-1 and relative to normal controls, the BFU-E of individuals with FA were IFNγ hypersensitive (p = 0.005). Furthermore, FA patients that had evolved to MDS or AML did not demonstrate a statistically significant difference from normal controls (p = 0.989). These findings represent an essential requirement for the validation of the proposed model of selective pressure in FA. Of particular interest, this FA patient cohort contained two full siblings from a multiplex pedigree of FA and MDS.

2-4: A multiplex pedigree of FA and MDS agrees with the adaptive model

Figure 2-2 illustrates an FA kindred of complementation group A (FA-A). Individuals II-1 (a female young-adult) and II-3 (an adolescent male) each have a history of congenital short stature for which individual II-1 received prior recombinant human growth hormone treatment. More recently, the kindred came to medical attention due to the effects of anemia in individual II-3. A differential diagnosis made at that time included Fanconi anemia which was confirmed via mitomycin C (MMC) induced chromosomal breakage and radial formation (100% of analyzed metaphases had radial forms). Bone marrow biopsy in II-3 revealed multilineage dysplasia, myeloid arrest, left-shifted erythroid maturation, oval

macrocytes, less than 5% blast forms, and increased abundance of mast cells (an increased number of mast cells has been associated with hematological malignancy).[169] G-banded karyotyping in II-3 revealed a clonal cytogenetic abnormality of 1q trisomy via translocation to the long arm of chromosome 18 [t(1;18)(q21;q23)]. These observations were consistent with a diagnosis of myelodysplastic syndrome. Individual II-1 was diagnosed with FA subsequent to the evaluation of her brother. She has positive MMC studies (100% of analyzed metaphases had radial forms). Bone marrow aspirates in II-1 demonstrated a hypocellular marrow (30%) with no dysplastic changes. The karyotype in II-1 is 46,XX. Individual II-2 demonstrated a negative MMC sensitivity test and is therefore, unaffected (though her likelihood of being a carrier is 2/3). To determine the IFNγ-sensitivity of HSC's/HPC's from individuals II-1 and II-3 relative to one another, bone marrow aspirates were obtained from each patient, CD34+ cells were isolated, and these were plated in colony-forming assays in varying doses of IFNγ.

As shown in figure 2-3, the outcome agrees with the predictions made in the adaptive evolution model in that CFU's from the sibling with FA/MDS are resistant to IFNγ (in both CFU-GM and BFU-E though only BFU-E are illustrated) while those from the sibling with FA alone remain hypersensitive. This observation not only supports the theory of adaptive evolution in FA leukemogenesis outlined in chapter one, but also presents a unique opportunity to evaluate genes that are seminal to the *initiation* of hematological malignancy in

FA. Ideally, if one were to design an experiment to determine initiating events in malignancy, it would be most instructive to obtain and compare serial specimens from the same individual as they progressed through distinct stages of malignancy. However, it was reasoned that such an analysis using this sibling pair (instead of serial observations in a single person) would provide an intriguing and much more timely alternative approach to the identification of MDS-initiating events in FA. Specifically, the siblings; (a) have identical gene mutations (the parents are asymptomatic), (b) are close to one another in age, (c) are from the same environmental background, and (d) present with clinically distinct stages of FA disease progression. To determine the identities of MDS-initiating genes in FA, gene expression microarray analysis was employed.

2-5: Comparative transcriptomal analysis in FA and FA/MDS

Gene expression microarray analysis is ideally suited to a problem such as that presented here. This is due in part to its ability to interrogate the expression levels of many thousands of different genes (probes) with a single, small input cRNA specimen. The fact that most children with FA have hypocellular marrows[170] by the time of diagnosis demands that high throughput microanalyses be used whenever possible. The Affymetrix methodology allows one to screen nearly 13,000 distinct transcripts on a single chip (HU 95A) with an input of only 5 μg of total RNA (or as little as 0.2 μg of poly-A+ mRNA).[171] Each gene is represented on the chip by clusters of what are known as "features" with

each feature containing 10^7 to 10^8 25-base oligomer probes each. Each feature corresponds to a different sub-section (5' to 3') of a gene to ensure maximal gene coverage per transcript. The total number of features per gene (40) is divided between two rows, the perfect-match (PM) row (20 features per gene) and the mismatch (MM) row (20 features per gene). The PM row is an exact sequence match of a 25-base section of the mRNA sequence while the MM row is this same sequence with a single base substitution. The MM allows quantitation of non-specific hybridization intensity for each gene. For a gene feature to be scored as positive, it must have a greater absolute signal intensity in the PM than in the MM. The relationship of all PM to all MM features for a given gene is termed the average difference (AD) and will be greater than one for true positive signals and less than one for spurious hybridization signals. The AD is the number used for all subsequent calculations regarding the data presented here.

LDBMC's were obtained from each of the aforementioned siblings and subjected to microarray analysis on two distinct microarray chips (Affymetrix chip HU 95A) each. These will be referred to hereafter as "replicates" and are intraspecimen hybridization and labeling controls. As shown in table 2-1, the quality and reproducibility noted between replicates and between specimens was quite high. The overall signal intensity for all four chips used (FA1, FA2, MDS1, and MDS2) was comparable (average of ~ 130), as was background (~ 45). As also summarized in table 2-1, the percent negative average difference was

approximately 20% for the entire chip, indicating that 1/5 features (thus, 4 per gene) experienced what was some degree of spurious hybridization. The percent presence calls were approximately 50% for all chips, indicating that though some spurious hybridization occurred, true positives were abundant. The noise (Q) for the chip (determined by a measurement of pixel intensity across the entire chip and compared to background cells using a complex, computational algorithm;[171] background cells being the lowest 2% of hybridization intensities for the whole chip) was an average of approximately 1.95 and is used along with the background to establish threshold values for assigning genes to either positive (present) or negative (absent) pools. The scaling factor (SF) is an arbitrary (here, set to 200), linear value applied to the data sets so that multiple experiments can be compared. As all data here have comparable, positive SF's, this indicates again, that hybridization intensity for all chips was comparable. Finally, the AD values for two housekeeping genes (actin and glyceraldehyde-3-phosphate dehydrogenase [GAPDH]) indicate comparable signal between chips though experimental cRNA for actin tended to be longer as evidenced by an average 3'/5' ratio of 1.01 compared to an average 3'/5' ratio in GAPDH of 1.51. Overall, these data indicate that excellent probe and chip quality was present, lending confidence to the data integrity. Next, pairwise comparisons were made between intrasample replicates.

Figure 2-4A illustrates a $\log_2$ scatter plot (base-2 was used as it directly plots fold differences between specimens) of intraspecimen hybridization

replicates in the sibling with FA alone while figure 2-4B is a similar plot in the sibling with FA plus evidence of clonal evolution and MDS. Pierson's correlation coefficients (r) for each sibling (r = 0.945 for FA1vs. FA2; r = 0.928 for MDS1 vs. MDS2) demonstrate a high degree of similarity in independent hybridization reactions. Correlation can also be determined in this instance by calculating the slope of a line plotted through the geographical mean of the combined data on an absolute (linear, Cartesian) scale. The result of slope determination indicates that in the sibling with FA alone, 99.14% of all data points were unchanged in the replicate (slope = 0.9914) while a similarly high percentage (98.72%) was present in the replicate from the sibling with FA/MDS. These figures indicate that the data has a high correlation of reproducibility within replicates. Data recoding (see methods) resulted in 7,906 genes that were expressed in each replicate, in each sibling.

Figure 2-5 shows a $\log_2$ scatter plot for the 7,906 genes that were expressed in each sibling relative to one another (sibling with FA alone on the X-axis and the sibling with FA/MDS on the Y-axis). This list of nearly 8,000 genes was narrowed to 327 genes differentially expressed with greater than a 2-fold difference. The list of 327 contains 131 genes more highly expressed in the sibling with FA/MDS and 196 genes more highly expressed in the sibling with FA alone. The individual identities of these genes is listed in table 2-2. This table indicates (from left): chromosomal assignment (where mapping data is available), Entrez unique identifier, the AD's for that gene in each chip (of 4), the absolute

call for each intersibling pair-wise comparison (I = increased, D= decreased, MI = marginally increased, MD = marginally decreased, NC = no change), the average fold change (rounded to a whole number), and finally, a short description or gene name. The genes are sorted in ascending order by fold change from negative (*i.e.* more highly expressed in the sibling with FA alone) to positive (more highly expressed in the sibling with FA/MDS). These 327 are candidate genes for factors contributing to the initiation of malignancy in Fanconi anemia.

2-6: Discussion

As summarized in chapter one, hematopoietic cells of children with Fanconi anemia (FA) are highly apoptotic.[134-136,147,164-167] I have proposed that this state of excessive cell death[134] represents a selective pressure contributing to the evolution of malignant clones.[137] While the publication by Lensch *et al* illustrated hypersensitive inhibition of CFU-GM by IFNγ in a single case of FA-A (see figure 1-2), the plot demonstrating IFNγ-resistance in malignancy was from a non-FA adult with secondary AML and monosomy 7 (we argued that the disease manifestations in FA-associated MDS/AML and adult, secondary MDS/AML are similar).[137] To provide proof-of-principle for the model of adaptive clonal evolution in an FA-specific cohort, additional patient data has been provided here. Analysis of these data support the clonal evolution model in that BFU-E from individuals with FA alone are hypersensitive to the inhibitory effects of IFNγ compared to normal controls (p = 0.005) while patients whose disease has

progressed to malignancy are interferon-resistant (p = 0.989 compared to normal controls). This patient cohort included a unique FA-A pedigree wherein two siblings have each been diagnosed with FA though one has also progressed to MDS [89,91] with cytogenetic signs of clonal evolution including trisomy 1q. The finding of abnormalities of chromosome 1 is not infrequent in MDS [85] or FA [113] including 1q trisomy specifically in each.[172,173] Colony-forming assays performed in each sibling correlated with predictions made by the adaptive evolution model in that the individual with evidence of MDS had IFN-resistant CFU-GM and BFU-E while the sibling with FA and no signs of malignancy was hypersensitive. These findings agree fully with the predictions made in the proposed adaptive evolution model.[137] Additionally, as the siblings in this rare pedigree represent distinct stages of FA disease progression on a shared genetic background and have identical FA gene mutations, it was thought that transcriptomal differences between them would indicate genes important to the initiation of malignancy in FA. Towards this end, microarray data was obtained and revealed a 2.6% transcriptional difference between them (327 of 12,625 genes).

The ability to compare gene expression differences on a genome-wide scale has become more approachable in recent years due in part to the increased attention given to the development of bioinformatics tools that allow interpretation of these massive data sets.[174,175] This technique has recently been applied to MDS in an attempt to define genes that contribute specifically to MDS but not AML.[176] In the present study, genes important for influencing the survival

of hematopoietic cells in FA were sought. The identities of the 327, FA malignancy-initiating candidate genes were diverse and some may be relegated to a low follow-up priority based either upon their function (such as beta tubulin, expressed 5-fold higher in the sibling without MDS) or chromosomal location (such as the Y-chromosome encoded ribosomal protein RPS4Y, increased 108-fold in the sibling [male] with MDS; no gender bias has ever been reported in malignant transformation in FA). However, among these 327 genes are also candidates that have previously been implicated in a variety of disease processes including the apoptosis-promoting protein BAX,[177,178] the c-ETS-1 proto-oncogene[41], and the L-3-phosphoserine phosphatase homolog.[179]

BAX is a member of the BCL-2 (BCL = B-cell lymphoma) protein family. BCL-2 is a major promoter of cell survival that when present in a homodimeric form, prevents cellular apoptosis.[178,180] BAX is a BCL-2 binding antagonist and when expressed, leads to inhibition of BCL-2 dimerization and cell death. In the study presented here, the sibling with MDS has a 13-fold reduction in *BAX* gene expression (table 2-2, row 13 of 343) relative to his sibling without malignancy. It seems plausible that an increased pool of homodimeric BCL-2 could then inhibit apoptosis. Even though the data is compatible with the notion that the sibling with FA alone (not MDS) may have a 13-fold increase in the level of *BAX*, the point that the MDS clone has potentially adapted to the consequences of *BAX* expression by suppressing it, is inescapable particularly in the light of the observations of Braun *et al*, demonstrating that knocking out *Bax* complements

the apoptotic defect in *Fancc*$^{-/-}$ germ cells.[139] The relationship of specific candidate genes to the disease state may be evaluated in other ways including Western blots. Indeed such studies are currently underway in our laboratory with regard to BAX. Once differential *BAX* expression is confirmed using real-time PCR and immunoblotting, linkage of *BAX* suppression and clonal evolution of this MDS clone will be formally tested by gain of function studies in which *BAX* expression is enforced in MDS cells seeking to restore their apoptotic phenotype. Clearly, because of the pitfalls in selecting for apoptotic cells, the enforced expression of *BAX* in MDS cells must be conditional.

The *c-ETS-1* proto-oncogene (a basic, helix-loop-helix transcription factor) has been implicated in a variety of malignancies including leukemia.[181] *c-ETS* was increased 5-fold in the sibling with MDS (table 2-2, row 306 of 343). The attractiveness of *c-ETS-1* as a candidate gene in this study is that *c-ETS* expression is linked to the control of several key hematopoietic growth factor genes (including those for IL-3 and GM-CSF)[41] important to primitive hematopoietic cell survival and proliferation. An increased abundance of the c-ETS transcription factor could be involved in the production of hematopoietic growth factors. Autocrine loops (self-controlled processes that in this case, promote prolferation) or processes that impair development have previously been implicated in the factor-independent growth of leukemic cells (see chapter four). Expression of c-ETS, IL-3 and GM-CSF genes will be tested in our laboratory at

the time of the next bone marrow examination using immunoblotting and quantitative RT-PCR.

The L-3-phosphoserine phosphatase is a gene about which little is known. It was first identified by Planitzer and colleagues in studies on Fanconi anemia fibroblasts (where it was found to be overexpressed relative to normal controls).[179] Of interest, the gene for this phosphatase was also mapped to chromosome 7q11.2,[179] a genomic region implicated in the pathogenesis of MDS and AML (see chapter 1). Here, however, the L-3-phosphoserine phosphatase homolog demonstrated a 7-fold increase in the sibling with MDS (table 2-2, row 321 of 343). This gene is of considerable interest as our laboratory has recently reported the involvement of the double-stranded RNA-dependent protein kinase (PKR) in the apoptotic hypersensitivity of FA cells.[136] In the report by Pang *et al*, constitutive activation (via serine phosphorylation) of PKR was observed, an activity that led to a high percentage of apoptosis in $FANCC^{-/-}$ cells treated with IFNγ and dsRNA or IFNγ and TNFα relative to normal controls.[136] This apoptotic hypersensitivity was blocked by overexpression of a dominant-negative inhibitor of PKR.[136] No serine phosphatase responsible for down-regulating PKR has yet been identified, the L-3-phosphoserine phosphatase homolog identified in this study is currently being evaluated (via enforced expression in $FANCC^{-/-}$ cells) for its ability to inhibit PKR activity *in vitro*. Should the L-3-like phosphatase be capable of downregulating the activity of PKR in FA cells, the role of L-3 activity reported here in the sibling with FA/MDS may potentially relate to an ability to

directly inhibit a known pro-apoptotic pathway in FA cells. Such an observation would define at the molecular level the very type of abnormality suggested previously for FA progressing to malignancy.[137]

Finally, how do gene expression differences in these siblings correlate to the cytogenetic defects noted in the sibling with FA/MDS (1q trisomy via duplication and translocation to the long arm of chromosome 18)? Trisomy suggests a dominant, gene dosage mechanism for a locus (loci) on chromosome 1q. While the gene expression data indicate large fold differences for some genes on chromosome 1, there are no "smoking guns" similar to the three genes described above. One interpretation of this is that if genes on chromosome 1q are pathogenic in this case of MDS (as opposed to simply the result of MDS), they are perhaps as yet unidentified (or rather, listed ambiguously as EST's) and not among the 12,625 genes on the Affymetrix HU-95A GeneChip. Additional studies making use of not only other microarrays but also the powerful techniques of molecular biology are currently underway. This report describes the index cases in a multi-center, Fanconi Anemia Transcriptome Consortium (FATC).

Figure 2-1

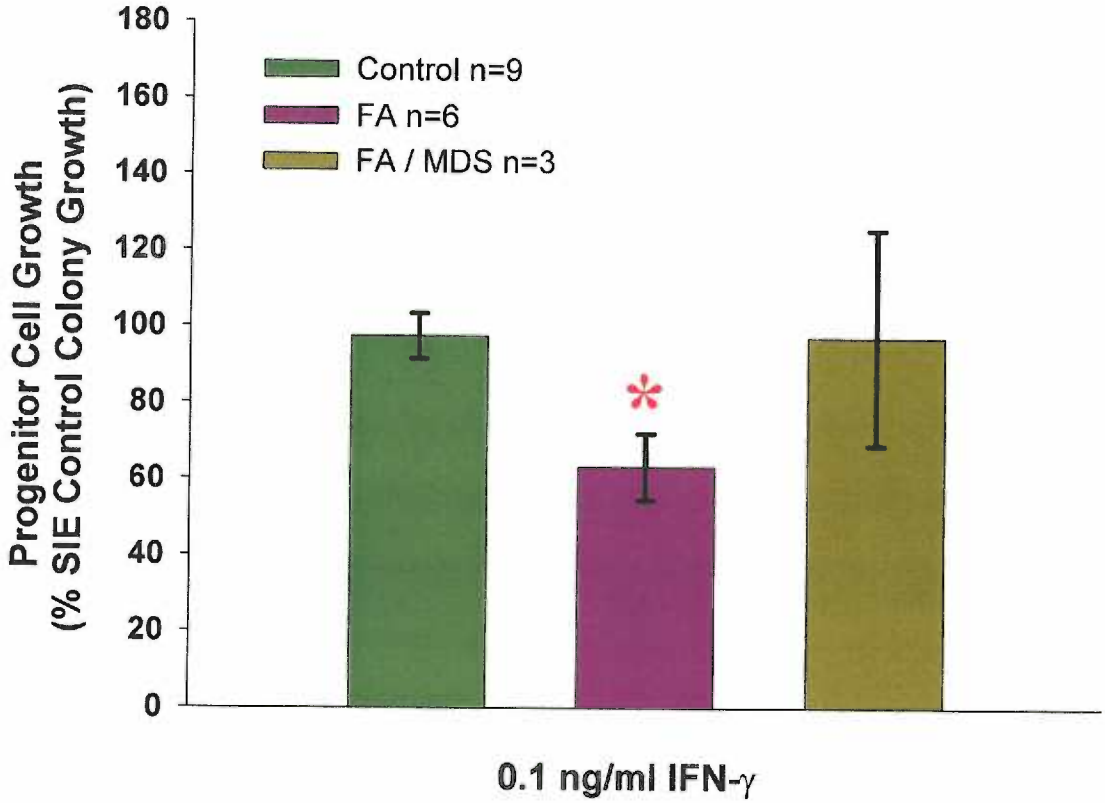

Progenitor Cell Growth
(% SIE Control Colony Growth)
180
160
140
120
100
80
60
40
20
0
Control n=9
FA n=6
FA / MDS n=3
*
0.1 ng/ml IFN-γ

Figure 2-1 Legend: IFNγ inhibition of CFU's associates with disease stage. Y-axis = myeloid progenitor cell growth (% control). To determine if myeloid colony inhibition correlated with disease stage, methylcellulose colony forming unit assays (CFU-A) were performed with bone marrow CD34+ cells in S.I.E. (hSCF (50 ng/ml), IL-3 (10 ng/ml), and erythropoietin (2 U/ml)) and 0.1 ng/ml of IFNγ. As shown here, compared to normal controls (green bar, n = 9), CFU's from patients in the progressive bone marrow failure (BMF) stage of FA (purple bar, n = 6) were significantly more sensitive (hypersensitive) to the inhibitory effects of IFNγ (* p = 0.005). CD34+ bone marrow cells from FA patients progressing to MDS/AML (olive bar, n = 3) did not demonstrate a statistically significant difference from normal controls (p = 0.989). Both of these observations agree with the theory that initial IFNγ hypersensitivity in the FA bone marrow[133-136,165] is a robust force of selective pressure to which a patient evolving to leukemia must become resistant as an adaptive mutation.[137] Error bars = standard error of the mean (SEM). Colony forming assays were performed in our lab by Keaney Rathbun.

Figure 2-2

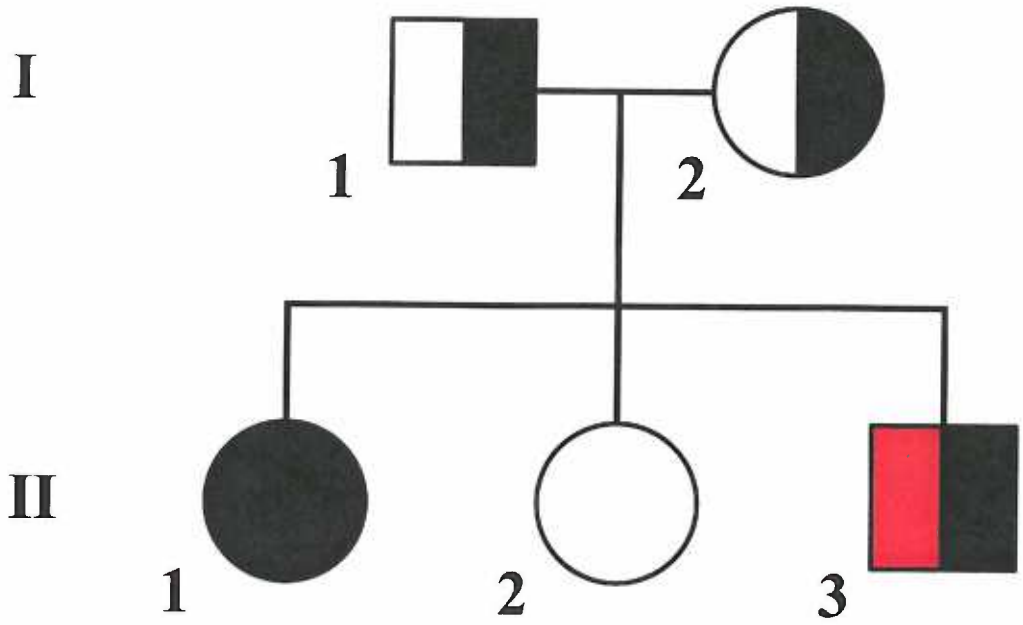


Figure 2-2 Legend: Multiplex FA-A pedigree of FA and MDS. To determine whether or not IFN-resistance correlated with clinical progression of MDS in FA within a single kindred, a multiplex pedigree of FA and FA evolving to MDS was ascertained. Individual II-3 is a juvenile male that came to medical attention due to the effects of anemia. He was diagnosed with FA based upon a positive MMC sensitivity test (100% of metaphases indicated radial forms, data not shown). He also has a history of short stature. Bone marrow biopsy revealed myeloid arrest, left-shift erythroid maturation, oval macrocytes, abundant mast cells,[169] multilineage dysplasia, and marrow blasts < 5% (data not shown). Cytogenetic analysis revealed functional chromosome 1q trisomy via translocation to the long arm of chromosome 18 [t(1;18)(q21;q23)]. These observations were consistent with a diagnosis of myelodysplastic syndrome (refractory anemia or RA) progressing from FA. Individual II-1 was ascertained subsequent to her sibling and was diagnosed on the basis of a positive MMC sensitivity test (100% of metaphases indicated radial forms, data not shown). She had a hypocellular marrow on biopsy, and short stature. She had no dysplastic changes in marrow and a normal 46, XX karyotype (data not shown). Individual II-2 is asymptomatic and has negative MMC sensitivity. The pedigree has been assigned to complementation group A (FA-A) via cell fusion studies (Markus Grompe,OHSU).

 = FA carrier (obligate)

 = Affected with FA

 = Affected with FA and MDS

Figure 2-3

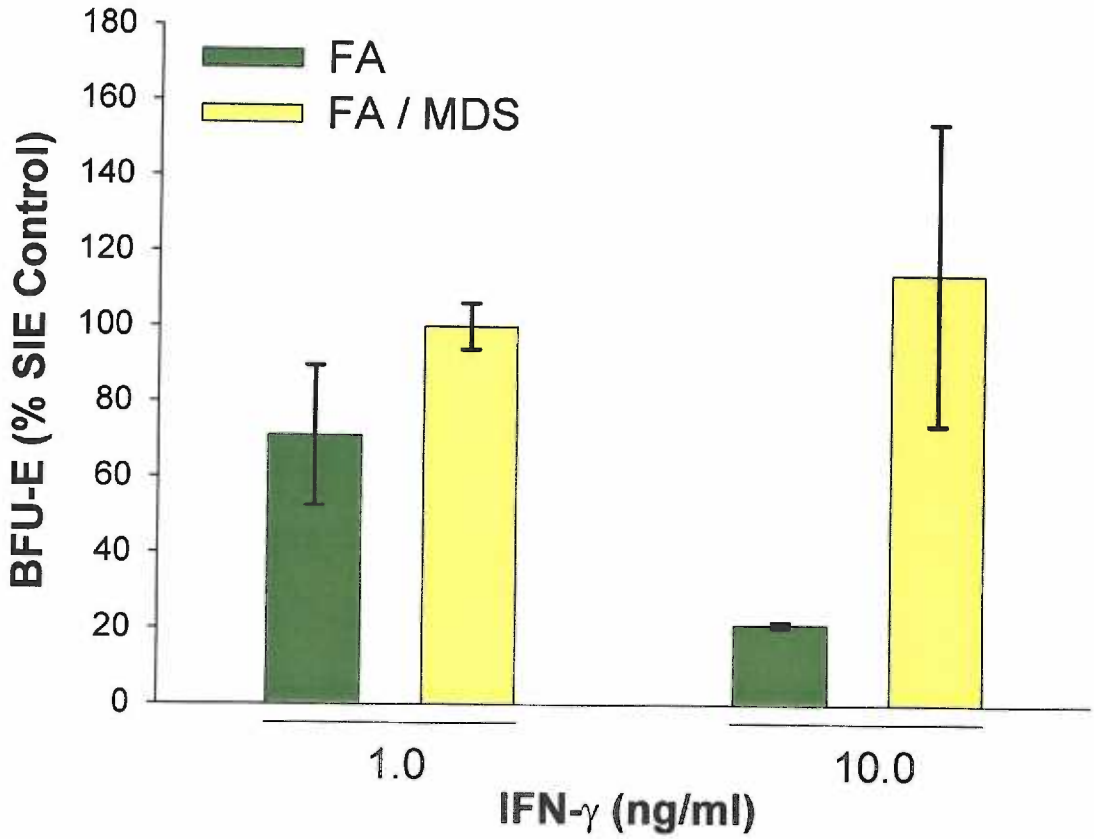

180
160
140
120
100
80
60
40
20
0
FA
FA / MDS
BFU-E (% SIE Control)
1.0
10.0
IFN-γ (ng/ml)

Figure 2-3 Legend: IFNγ sensitivity within a multiplex pedigree correlates with disease stage. The Y-axis shows burst-forming unit erythroid (BFU-E) percent control. Bone marrow CD34+ cells were obtained from individuals II-1 and II-3 in the multiplex FA/MDS pedigree and plated in methylcellulose colony forming unit assays in SIE and two doses of IFNγ (X-axis = 1.0 and 10.0 ng/ml). While the sibling with FA alone (II-1, green bars) demonstrates a dose-dependent, hypersensitive decrease in BFU-E forming potential with increasing IFNγ concentration, her sibling with FA/MDS (II-3, yellow bars) remains IFN-resistant at both doses. This agrees with predictions made in the adaptive mutation model.[137] Error bars are standard deviation (SD). CFU-A were performed by Keaney Rathbun in our lab.

Table 2-1: Quality control for Affymetrix analysis

Quality Parameter	FA1	FA2	MDS1	MDS2
Average Intensity	134.3	144.6	124.6	113
Average Background	42.3	44.18	45.85	46.51
% Negative Average Difference	19.26%	19.56%	21.58%	21.30%
% Presence Calls	53.80%	53.40%	48.10%	48.60%
Q	1.84	1.88	2.17	1.98
SF*	2.26	2.09	2.50	2.80
Actin Intensity (All)	4875.63	5162.55	5034.16	4548.41
Actin 3′/5′ Ratio	1.04	1.08	1.02	0.9
GAPDH Intensity (All)	2733.73	3002.63	2144.01	2006.18
GAPDH 3′/5′ Ratio	1.55	1.59	1.47	1.43

* = the target intensity was set to 200

Figure 2-4A

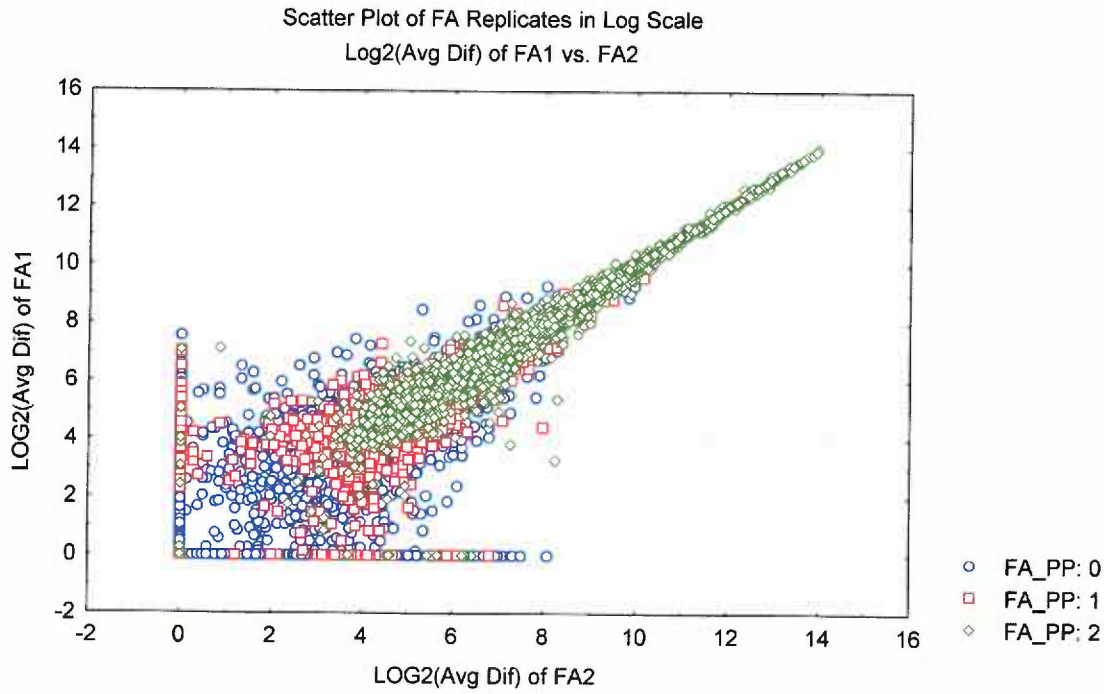


Figure 2-4B

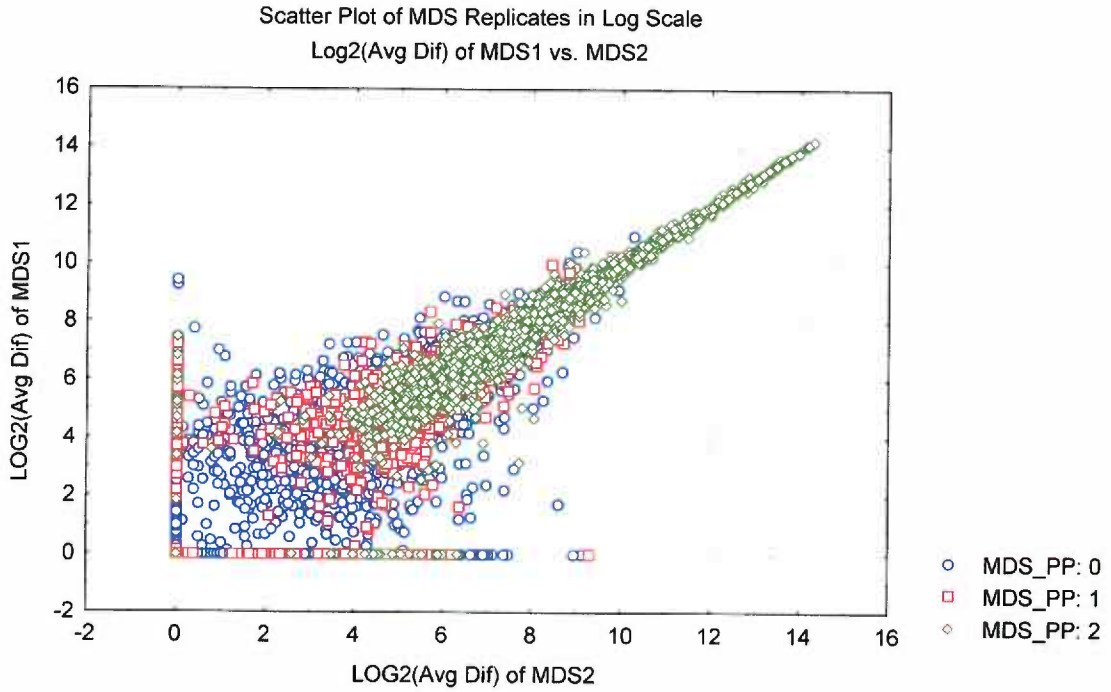


Figure 2-4A and 2-4B Legend: Gene-expression microarray replicates demonstrate a high degree of correlation. X and Y-axes represent independent hybridizations with identical RNA. To define candidate genes that influence the survival of FA bone marrow progenitor cells, expression microarray analysis was performed on total RNA obtained from low-density bone marrow cells using the Affymetrix method and microarray HU-95A GeneChip (12,625 genes)(Affymetrix, Santa Clara, CA). Two intraspecimen cRNA hybridizations were performed per individual and are referred to as replicates. Signal intensities were required to agree between replicates in order for a given data point to be included in the final intersibling comparison. Two-dimensional, $\log_2$ scatter plots were constructed for each replicate and are illustrated here. Figure 2-4A is the sibling with FA alone while figure 2-4B is the sibling with FA/MDS. In each figure, green symbols represent RNA present in each replicate, red symbols are those present in only one replicate, and blue symbols are those falling under any other heading (such as absent in both or discordantly or marginally present). Pearson's correlation coefficients (r) in each replicate indicate a high degree of hybridization reproducibility with r = 0.945 (99.14% unchanged on the absolute scale) in the sibling with FA alone and r = 0.928 (98.72% unchanged on the absolute scale) in the sibling with FA/MDS. Microarray hybridizations and $\log_2$ plots were obtained from the OHSU Microarray Shared Resource.

◊ = RNA present in each replicate □ = RNA present in only one replicate

O = any other situation (such as absent in both or discordant between replicates)

Figure 2-5

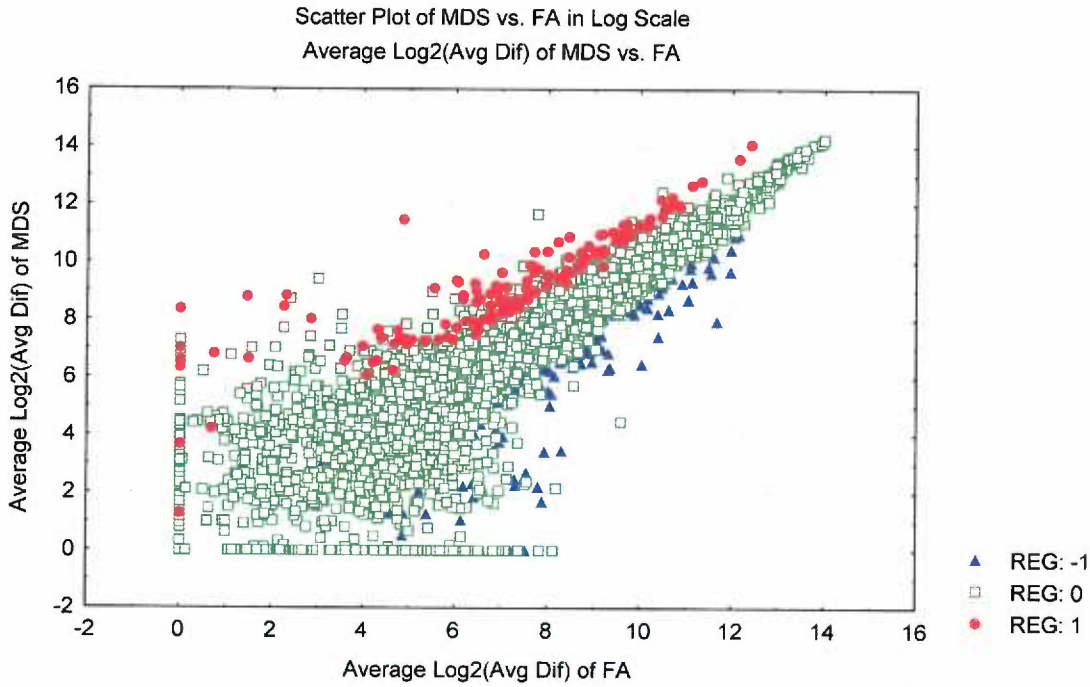

Scatter Plot of MDS vs. FA in Log Scale
Average Log2(Avg Dif) of MDS vs. FA
Average Log2(Avg Dif) of MDS
Average Log2(Avg Dif) of FA
16
14
12
10
8
6
4
2
0
-2
-2
0
2
4
6
8
10
12
14
16
REG: -1
REG: 0
REG: 1

Figure 2-5 Legend: Data recoding suggests a list of 327 differentially expressed genes. The sibling with FA/MDS is plotted on the Y-axis while the sibling with FA alone in plotted on the X-axis. The data plotted in the average difference in replicates. The complete list of 12,625 genes on the HU-95A chip was recoded to exclude data points that were irreproducible in replicates (70 genes excluded), or were completely absent (4,649 genes). This resulted in a list of 7,906 genes that are shown here in a two-dimensional $\log_2$ scatter plot. Green symbols are those genes (7,579) that did not demonstrate greater than a 2-fold change between siblings. Symbols in red (131 genes) represent genes more highly expressed in the sibling with FA/MDS while symbols in blue (196 genes) are those suppressed in this same individual.

□ = genes with less than a 2-fold expression difference between siblings

● = genes more highly expressed in the sibling with FA/MDS

▲ = genes with reduced expression in the sibling with FA/MDS

Table 2-2 (page 1 of 8): Spreadsheet of 327 differentially expressed genes

	A	B	C	D	E	F	G	H	I	J	K	L
1			AD	AD	AD	AD	R1	R1	R2	R2		
2	chr	Entrez	JP1	JP2	RP1	RP2	J1	J2	J1	J2	ave	Description
3	17	M57506	242	230	-90	11	D	D	D	D	-20	M57506: secreted protein (I-309) gene
4	14	Y14737	3241	3263	220	281	D	D	D	D	-18	Y14737: mRNA for immunoglobulin lambda heavy chain
5	10	D13900	14	153	-312	-57	MD	D	D	D	-17	D13900: mitochondrial short-chain enoyl-CoA hydratase
6	19	X74039	253	238	8	15	D	D	D	D	-16	X74039: urokinase plasminogen activator receptor
7	15	M55150	109	126	-229	-199	D	D	D	D	-16	M55150: fumarylacetoacetate hydrolase mRNA
8	X	AD001530	110	115	-127	-96	D	D	D	MD	-16	AD001530: XAP-5 mRNA
9	?	AC004770	41	114	-277	-19	D	D	D	D	-15	AC004770: chrom 11, BAC CIT-HSP-311e8 cont hFEN1
10	19	AA976838	146	152	-70	-71	D	D	D	D	-15	AA976838 oq35c12.s1 cDNA
11	17	M89957	136	133	-70	-57	D	D	D	D	-15	M89957 Ig suptam membBcell recepcmplx surf glycoprot(IGB)
12	9	U91512	156	218	-71	-22	D	D	D	D	-14	U91512: adhesion molecule ninjurin mRNA
13	19	U19599	300	330	-5	120	D	D	D	D	-13	U19599 HSU19599 (BAX delta) mRNA
14	17	AF038961	110	81	-63	-60	D	D	D	D	-11	AF038961: SL15 protein mRNA
15	19	U05259	72	69	-70	-56	D	D	D	MD	-11	U05259: MB-1 gene
16	19	D38047	136	162	-27	-13	D	D	D	D	-11	D38047: mRNA for 26S proteasome subunit p31
17	19	M15059	1064	1044	95	83	D	D	D	D	-11	M15059: Fc-epsilon receptor (IgE receptor)(H107 epitope)
18	17	AF082657	82	98	-31	-61	D	D	D	NC	-10	AF082657: Era GTPase A protein (HERA-A) mRNA
19	?	U95626	138	182	23	-5	D	D	D	D	-10	U95626: ccr2b,ccr2a,ccr5andccr6genes& lactoferrin
20	17	W28508	136	94	17	7	D	D	D	D	-9	W28508 48f12 cDNA
21	9	U05340	235	211	1	21	D	D	D	D	-9	U05340: p55CDC mRNA
22	11	X07203	289	241	37	29	D	D	D	D	-9	X07203: mRNA for CD20 receptor (S7)
23	X	AA149307	45	31	-86	-75	D	MD	D	D	-9	AA149307:zl25h05.s1 cDNA
24	14	X55989	99	91	-19	-21	D	D	D	D	-8	X55989: ECRP gene for eosinophil cationic related protein
25	19	U05259	656	615	97	64	D	D	D	D	-8	U05259: MB-1 gene
26	8	J04621	138	93	11	11	D	D	D	D	-8	J04621: heparan sulfate proteoglycan (HSPG) core protein
27	7	AB028974	97	78	-9	-11	D	D	D	D	-7	AB028974: mRNA for KIAA1051 protein
28	19	X00734	423	417	45	180	D	D	D	D	-7	X00734 beta-tubulin gene (5-beta) w/ten Alu fam memb
29	7	AJ001902	147	237	-17	43	D	D	D	D	-7	AJ001902: TRIP6 (thyroid receptor interacting protein)
30	14	X58529	1341	1344	162	184	D	D	D	D	-7	X58529:Ig mRNA for mu heavy chain enhan & const reg
31	?	M64936	61	48	-49	-17	D	D	NC	D	-7	M64936 HUMRIRT retinoic acid-induc endogs retrovr
32	19	M15059	261	281	55	34	D	D	D	D	-7	M15059: Fc-epsilon receptor (IgE receptor) (H107 epitope)
33	19	M74093	129	134	13	19	D	D	D	D	-6	M74093: cyclin mRNA
34	1	X63097	114	124	14	24	D	D	D	D	-6	X63097: rhesus polypeptide (RhXIII)
35	19	X71129	157	336	59	46	D	D	D	D	-6	X71129: electron transfer flavoprotein beta subunit
36		U80114	-5	77	72	-4	D	D	D	D	-6	U80114: immunoglobulin heavy chain variable region (V4-31)
37	20	AF011468	135	115	5	37	D	D	D	NC	-6	AF011468: serine/threonine kinase (BTAK) mRNA
38	7	AC005162	77	65	4	-3	D	D	D	D	-6	AC005162: BAC clone RG113D17 from 7p14-p15
39	11	AF007893	63	46	-36	-4	D	D	D	NC	-6	AF007893: P2Y6 receptor pseudogene
40	11	W26480	58	82	-21	-4	NC	D	D	D	-6	W26480:30b8 cDNA
41		AF039029	30	10	-41	-32	D	NC	D	D	-6	AF039029: snurportin1 mRNA
42	22	AJ006973	199	124	31	-21	D	D	D	D	-6	AJ006973: mRNA for TOM1 protein
43	16	L07765	114	97	55	53	D	D	D	D	-6	L07765: carboxylesterase mRNA
44	4	U81234	53	64	-13	-7	D	D	D	D	-6	U81234: chemokine alpha 3 (CKA-3) mRNA
45	?	HG4322-HT	623	644	115	115	D	D	D	D	-6	Tubulin, Beta
46	15	AL080146	274	242	30	72	D	D	D	D	-5	AL080146: mRNA, cDNA DKFZp434B174
47	2	U29332	2	9	-17	-11	D	D	D	D	-5	U29332: heart protein (FHL-2) mRNA

Table 2-2 (page 2 of 8): Spreadsheet of 327 differentially expressed genes

	A	B	C	D	E	F	G	H	I	J	K	L
48			AD	AD	AD	AD	R1	R1	R2	R2		
49	chr	Entrez	JP1	JP2	RP1	RP2	J1	J2	J1	J2	ave	Description
50	X	Y10043	60	44	-16	-3	D	D	D	D	-5	Y10043: mRNA for high mobility group protein HMG2a
51	19	L01664	538	453	85	105	D	D	D	D	-5	L01664 eosinoCharcot-Lydncryst(CLC)(lysophospholipase)
52	13	AF097021	1326	1330	347	261	D	D	D	D	-5	AF097021: GW112 protein (GW112) mRNA
53	?	AL021366	44	61	-17	0	D	D	D	D	-5	AL021366 dICK0721Q 3 (Kinesin related protein)
54	2	N95229	83	88	-13	13	D	D	D	D	-5	N95229 zb53g09.s1 cDNA, 3 end
55	16	D43767	2122	2105	419	429	D	D	D	D	-5	D43767 HUMAR mRNA for chemokine
56	2	X16302	92	81	25	10	D	NC	D	D	-5	X16302: insulin-like growth factor binding protein (IGFBP-2)
57	13	U11700	30	35	-4	0	D	D	D	MD	-5	U11700: copper transporting ATPase mRNA
58	14	X67301	4098	3867	813	838	D	D	D	D	-5	X67301: IgM heavy chain constant region (Ab63)
59	6	X79535	590	566	139	114	D	D	D	D	-5	X79535: beta tubulin, clone nuk
60	11	Z49194	227	205	49	44	D	D	D	D	-5	Z49194: oct-binding factor
61	10	AF068180	80	66	22	-2	NC	D	D	D	-5	AF068180: B cell linker protein BLNK alternatively spliced
62	6	M16276	382	360	66	106	D	D	D	D	-5	M16276: MHC class II HLA-DR2-Dw12 mRNA DQw1-beta
63	14	X82240	459	511	121	116	D	D	D	D	-5	X82240: Tcell leukemia/lymphoma 1
64	16	M30038	65	108	-17	25	D	MD	D	D	-5	M30038: tryptase mRNA
65	22	M27749	-3	46	-30	-27	D	D	D	D	-4	M27749: immunoglobulin-related 14.1 protein mRNA
66	20	D80008	123	73	37	19	D	D	D	D	-4	D80008: mRNA for KIAA0186 gene
67		D64142	291	220	128	145	D	NC	D	D	-4	D64142 D64142 mRNA for histone H1x
68	1	M13452	61	57	22	23	D	D	D	D	-4	M13452: lamin A 3end
69	?	U93305	1597	1552	328	339	D	D	D	D	-4	U93305:A4 diff-dep prot(A4),(LMO6),(SYP)(CACNA1F)
70	?	AL008726	237	274	123	49	D	D	D	D	-4	AL008726: LysosomProtecProtPrecur(CathepA,CarboxypepC)
71	21	U89606	298	266	65	68	D	D	D	D	-4	U89606: pyridoxal kinase mRNA
72	15	X67155	32	26	2	-15	D	D	D	D	-4	X67155: mitotic kinesin-like protein-1
73	?	Z82244	86	98	23	48	D	D	D	NC	-4	Z82244: (Heme Oxygenase 1 (HO-1, EC 1.14.99.3))
74	7	U61145	152	162	25	87	D	D	D	D	-4	U61145: enhancer of zeste homolog 2 (EZH2) mRNA
75	6	W26667	57	42	6	8	D	D	D	NC	-4	W26667:11a1 cDNA
76	?	HG4322-HT	467	477	127	106	D	D	D	D	-4	Tubulin, Beta
77	14	X67301	3002	2971	780	808	D	D	D	D	-4	X67301: IgM heavy chain const region (Ab63)
78	X	Y07867	54	47	5	4	D	D	D	D	-4	Y07867: Pirin, isolate 1
79	5	D86957	33	43	18	-21	D	D	D	D	-4	D86957: mRNA for KIAA0202 gene
80	10	M91669	215	202	61	45	D	D	D	D	-4	M91669: Bullous pemphigoid autoantigen BP180 gene, 3 end
81	19	D50922	159	144	30	56	D	D	D	D	-4	D50922: mRNA for KIAA0132 gene
82	17	U77949	30	58	6	-3	D	D	D	D	-4	U77949 HSU77949 Cdc6-related protein (HsCDC6) mRNA
83	22	AB023147	61	31	23	19	D	D	NC	D	-4	AB023147: mRNA for KIAA0930 protein
84	10	D17793	535	550	152	143	D	D	D	D	-4	D17793: mRNA for KIAA0119 gene
85	?	HG4074-HT	627	670	26	263	D	D	D	D	-4	Rad2
86	7	L05515	33	34	-9	-8	D	D	D	D	-4	L05515 cAMP response element-binding protein (CRE-BP1)
87	9	AB023137	247	279	70	81	D	D	D	D	-4	AB023137: mRNA for KIAA0920 protein
88	9	X02544	1141	1137	397	305	D	D	D	D	-4	X02544: mRNA for alpha1-acid glycoprotein (orosomucoid)
89	22	X92997	594	465	216	148	D	D	D	D	-4	X92997: IgG lambda light chn V-J-C reg (clone Tgl4)
90	4	D30783	61	41	6	4	D	D	D	NC	-4	D30783: mRNA for epiregulin
91	?	U05861	449	458	129	135	D	D	D	D	-4	U05861: hepatic dihydrodiol dehydrogenase gene
92		X02344	975	900	301	287	D	D	D	D	-4	X02344: beta 2 gene
93	6	U52682	295	319	75	116	D	D	D	D	-4	U52682: lymphocyte spec IFN reg fac (LSIRF/IRF4)
94	5	D83597	42	47	19	-1	D	D	D	D	-3	D83597: mRNA for RP105

Table 2-2 (page 3 of 8): Spreadsheet of 327 differentially expressed genes

	A	B	C	D	E	F	G	H	I	J	K	L
95			AD	AD	AD	AD	R1	R1	R2	R2		
96	chr	Entrez	JP1	JP2	RP1	RP2	J1	J2	J1	J2	ave	Description
97	9	U80811	50	34	8	15	D	D	D	D	-3	U80811: lysophosphatidic acid receptor homolog
98	17	X68487	45	50	13	7	MD	D	D	D	-3	X68487: A2b adenosine receptor
99	2	X72475	156	136	57	60	D	MD	D	D	-3	X72475: rearr ig kappa light chn vari reg (l.114)
100	3	M24902	96	89	25	32	D	D	D	D	-3	M24902 HUMPAPA prostatic acid phosphatase
101	6	U81375	255	241	122	69	D	D	D	D	-3	U81375: placent equilibr nucleosid transp1(hENT1)
102	6	D14678	138	150	50	38	D	D	D	D	-3	D14678 HUMMHCB mRNA for kinesin-related protein
103	11	X62048	185	193	76	32	D	D	D	D	-3	X62048 H.sapiens Wee1 hu gene
104	1	AA733050	119	125	33	38	D	D	D	D	-3	AA733050:zg79b05.s1 cDNA, 3 end
105	3	AB006537	312	332	96	103	D	D	D	D	-3	AB006537: interleukin 1 receptor accessory protein
106	14	AF027150	17	29	-14	-3	D	D	D	D	-3	AF027150:survival motor neur prot interact prot 1(SIP1)
107	18	Y00630	2256	2237	696	603	D	D	D	D	-3	Y00630: Arg-Serpin(plasminogen activ-inhib 2, PAI-2)
108	3	AL045811	169	176	58	43	D	D	D	NC	-3	AL045811:DKFZp434H166_r1 cDNA, 5 end
109	7	M55067	1176	1116	404	462	D	D	D	D	-3	M55067: 47-kD autosomal chron granulomatous disease
110	7	AF001601	76	85	33	20	D	D	D	D	-3	AF001601: paraoxonase (PON2) mRNA
111	6	U03106	1359	1359	470	522	D	D	D	D	-3	U03106 HSU03106 wt p53 activ frag-1 (WAF1)
112	12	U55766	17	30	4	2	D	NC	D	D	-3	U55766: Rev interacting protein Rip-1 mRNA
113	?	AC002073	461	369	109	137	D	D	D	D	-3	AC002073: PAC clone DJ515N1 from 22q11.2-q22
114	9	K03000	109	107	30	41	D	NC	D	D	-3	K03000: aldehyde dehydrogenase 1 mRNA
115	2	X76220	2852	2881	895	945	D	D	D	D	-3	X76220 H.sapiens MAL gene exon 1 (and joined CDS)
116	5	X15998	255	241	84	77	D	D	D	D	-3	X15998: chondroitin sulph proteogly versican, V1 spl-var
117	1	AB007928	56	42	15	21	D	D	D	D	-3	AB007928: mRNA for KIAA0459 protein
118	5	U53446	139	112	39	53	D	D	MD	D	-3	U53446 HSU53446 mitogen-respon phosphoprot DOC-2
119	17	AF029669	49	46	2	22	D	D	D	D	-3	AF029669: Rad51C (RAD51C) mRNA
120	1	AJ006288	65	61	24	10	D	D	D	D	-3	AJ006288: mRNA for bcl-10 protein
121	9	W28944	160	147	42	74	D	D	D	D	-3	W28944:54h12 cDNA
122	10	J04076	518	491	167	161	D	D	D	D	-3	J04076: early growth response 2 protein (EGR2) mRNA
123	11	AB014540	252	181	75	74	D	D	D	D	-3	AB014540: mRNA for KIAA0640 protein
124	11	M27394	178	184	52	46	D	D	D	D	-3	M27394 B-lymphocyte cell-surface antigen B1 (CD20)
125	1	M29877	1097	953	403	348	D	D	D	D	-3	M29877: alpha-L-fucosidase
126	16	AB007879	94	94	35	47	D	D	D	NC	-3	AB007879: KIAA0419 mRNA
127	15	X70297	67	44	33	35	D	D	D	NC	-3	X70297: neuron nicotinic acetylchol recep a-7 su
128	2	AJ001381	30	30	-13	5	D	D	D	D	-3	AJ001381:mutated allele of a myosin class I, myh-1c
129	9	X71345	21	22	15	-8	D	D	D	D	-3	X71345: trypsinogen IV b-form
130	12	Y07909	147	134	75	41	D	D	D	D	-3	Y07909: Progression Associated Protein
131	2	U43959	171	146	53	57	D	D	D	D	-3	U43959: beta 4 adducin alternatively spliced
132	6	Y18046	71	111	28	25	D	D	NC	D	-3	Y18046: mRNA for FOP (FGFR1 oncogene partner)
133	5	M25753	340	364	101	111	D	D	D	D	-3	M25753: cyclin B 3 end
134	2	M63438	3958	4057	1440	1348	D	D	D	D	-3	M63438: Ig rearranged gamma chain V-J-C region
135	21	AJ006291	122	90	47	36	D	D	D	D	-3	AJ006291: mRNA for leucine rich protein
136	11	L23808	140	135	52	46	D	D	D	D	-3	L23808 HUMHME metalloproteinase (HME)
137	19	M24283	377	335	127	140	D	D	D	D	-3	M24283: major group rhinovirus receptor (HRV)
138	6	Z11697	915	831	340	354	D	D	D	D	-3	Z11697: mRNA for HB15
139	6	M11717	1335	1513	573	573	D	D	D	D	-3	M11717 HUMHSP70D heat shock protein (hsp 70)
140	10	X76488	2984	3218	1297	1074	D	D	D	D	-3	X76488: lysosomal acid lipase
141	7	AI432401	874	836	321	310	D	D	D	D	-3	AI432401:tg73b09.x1 cDNA

Table 2-2 (page 4 of 8): Spreadsheet of 327 differentially expressed genes

	A	B	C	D	E	F	G	H	I	J	K	L
142			AD	AD	AD	AD	R1	R1	R2	R2		
143	chr	Entrez	JP1	JP2	RP1	RP2	J1	J2	J1	J2	ave	Description
144	10	X02419	553	575	198	177	D	D	D	D	-3	X02419:H.sapiens uPA gene
145	11	AF001294	247	253	92	75	D	D	D	D	-3	AF001294: IPL (IPL) mRNA
146	17	X79865	181	129	57	98	D	D	D	D	-3	X79865:H.sapiens Mrp17 mRNA
147	22	AI932613	1930	1940	578	669	D	D	D	D	-3	AI932613:wo05c02.x1 cDNA
148	7	U41518	178	167	74	58	D	D	D	D	-3	U41518: channel-like integral membrane protein (AQP-1)
149	X	AF000993	85	64	25	32	D	D	D	D	-3	AF000993: ubiquitous TPR motif, X isoform (UTX)
150	5	AF032862	225	256	75	86	NC	D	D	D	-3	AF032862: intracell hyaluronic acid bnd prot (IHABP)
151	11	U24152	102	81	38	32	D	NC	D	D	-3	U24152 HSU24152 p21-activated protein kinase (Pak1)
152	?	U95626	158	132	72	57	D	D	D	D	-3	U95626:ccr2b,ccr2a,ccr5andccr6genes & lactoferri
153	3	D87448	169	167	74	49	D	D	D	D	-3	D87448: mRNA for KIAA0259 gene
154	11	L23808	245	246	82	77	D	D	D	D	-3	L23808 HUMHME metalloproteinase (HME) mRNA
155	15	W28743	311	280	116	116	D	D	D	D	-3	W28743:51a9 cDNA
156	20	AF011468	133	97	63	49	D	D	D	D	-3	AF011468: serine/threonine kinase (BTAK) mRNA
157	?	U78027	427	418	264	178	D	D	D	D	-3	U78027: (BTK)(GLA),L44-like ribosprot(L44L)(FTP3)
158		U95006	423	390	94	140	D	D	D	D	-3	U95006: D9 splice variant A mRNA
159	1	W27050	142	153	59	60	D	D	D	D	-3	W27050:19f7 cDNA
160	?	M76665	466	461	182	183	D	D	D	D	-3	M76665: 11-beta-hydroxysteroid dehydrogenase (HSD11)
161	5	X15998	135	134	48	62	D	D	D	MD	-3	X15998: chondroitin sulph proteoglyc versican, V1 spl-var
162	17	X17620	502	497	195	211	D	D	D	D	-3	X17620 mRNA HSNM23 mRNA for Nm23 protein
163	22	M18645	4328	4171	1834	1978	D	D	D	D	-3	M18645:Ig rearr lamb-chn VJC-reg subgr lmbda-IV
164	X	D11139	2168	2198	847	944	D	D	D	D	-2	D11139 exons#1-4 HUMTIMP tissue inhib metalloprot
165	21	AI984234	228	216	89	108	D	D	D	D	-2	AI984234:wz57e04.x1 cDNA
166		U80114	207	196	91	75	D	D	D	D	-2	U80114: immunoglob hvy chn vari reg (V4-31)
167	2	AB000450	152	143	55	69	D	D	D	NC	-2	AB000450: mRNA for VRK2
168	6	U73304	16	19	-1	-8	D	D	D	D	-2	U73304: CB1 cannabinoid receptor (CNR1) gene
169	7	Z36531	516	543	224	211	D	D	D	D	-2	Z36531: fibrinogen-like protein (pT49 protein)
170	?	AL021977	493	443	189	193	D	D	D	D	-2	AL021977 (v-maf fibrsarc(avian)onco fam, prot F)LIKE prot
171	8	AF091433	99	84	35	42	D	D	D	D	-2	AF091433: cyclin E2 mRNA
172	14	AI147237	896	942	347	423	D	D	D	MD	-2	AI147237:qb36f02.x1 cDNA
173	6	M59830	678	704	283	303	D	D	D	D	-2	M59830: MHC class III HSP70-2 gene (HLA)
174	16	X52425	1609	1696	704	714	D	D	D	D	-2	X52425 HSIL4R IL-4-R mRNA for the interleukin 4 receptor
175	20	U73379	572	569	174	182	D	D	D	D	-2	U73379 HSU73379 cyclin-selective ubiquitin carrier protein
176	22	X57809	4325	4665	1993	2027	D	D	D	D	-2	X57809: rearranged immunoglobulin lambda light chain
177	4	AL080110	35	25	4	11	D	D	D	NC	-2	AL080110: mRNA; cDNA DKFZp586G1922
178	5	M25753	255	266	140	103	D	D	D	D	-2	M25753 HUMCYCB cyclin B 3 end
179	8	U48807	78	67	25	32	D	NC	D	D	-2	U48807 HSU48807 MAP kinase phosphatase (MKP-2)
180	?	M21535	81	72	39	29	D	NC	D	D	-2	M21535 HUMERG11 erg protein (ets-related gene)
181	22	X77094	166	188	72	78	D	D	D	NC	-2	X77094: p40phox
182	14	X55990	1334	1323	588	615	D	D	D	D	-2	X55990: ECP gene for eosinophil cationic protein
183	17	L19183	95	72	29	56	D	D	D	D	-2	L19183: MAC30 mRNA
184	22	Y07846	97	91	44	10	D	NC	D	D	-2	Y07846: GAR22 protein
185	1	M28825	380	387	144	133	D	D	D	D	-2	M28825: thymocyte antigen CD1a mRNA
186	1	M28826	592	582	256	282	D	D	D	D	-2	M28826: thymocyte antigen CD1b
187	2	U03688	2249	2161	992	1023	D	D	D	D	-2	U03688 dioxin-inducible cytochrome P450 (CYP1B1)
188	3	D10925	1542	1385	618	650	D	D	D	D	-2	D10925: mRNA for HM145

Table 2-2 (page 5 of 8): Spreadsheet of 327 differentially expressed genes

	A	B	C	D	E	F	G	H	I	J	K	L
189			AD	AD	AD	AD	R1	R1	R2	R2		
190	chr	Entrez	JP1	JP2	RP1	RP2	J1	J2	J1	J2	ave	Description
191	6	AL031781	238	212	112	126	D	D	D	D	-2	AL031781 ortholog mouse KH Dom RNA Bind prot QKI-7
192	9	X54942	511	513	230	233	D	D	D	D	-2	X54942:H.sapiens ckshs2 Cks1 protein homol
193	10	U37426	277	253	113	131	D	D	D	D	-2	U37426: kinesin-like spindle protein HKSP (HKSP)
194	14	D13633	221	190	111	98	D	D	D	D	-2	D13633: mRNA for KIAA0008 gene
195	22	AF042083	21	29	3	8	D	D	NC	D	-2	AF042083: BH3 interacting domain death agonist (BID)
196		U41766	238	234	97	87	D	D	D	D	-2	U41766: metalloprot/disintegrin/cys-rich prot precur(MDC9)
197	6	X94323	435	437	202	197	D	D	D	D	-2	X94323: SGP28 protein
198	7	U96876	466	558	227	220	D	D	D	D	-2	U96876: insulin induced protein 1 (INSIG1) gene
199	1	AB007946	25	24	5	1	NC	D	D	D	-2	AB007946: mRNA for KIAA0477 protein
200	5	AB007893	174	162	80	72	D	D	D	D	-2	AB007893: KIAA0433
201	6	U15085	1231	1275	626	607	D	D	D	D	-2	U15085: HLA-DMB
202	1	M28696	559	535	254	262	D	D	D	D	-2	M28696: low-affinity IgG Fc receptor (beta-Fc-gamma-RII)
203	6	X64594	390	390	146	150	D	D	D	D	-2	X64594: 50 kDa erythrocyte plasma membrane glycoprot
204	7	AF052728	316	305	166	158	D	D	D	D	-2	AF052728: HERG-USO (HERG) alternatively spliced
205	?	AC004770	711	666	305	332	D	D	D	D	-2	AC004770.chrom11, BAC CIT-HSP-311e8 cont hFEN1
206	12	U72515	157	137	71	80	D	D	D	D	-2	U72515: C3f
207	16	AI565760	1848	1861	3817	3866	I	I	I	I	2	AI565760:tn20b01.x1 cDNA
208	19	U39412	604	568	915	968	I	I	I	I	2	U39412: alpha SNAP
209	9	Z35491	341	334	610	649	I	I	I	I	2	Z35491:novel glucocorticoid receptor-associated protein
210	5	AL050141	1159	1106	2409	2389	I	I	I	I	2	AL050141: mRNA; cDNA DKFZp586O031
211	11	AB018293	306	333	694	676	I	I	I	I	2	AB018293: mRNA for KIAA0750 protein
212	17	X58851	141	147	311	311	I	I	I	NC	2	X58851:MLC1emb embryonic myosin alk light chain
213	X	U41315	1407	1434	3123	3051	I	I	I	I	2	U41315: ring zinc-finger prot (ZNF127-Xp) gene
214	4	AL049409	595	600	1299	1295	I	I	I	I	2	AL049409:cDNA DKFZp586H0919
215	5	X59871	955	817	1814	2104	I	I	I	I	2	X59871: TCF-1 mRNA for T cell factor 1 (splice form C)
216	8	X57352	1126	1172	2397	2688	I	I	I	I	2	X57352: 1-8U from interferon-inducible gene family
217	9	W80399	415	411	921	923	I	I	I	I	2	W80399:zh49e04.s1 cDNA
218	22	AL050254	1850	1843	4004	4106	I	I	I	I	2	AL050254:Novel gene mapping to chomosome 22
219	?	L31584	1413	1404	3024	3162	I	I	I	I	2	L31584 HUMEBI103 G protein-coupled receptor (EBI 1)
220	19	AL047596	216	165	380	466	I	I	I	I	2	AL047596.DKFZp586G0421_s1 cDNA
221	?	M27504	96	86	215	190	I	I	I	I	2	M27504 HUMTOPIIX topoisomerase type II (Topo II)
222	X	M64925	804	764	1767	1607	I	I	I	I	2	M64925palmitoylated erythrocyte membr prot (MPP1)
223	1	Y10055	153	166	483	355	I	I	I	I	2	Y10055 HSP110DEL phosphoinositide 3-kinase
224	3	AF052160	84	89	196	164	I	I	I	I	2	AF052160: clone 24629 mRNA sequence
225	19	L22005	353	362	779	833	I	I	I	I	2	L22005 HUMCDC34H ubiquitin conjugating enzyme
226	X	L19161	195	188	455	440	I	I	I	I	2	L19161: translation initiation factor eIF-2 gamma subunit
227	X	Z32684	407	423	941	965	I	I	I	I	2	Z32684:membrane transport protein (XK gene)
228		AF038186	381	449	1024	1020	I	I	I	I	2	AF038186: clone 23914 mRNA sequence
229	3	M11507	148	133	354	339	I	I	I	I	2	M11507 transferrin receptor
230	6	Z35102	164	171	325	362	I	I	I	I	2	Z35102 HSPROKINX Ndr protein kinase
231	10	S78798	112	106	256	252	I	I	I	I	2	S78798:1-phosphatidylinositol-4-phos 5-kin isof C
232	14	S87759	253	247	586	586	I	I	I	I	2	S87759 S87759 protein phosphatase 2C alpha
233	16	X74594	337	314	742	774	I	I	I	I	2	X74594 HSRB2P130 Rb2/p130 protein
234	16	X90999	582	588	1399	1418	I	I	I	I	2	X90999: Glyoxalase II
235	18	AL050225	205	218	516	497	I	I	I	I	2	AL050225: mRNA

Table 2-2 (page 6 of 8): Spreadsheet of 327 differentially expressed genes

	A	B	C	D	E	F	G	H	I	J	K	L
236			AD	AD	AD	AD	R1	R1	R2	R2		
237	chr	Entrez	JP1	JP2	RP1	RP2	J1	J2	J1	J2	ave	Description
238	?	U50648	740	739	1955	2033	I	I	I	I	2	U50648 IFN-inducible RNA-dependent prot kin (Pkr)
239	Y	Y15801	164	164	398	401	I	I	I	I	2	Y15801: mRNA for PRKY protein
240	8	U28389	1599	1568	3842	3917	I	I	I	I	2	U28389: dematin 52 kDa subunit
241	4	W04490	731	672	1653	1744	I	I	I	I	2	W04490:za57h12.r1 cDNA, 5 end
242	5	L05072	542	551	1374	1314	I	I	I	I	2	L05072 IRF-1
243	18	AA725102	705	978	2306	1776	I	I	I	I	2	AA725102:ai08h05.s1 cDNA
244	X	L19161	131	126	322	279	I	I	I	I	2	L19161 HUMIEF2G translation init factor eIF-2 gamma su
245	3	X13710	2561	2595	7075	7335	I	I	I	I	3	X13710: glutathione peroxidase
246	10	L07648	1434	1515	3592	3757	I	I	I	I	3	L07648: MXI1
247	10	L07648	807	960	2041	2100	I	I	I	I	3	L07648 HUMMXI1A MXI1
248	17	L22075	85	96	229	220	I	I	NC	I	3	L22075 HUMG13A guanine nucleotide reg prot (G13)
249	1	L76200	1137	1210	2885	3064	I	I	I	I	3	L76200 HUMGUK1R guanylate kinase (GUK1)
250	12	AJ001685	79	128	277	255	I	I	I	I	3	AJ001685: NKG2E gene
251	3	U20350	111	109	291	277	I	I	I	I	3	U20350: G protein-coupled receptor V28
252	7	U40462	534	514	1217	1307	I	I	I	I	3	U40462: Ikaros/LyF-1 homolog (hlk-1)
253	4	X02530	3	-9	22	16	I	I	NC	I	3	X02530 cdsHSINFGER IFNg induc early resp gene
254	17	L36719	505	530	1331	1361	I	I	I	I	3	L36719 HUMMKK3A MAP kinase kinase 3 (MKK3)
255	14	S87759	270	249	650	894	I	I	I	I	3	S87759 protein phosphatase 2C alpha [, teratocarcinoma
256	16	X17644	443	452	1192	1063	I	I	I	I	3	X17644: GST1-Hs mRNA for GTP-binding protein
257	12	M24069	1010	1002	2533	2438	I	I	I	I	3	M24069: DNA-binding protein A (dbpA) gene
258	1	D63789	99	95	241	284	I	I	I	I	3	D63789: DNA for SCM-1beta precursor
259	2	X53586	329	341	785	922	I	I	I	I	3	X53586: mRNA for integrin alpha 6
260	X	X60364	4548	4515	11944	12687	I	I	I	I	3	X60364: ALAS 5-aminolevulinate synth precur
261	4	L27071	140	156	400	410	I	I	I	I	3	L27071 HUMTYRKINA tyrosine kinase (TXK)
262	12	AJ001683	51	49	137	186	MI	I	I	I	3	AJ001683: NKG2F mRNA
263	3	AF042166	77	49	178	140	I	I	NC	I	3	AF042166: beta-filamin)
264	8	AF079221	2166	2305	6455	6550	I	I	I	I	3	AF079221: BCL2/adenovirus E1B 19kDa-interact prot 3a
265	6	W28616	43	74	178	163	I	MI	I	I	3	W28616:49b9 cDNA
266	12	D83018	756	817	2548	2554	I	I	I	I	3	D83018: mRNA for nel-related protein 2
267	1	U29091	1646	1662	4791	5046	I	I	I	I	3	U29091: selenium-binding protein (hSBP)
268	2	M28212	72	91	250	245	I	I	I	I	3	M28212 HUMRAB6A GTP-binding protein (RAB6)
269	11	AF013611	198	170	561	540	I	I	I	I	3	AF013611: lymphopain
270	2	M97935	900	777	2597	2664	I	I	I	I	3	M97935: transcription factor ISGF-3
271	20	Z23115	287	311	721	690	I	I	I	I	3	Z23115:H.sapiens bcl-xL mRNA
272	6	U90546	348	343	1045	1337	I	I	I	I	3	U90546: butyrophilin (BTF4)
273	2	M85276	700	649	2104	2141	I	I	I	I	3	M85276: NKG5 gene
274	19	L37033	473	366	1219	1412	I	I	MI	I	3	L37033: FK-506 binding protein homologue (FKBP38)
275	3	U37518	123	139	433	403	I	I	I	I	3	U37518 HSU37518 TNF-related apopt induc lig TRAIL
276	11	X82200	237	273	831	673	I	I	I	I	3	X82200:H.sapiens Staf50 mRNA
277	?	M91036	5386	5449	17221	17661	I	I	I	I	3	M91036:H.sapiens G-gamma globin and A-gamma globin
278	7	U40462	465	472	1529	1547	I	I	I	I	3	U40462 HSU40462 Ikaros/LyF-1 homolog (hlk-1)
279	18	AF013591	65	66	244	186	I	I	I	I	3	AF013591: homolog of the Aspergillus nidulans sudD
280	11	J04164	1400	1389	4486	4818	I	I	I	I	3	J04164 HUM927A interferon-inducible protein 9-27
281	2	M97935	607	553	2010	2036	I	I	I	I	3	M97935 transcription factor ISGF-3
282	7	X00351	96	122	366	363	NC	I	I	I	3	X00351 mRNA for beta-actin

Table 2-2 (page 7 of 8): Spreadsheet of 327 differentially expressed genes

	A	B	C	D	E	F	G	H	I	J	K	L
283			AD	AD	AD	AD	R1	R1	R2	R2		
284	chr	Entrez	JP1	JP2	RP1	RP2	J1	J2	J1	J2	ave	Description
285	17	Y16645	-10	-6	11	15	I	NC	I	I	3	Y16645: mRNA for monocyte chemotactic protein-2
286	3	U57317	77	74	268	232	MI	I	I	I	4	U57317 HSU57317 p300/CBP-associated factor (P/CAF)
287	?	L17418	51	32	148	155	NC	I	I	I	4	L17418: compl recept type 1 (alleles SandF) enhancer
288	7	AL080170	559	554	2000	1980	I	I	I	I	4	AL080170: mRNA; cDNA DKFZp434C091
289	21	M30818	125	126	423	414	I	I	I	I	4	M30818 IFN-ind cellular resist mediator prot (MxB)
290	12	M87434	211	236	931	826	I	I	I	I	4	M87434: 71 kDa 25 oligoadenylate synth (p69 2-5A)
291	11	AB018293	167	210	682	639	I	I	I	I	4	AB018293: mRNA for KIAA0750 protein
292	5	D50810	34	18	75	75	I	I	I	I	4	D50810: mRNA for placental leucine aminopeptidase
293	20	Z23115	125	132	399	364	I	I	I	I	4	Z23115 HSBCLXL H.sapiens bcl-xL mRNA
294	?	D28915	134	143	581	555	I	I	I	I	4	D28915: hepatitis C-asso microtub aggregate prot p44
295	2	AF026941	136	105	508	512	I	I	I	I	4	AF026941: cig5 partial sequence
296	2	M97935	208	205	827	811	I	I	I	I	4	M97935 transcription factor ISGF-3
297	17	X99699	-54	-48	-7	6	I	I	I	I	4	X99699: XIAP associated factor-1
298	7	X04327	386	317	1846	1937	I	I	I	I	4	X04327: erythrocyte 2,3-bisphosphoglycerate mutase
299	11	AW006742	22	13	75	60	I	I	I	I	4	AW006742:wr28g10.x1 cDNA
300	13	U01134	39	78	224	238	I	NC	I	I	4	U01134 U01134 solubleVEGF receptor (sflt)
301	1	M55542	111	104	466	506	I	I	I	I	5	M55542: guanylate binding protein isoform I (GBP-2)
302	18	D00726	102	136	612	564	I	I	I	I	5	D00726: mRNA for ferrochelatase
303	9	M77016	105	81	424	426	I	I	I	I	5	M77016: tropomodulin
304	2	M97935	32	35	158	160	I	I	I	I	5	M97935: transcription factor ISGF-3
305	10	M24594	189	205	965	960	I	I	I	I	5	M24594: interferon-inducible 56 Kd protein
306	11	X14798	15	25	106	86	I	I	I	I	5	X14798 cds#1 HSCETS1 c-ets-1 proto-oncogene
307	11	N55205	25	29	225	175	I	I	I	I	5	N55205:yv44g05.s1 cDNA
308	1	M13755	242	267	1278	1387	I	I	I	I	5	M13755 HUMIFN15K IFN-induced 17-kDa/15-kDa
309	?	K02882	75	69	486	371	I	I	I	I	5	K02882: germline IgD chain C-region, C-delta-1 dom
310	2	S66213	29	32	160	164	I	I	I	I	5	S66213:integrin alpha 6B [, mRNA Partial, 528 nt]
311	10	D63940	94	81	376	420	I	I	I	I	6	D63940 HUMHMXI1HL mRNA for Mxi1 protein
312	12	M98776	290	303	1677	1660	I	I	I	I	6	M98776 HUMKRT1X keratin 1 gene
313	?	U57452	26	25	144	144	I	MI	I	I	6	U57452 HSU57452 SNF1-like protein kinase
314	?	HG3543-HT	25	31	131	191	I	I	NC	I	6	Insulin-Like Growth Factor 2
315	10	AF026939	87	87	534	496	I	I	I	I	6	AF026939: CIG49 (cig49)
316	2	S66213	13	11	84	107	I	I	I	I	6	S66213:integrin alpha 6B [, mRNA Partial, 528 nt]
317	2	M97936	207	209	1281	1375	I	I	I	I	6	M97936: transcription factor ISGF-3 mRNA sequence
318	?	M10098	35	10	97	87	I	I	I	I	6	M10098 18S rRNA gene, complete
319	?	J03796	66	79	475	458	I	I	I	I	7	J03796: erythroid isoform protein 4.1
320	?	M14660	46	9	191	213	MI	I	I	I	7	M14660 ISG-54K gene (IFN stim gene) 54 kDA prot
321	7	AJ001612	13	12	103	102	I	I	I	I	7	AJ001612: L-3-phosphoser-phosphatase homo
322	12	X04371	-6	8	91	111	I	I	I	I	7	X04371: 1.6Kb 2-5A synthetase IFN induc
323	1	M61733	35	27	138	148	I	I	I	I	7	M61733: erythroid membrane protein 4.1
324	2	M97936	125	133	777	829	I	I	I	I	8	M97936: transcription factor ISGF-3
325	2	M10905	11	22	127	144	I	I	I	I	8	M10905: cellular fibronectin mRNA
326	2	X02761	-16	-21	83	78	I	I	I	I	8	X02761: mRNA for fibronectin (FN precursor)
327	?	M14660	28	16	160	170	I	I	MI	I	8	M14660 HUMISG2 ISG-54K gene (interferon stim)
328	?	HG3044-HT	-20	-11	108	85	I	I	I	I	8	Fibronectin, Alt. Splice 1
329	2	M97935	96	77	583	656	I	I	I	I	8	M97935 transcription factor ISGF-3

Table 2-2 (page 8 of 8): Spreadsheet of 327 differentially expressed genes

	A	B	C	D	E	F	G	H	I	J	K	L
330			AD	AD	AD	AD	R1	R1	R2	R2		
331	chr	Entrez	JP1	JP2	RP1	RP2	J1	J2	J1	J2	ave	Description
332	1	AB000115	67	67	640	638	I	I	I	I	9	AB000115: mRNA expressed in osteoblast
333	Y	AF000994	3	-32	107	119	I	I	I	I	10	AF000994: ubiquitous TPR motif, Y isoform (UTY)
334	Y	AF000986	-5	-3	127	131	I	I	I	I	10	AF000986: Drosophila fat facets related Y prot (DFFRY)
335	7	Z29331	65	65	646	673	I	I	I	I	10	Z29331 HSUCEH3 (23k/3) ubiq-conj enz UbcH2
336	10	M24594	45	48	538	560	I	I	I	I	12	M24594 HUMII56KD interferon-inducible 56 Kd protein
337	21	M33882	105	91	1256	1220	I	I	I	I	13	M33882: p78 protein
338	2	M97935	19	3	266	255	I	I	I	I	15	M97935: transcription factor ISGF-3
339	Y	AF000987	4	6	360	343	I	I	I	I	18	AF000987: eIF-1A, Y isoform (EIF1AY)
340	Y	U52191	5	5	458	466	I	I	I	I	29	U52191: SMCY (H-Y)
341	Y	AF000984	0	8	459	431	I	I	I	I	30	AF000984: dead box, Y isoform (DBY) alt transcr 2
342	14	X67325	-63	-119	246	447	I	I	I	I	34	X67325 HSP27 H.sapiens p27 mRNA
343	Y	M58459	37	23	2878	2776	I	I	I	I	108	M58459: ribosomal protein (RPS4Y) isoform

Footnote: The predominance of transcripts from genes located on the Y chromosome at the extreme end of this table (rows 333 to 343; more highly expressed in the sibling with FA/MDS) reflects that the pairwise comparison was of female versus male siblings and serves as a validation of the sensitivity of the gene microarray approach.

Chapter Three:

Acquired FANCA dysfunction and cytogenetic instability in adult acute myelogenous leukemia

3-1 Introduction

A variety of molecular defects have been discovered in patients with myeloproliferative syndromes and acute myelogenous leukemia, many of which result in the activation of autonomous signal transduction pathways for growth and survival.[182-186] While oncogenic mutations in such pathways are sufficient to induce myeloproliferative disorders in transgenic mice,[183,184] the development of acute leukemia requires additional mutations that ultimately interfere with myeloid differentiation.[187] The risk of this second type of mutation would be necessarily enhanced by cytogenetic instability, a manifestation of which would be the exhibition of multiple cytogenetic defects in the leukemic clone. That such complex cytogenetic defects universally occur in the myeloid leukemic clones of children with Fanconi anemia, a cytogenetic instability syndrome, supports this notion. In fact, because the clonal abnormalities found in this clinical context exactly recapitulate those found in patients with secondary MDS and AML,[137,188] and high-risk MDS,[69] we hypothesized that Fanconi anemia protein dysfunction might serve as a progression factor for the evolution of cytogenetically unstable AML clones, even in patients without hereditary evidence of Fanconi anemia.

To test this notion, a variety of AML cells and cell lines were examined, including a cell line from a 68 year-old patient with cytogenetic defects characteristic of secondary AML. The patient's lymphoblasts were normal but the leukemic cell population exhibited hypersensitivity to mitomycin C, a feature of Fanconi anemia. Nuclear FANCC, FANCG, and FANCA levels were markedly reduced and retroviral complementation indicated that the leukemic cells exhibited a secondary defect of FANCA function. Because lymphoblasts from this same patient showed normal levels of nuclear Fanconi proteins, we propose that this acquired defect of FANCA function occurred during the course of leukemic clonal evolution and may have been the cause of the cytogenetic instability.

3-2: Materials and methods

Patient clinical history – A brief clinical history from this individual has been previously reported.[189] There was no family history of Fanconi anemia, bone marrow failure, or acute myelogenous leukemia. The patient had no known exposure to alkylating agents, radiation or chemical leukemogens. The patient had no pre-existing blood disorder, no *café-au-lait* spots, or developmental defects of the skeletal, renal, or nervous systems.

Origins and maintenance of cell lines – The establishment of UoC-M1, a spontaneously immortalized, factor-independent, myeloid cell line has been

previously reported.[189] The cell line 4081 was provided by Richard Larson, M.D. (Chicago, IL) and was obtained by EBV-mediated immortalization of peripheral blood, mononuclear cells from the same patient as UoC-M1 and is maintained in RPMI-1640 supplemented with 15% fetal calf serum (FCS)(Hyclone Labs, Logan, UT), penicillin and streptomycin. The human, cervical adenocarcinoma cell line HeLa was obtained from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in DMEM + 10% FCS. The factor-dependent, myeloid cell line Mo7e was a generous gift from Hal E. Broxmeyer (Indiana University) and was maintained in RPMI 1640 + 10% FCS and 100 U/ml of recombinant-human granulocyte-macrophage colony stimulating factor (rhGM-CSF).

Flow cytometry - Flow cytometry analysis was performed to verify and add to reported cell surface markers in the UoC-M1 cell line.[189] Cells were cultured and washed twice in PBS prior to staining. $5x10^5$ cells were stained (in duplicate tubes) in PBS + 0.1% bovine serum albumin (BSA) for each marker analyzed including CD34, CD38, CD95 (FAS), CD117 (c-KIT or SCFR), interferon gamma receptor beta subunit (IFNγRβ), and tumor necrosis factor receptors 1 and 2 (TNFR1/2) using either FITC-conjugated, rabbit anti-human primary antibodies or rabbit-anti human primary antibodies and FITC-conjugated, goat-anti rabbit secondary antibodies (Pharmingen, San Diego, CA, U.S.A.). FITC-conjugated, goat-anti rabbit immunoglobulin (Ig) isotype controls were also incubated and analyzed as staining specificity controls. 10,000 cells were analyzed per

condition by flow cytometry using a FACSCaliber machine (Becton-Dickinson, Mansfield, MA, U.S.A.) and plots obtained via Cell Quest software (v. 3.3, Becton Dickinson, Mansfield, MA, U.S.A.).

Breakage analysis – Cultures were treated with 40 ng/ml mitomycin C (MMC) or 200 ng/ml diepoxybutane (DEB) for 72 hours in the dark, at 37°C. Cultures were then harvested after a two-hour exposure to 0.25 μg/ml colcemid (Sigma, St. Louis, MO). After a 10-minute treatment with 75 mM KCl, the cells were fixed with a 3:1 mixture of methanol and acetic acid (respectively). Wet-mount slides were prepared, air-dried, and treated with Wright's stain. At least 50 metaphase figures from each culture were scored for DNA breaks and radial formations per experiment.

Quantitative, real-time RT-PCR – QRT-PCR analysis was performed in triplicate (or more in some instances) for each primer set and in each cell line. The overall number of UoC-M1 amplification cycles per primer set was calibrated against an internal control of 18S cDNA. The cycle number was then standardized against the cycle number for the same primer pair in a concurrently amplified reaction in the myeloid cell line Mo7e. For each FA gene, the amplification threshold obtained with Mo7e was defined as a fold value of "one". Total cellular RNA was isolated using TriReagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's instructions. cDNA synthesis was performed with 1 μg total RNA, 40 nM random hexanucleotide oligomers

5'-TCCTGGATAGCTTTGGGCAA-3'; *hFANCE* (dual-labeled probe) 5'-FAM-AAGACTGGCGAGGACGGTTCGAATC-TAM-3'; *hFANCF* (forward primer) 5'-TTCCTGAAGGTGATAGCGGTG-3'; *hFANCF* (reverse primer) 5'-CTCCCCTCTCCAGGTGATTTG-3'; *hFANCF* (dual-labeled probe) 5'-FAM-CTGTTGCAGCCGCCTTTGTCTCGT-TAM-3'; *hFANCG* (forward primer) 5'-CTCCCTGCAGCTGTTCCTGT-3'; *hFANCG* (reverse primer) 5'-GCCTGATCCTCTGTGAAACCC-3'; *hFANCG* (dual-labeled probe) 5'-FAM-CCTTGGAGCTGACTGTCACCTGCAACT-TAM-3'; *h18S* (forward primer) 5'-CGGCTACCACATCCAAGGAA-3'; *h18S* (reverse primer) 5'-GGGCCTCGA AAGAGTCCTGT-3'; and *18S* (dual-labeled probe) 5'-VIC-CAGCAGGCGCGCAAATTACCCA-TAM-3'.

Cell fractionation and immunoblot analysis – Whole-cell extracts were obtained as previously described.[146] Sub-cellular fractions were prepared by hypotonic lysis (TECK buffer: 10 mM Tris-HCl, pH 7.8, 1 mM EDTA, 3 mM $CaCl_2$, 10 mM KCl, 1 μg/ml leupeptin, 1 mM pepstatin A, 1 mM phenylmethylsulfonylfluoride (PMSF), 1% aprotinin, and 2 mM sodium orthovanadate (Na_3VO_4)), Dounce homogenization, and sucrose density-gradient ultracentrifugation as previously described.[190] Proteins were resolved via denaturing, SDS-polyacrylamide gel electrophoresis (PAGE) (7.5%) and immobilized to nitrocellulose membranes (Bio-Rad, Hercules, CA) by electroblotting as previously described.[191] Filters were blocked for one hour in a suspension of 5% (w/v) non-fat powdered milk (Nestle, Solon, OH) in TBS (10

immunoblot analysis of HeLa, HeLa/FANCD2, and PD20L lysates using antisera blocked or unblocked with peptide prior to immunoblotting. PD20 is an FA-D2 cell line provided to us by the Fanconi Anemia Cell Repository at Oregon Health & Science University, Portland, OR. FANCD2 positive antisera were affinity purified with the immunizing FANCD2 peptide bound to a HiTrap NHS-activated column (Amersham Pharmacia Biotech AB, Piscataway, NJ). Purified antibody was eluted from the column according to the manufacturer's recommendations.

Retroviral transduction – The retroviral constructs used in these studies[192,193] were kindly provided by Alan D'Andrea, M.D. (Dana-Farber Cancer Institute, Boston, MA) and Markus Grompe, M.D. (OHSU). Each vector contains a puromycin-resistance gene. High-titer retroviral supernatants containing one of the four Fanconi anemia cDNAs (pMMP-FAA IRES/PURO, pMMP-FAC IRES/PURO, pMMP-FAF IRES/PURO, and pMMP-FAG IRES/PURO) were obtained. Briefly, $1x10^6$ UoC-M1 cells were washed three times in cold PBS and resuspended in 1ml of cell culture medium (RPMI-1640 (Gibco, Carlsbad, CA), 10% heat-inactivated fetal calf serum (Hyclone, Logan, UT), penicillin and streptomycin) and placed in one well of a 6-well tissue culture dish. 8 μg/ml of polybrene (Sigma, St. Louis, MO) in PBS (Gibco, Carlsbad, CA) was added to each well along with 1 ml of retroviral supernatant and the plates were incubated for 4 hours at 37°C. At the end of incubation, the cells were pelleted and resuspended in 2 ml of the aforementioned media per well and allowed to recover for 24 hours before being placed in 1 μg/ml of puromycin. The resulting

puromycin-resistant, retrovirally transduced lines were subsequently identified as UoC-M1/FA, UoC-M1/FC, UoC-M1/FF, and UoC-M1/FG.

Statistical analysis – The statistical significance of differential chromosomal breakage and radial formation after exposure to mitomycin C (MMC) or diepoxybutane (DEB) was determined via contingency table analysis (chi-square) on pooled data from multiple, independent experiments. Two different MMC sensitivity comparisons were: (a) between UoC-M1 controls and cell lines retrovirally transduced with constructs encoding four different Fanconi genes (UoC-M1/FA, UoC-M1/FC, UoC-M1/FF. and UoC-M1/FG) in pair-wise comparisons, and (b) between UoC-M1 controls and an EBV-transformed lymphoblastoid cell line from the same patient (4081). Yates correction for continuity was employed.[194] In each analysis, the null hypothesis (H_o) was defined as "no difference" between the data sets compared. Cutoffs were established wherein differential sensitivities were required to meet a $p<0.05$ to be labeled as significant. The standard deviation (SD) was calculated based on pooled data from all experiments for each cell type tested.

Gene dosage – For FANCA, the gene dosage, multiplex assay was based on an assay previously described by Morgan *et al.*[195] This uses five different multiplexes to PCR amplify exons 5,11,17,19/20, 31 together with either exon 1 of the Myelin Protein Zero *(MPZ)* gene or exon 1 of the *NCX1* gene (a sodium channel exchanger) as external controls. A multiplex assay has also been

designed for FANCG using exons 1 and 14. Primer sequences have been previously published [195] with the following exceptions: *FANCA*, exon 19/20, forward, 5'-GAAACACCGGTCACCGTCTGTG-3'; *FANCA*, exon 19/20, reverse, 5'-AGATCCACGATTCTTCGCATTGTC-3'; *FANCG*, exon 1, forward, 5'-CCTTCCTAAGTCCGCTTCTG-3'; *FANCG*, exon 1, reverse, 5'-CACATGAGGGAGGGGTTGTCAC-3'; *FANCG*, exon 14, forward, 5'-CCAGCTTTCTGCCAATCTTT-3'; *FANCG*, exon 14, reverse, 5'-CACAGAGAGACAGCCCACTG-3'; *NCX1*, exon 1, forward, 5'-CACTGTTCTCTGTTCCCAGG-3'; *NCX1*, exon 1, reverse, 5'-TGACATGTTTCAAAAGTGGC-3'. The sizes in base-pairs (bp) of the expected amplicons from each primer set are: *FANCA*, exon 5, (250 bp); *FANCA*, exon 11, (301 bp); *FANCA*, exon 17, (207 bp); *FANCA*, exon 19/20, (348 bp); *FANCA*, exon 31, (308 bp); *FANCG*, exon 1, (234 bp); *FANCG*, exon 14, (214 bp); *MPZ*, exon 1, (385 bp); *NCX1*, exon 1, (226 bp). PCR reactions were performed in 25 µl reactions with 125 ng of genomic DNA, 1x *Taq* Polymerase buffer (67 mM Tris-HCl, pH 8.8, 16.6 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 170 µg BSA/ml, 10 mM ß-mercaptoethanol), 200 µM each dNTP (Amersham Biosciences, Corp., Piscataway, NJ) and 0.2 µM of each primer. After an initial denaturation at 94°C for 3 minutes, 1.5 U *Taq* DNA Polymerase (Promega, Madison, WI) was added (hot start), and using a PE 480 thermocycler, 22 cycles of 93°C for 1 minute, annealing at 57°C for 1 minute (*FANCA* multiplexes) or 53°C for one minute (*FANCG* multiplexes) and extension for 2 minutes at 72°C were performed, followed by a final extension for 5 minutes at 72°C. Only 22 PCR cycles were

performed to keep the reaction within the exponential phase.[196] An aliquot of the PCR product (3 µl) was added to 16.5 µl formamide and 0.5 µl internal lane size standard (Genescan-500 Rox; ABI, Foster City, CA). The samples were denatured for 5 minutes at 94°C, snap-cooled on ice and transferred to an ABI 310 capillary-based system (ABI, Foster City, CA). Data were then analyzed by means of Genescan software to obtain electrophoretograms for each sample. The position of the peaks indicates the size (in bp) of the exons amplified, and the areas under the peaks indicate the amount of fluorescence from the product. At least four control DNA's were also included in each series of reactions. The copy number of each exon relative to all other amplified exons in patients and controls was established by importing the peak area values into spreadsheets and calculating a dosage quotient for each exon relative to all the other amplified exons in patients and controls. Peak areas for two chosen samples with approximately equal values were chosen and the dosage quotient was calculated for them; values are typically within the range 0.77-1.25.[196] An average peak area of these controls was taken and compared with the peak area of the exon from the experimental samples.

Fanconi gene sequencing - Sequences of interest were amplified using PCR and the product was cleaned by running on a 1% agarose gel, cutting out the band which corresponded to the predicted molecular weight, and extracting the product using a commercial kit (Qiagen Inc., Valencia, CA). 5 µl of purified product was then added to 4 µl of commercial sequencing kit (ABI, Foster City,

CA), 1.6 pmol of either the forward of reverse primer and using a Perkin-Elmer 480 thermocycler, 25 amplification cycles of 96°C for 30s, 50°C for 15s and 60°C for 4 minutes were carried out. Salt precipitation was achieved by adding 10 μl of product to 37 μl of 0.5 mM $MgCl_2$/70% EtOH and leaving to stand for 10-15 minutes after which the mixture was centrifuged at 13,000 rpm for 20 minutes. The supernatant was removed and the pellet was left to air dry for 20 minutes before being resuspended in 12 μl of Template Suppression Agent (ABI, Foster City, CA), denatured (5 minutes at 94°C), snap cooled on ice and transferred to an ABI 310 capillary based system. Sequence data was analyzed using Sequence Navigator software (ABI, Foster City, CA).

Haplotype analysis – To verify the genetic identity of the UoC-M1 myeloid and 4081 lymphoblastoid cell lines, haplotypes were constructed using PCR-based genomic mapping panels for human chromosomes 7 and 8 (ABI, Foster City, CA). Two CEPH reference DNAs were used as standards for individual allele size calculations (CEPH 1347-02 and 1347-15) (http://www.cephb.fr/cephdb/). Each DNA specimen was amplified individually with one of twelve sets of fluorescently labeled oligonucleotide primers corresponding to a unique di-nucleotide repeat with a high frequency of heterozygosity according to the manufacturer's specifications (ABI, Foster City, CA). PCR reactions were then subjected to capillary-gel electrophoresis in an ABI-Prism 310 machine (ABI, Foster City, CA) to resolve and detect individual amplicons. Alleles were labeled according to the calculated amplicon sizes (GeneScan v. 3.1 and Genotyper v.

2.5, ABI, Foster City, CA) obtained for each primer set as compared to concurrently amplified and electrophoresed CEPH genomic DNA controls (CEPH pedigree 1347) (http://www.cephb.fr/cephdb/) and a concurrently electrophoresed Genescan-400 Rox size standard (ABI, Foster City, CA). An estimate of the overall haplotype frequency was obtained by multiplying the individual allele frequencies reported for each marker as indicated in the CEPH marker database (http://www.cephb.fr/cephdb/).

Hematopoietic Progenitor Cell (colony forming unit) assays – UoC-M1 cells were suspended in a 1:3 solution of RPMI 1640 and methylcellulose (Methocult H4230, Stemcell Technologies, Vancouver, B.C.) to a final concentration of 20,000 cells per ml. One ml of medium containing cells and exogenously added cytokines was placed into 35x10 mm cell culture dishes, in triplicate for each experimental condition. To assess cytokine-mediated colony inhibition, recombinant human interferon-gamma (IFNγ) (R&D Systems, Minneapolis, MN, U.S.A.) or recombinant human tumor necrosis factor-alpha (TNFα) (R&D Systems, Minneapolis, MN, U.S.A.) were added to experimental conditions at concentrations ranging from 0.01 ng/ml to 100.0 ng/ml. Assays were allowed to incubate for seven days in a humidified chamber at 37°C in 5% CO2. Colonies were counted using a dissecting microscope and plotted as average absolute colony numbers per condition with standard deviations.

Apoptosis detection flow cytometry – To investigate cytokine-mediated apoptotic induction in UoC-M1, 500,000 cells were assayed per condition in duplicate experiments via flow cytometry against activated caspase-3. Cells were exposed to IFNγ (100.0 ng/ml) or TNFα (10 ng/ml) for 24 hours in suspension at 37°C. Following exposure, cells were rinsed 3 times in PBS + 0.5% BSA before being fixed and permeabilized by the Cytofix/Cytoperm kit (Pharmingen, San Diego, CA, U.S.A.) according to the manufacturer's instructions. Cells were then doubly stained with anti-CD34-FITC (Pharmingen, San Diego, CA, U.S.A.) and anti-active caspase-3-PE (Pharmingen, San Diego, CA, U.S.A.). Positive controls for caspase-3 staining were obtained by treating an independent specimen of UoC-M1 with 60 μM campothecin (Sigma, St. Louis, MO) for 24 hours. 10,000 cells were analyzed per condition by flow cytometry using a FACSCaliber machine (Becton-Dickinson, Mansfield, MA, U.S.A.) and plots obtained via Cell Quest software (v. 3.3, Becton Dickinson, Mansfield, MA, U.S.A.).

Results

3-3: UoC-M1 is morphologically primitive

As shown in figure 3-1, UoC-M1 has the appearance of a primitive, myeloid blast. This included a high nuclear to cytoplasmic ratio and abundant mitotic figures. Though the membrane is ruffled (UoC-M1 is in the megakaryoblastoid lineage [see section 3-4 below]),[189] platelet production has

never been observed. This is despite attempts to stimulate platelet production in UoC-M1 with thrombopoietin (TPO) or TPO plus all-trans retinoic acid (a differentiating agent)(data not shown).

3-4: Surface antigen presentation in UoC-M1 suggests a myeloid blast

The surface antigen phenotype has been reported in UoC-M1 with a limited set of markers including CD34 and CD61 (platelet glycoprotein IIIa), placing UoC-M1 in the megakaryoblastoid lineage.[189] Flow cytometry was performed to further characterize surface markers. Figure 3-2 indicates that UoC-M1 is positive for CD34, CD38, and CD117. As CD34 is an early-stage hematopoietic marker (as is CD117) and CD38 is a later-stage antigen, UoC-M1 may be considered to be of early-intermediate differentiation. This antigen phenotype is in agreement with the finding that UoC-M1 also maintains a near diploid chromosomal content (see section 3-5 and figure 3-3). As megakaryocytes mature their nuclei replicate though the cells do not divide. They are thus progressively hyperploid (up to 128C or 128 haploid equivalents) as they mature.[197] Additionally, as illustrated in figure 3-3, UoC-M1 cells express the interferon receptor beta chain, though they do not present the surface receptor FAS or the tumor necrosis factor receptors 1 or 2.

3-5: UoC-M1 has complex cytogenetics including deletions of chromosome 7q

As outlined in chapter one, complex cytogenetics are noted in karyotypes associated with myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML). These include recurrent findings such as 5q-, monosomy 7, and 20q-. As shown in figure 3-3, UoC-M1 has a complex karyotype including many translocations and several numerical or structural losses including 5q-, 7q-, monosomy 11, nullisomy 19, monosomy 21, -Y, and marker chromosomes.[189] Such a complex karyotype is frequently seen in individuals presenting with MDS/AML derivative of prior therapy or a genetic syndrome such as seen in Fanconi anemia, though the patient's medical history is unremarkable for each of these categories of AML predisposition.

3-6: UoC-M1 cells are hypersensitive to MMC

Upon exposure to the bi-functional alkylating agents mitomycin C (MMC) or diepoxybutane (DEB), the myeloid cell line UoC-M1 demonstrated extensive chromosomal breakage and unusual end-to-end chromosomal fusions termed radial forms (figure 3-4). Even in the absence of clastogens, the cells routinely exhibited chromosomal breakage (data not shown). These experiments were performed against an independent myeloid leukemic cell line (FA-AML1) and the sample identities were re-confirmed by virtue of their distinct G-banded karyotypes (data not shown). Though the percentage of clastogen-induced radial formation in UoC-M1 varied across multiple experiments, it never fell below the

diagnostic cutoff employed in our laboratory (20%). The fraction of metaphases exhibiting radial forms was 29.3%±7.6% (n=300; range 20% to 38%) (see figure 3-5, column 1). To rule out the possibility that UoC-M1 cells represent material from a person that truly had hereditary Fanconi anemia (the patient's advanced age and unexceptional prior medical and family history make this unlikely) an EBV immortalized lymphoblastoid cell line derived from the same individual (cell line 4081) was obtained and subjected to MMC/DEB sensitivity testing. As shown in figure 3-5, the percentage of radial formation in 4081 (8%) is significantly lower than that found in UoC-M1 (chi-square value of 8.995, 1 degree of freedom, p=0.003) and within the normal range (less than 10%). As increased cellular sensitivity to chromosomal damage following exposure to MMC and DEB represents the current diagnostic laboratory standard for Fanconi anemia,[198] experiments were conducted to determine whether the clastogen sensitivity demonstrated by UoC-M1 could be the result of acquired abnormalities in the Fanconi anemia pathway.

3-7: FA gene transcripts are quantitatively normal

To date, six FA genes have been cloned and the sequences of the representative cDNA's for the *FANCA*,[96,97] *FANCC*,[98] *FANCD2*,[94] *FANCE*,[99] *FANCF*,[100] and *FANCG*[101] genes are available. Quantitative, real-time, reverse-transcription polymerase chain reaction (QRT-PCR) was employed to quantify transcripts from each of these genes relative to an internal amplification standard

(18S RNA) and also compared to the myeloid cell line Mo7e (figure 3-6). Mo7e was used as a control for three reasons: (1) it is not MMC hypersensitive, (2) it is in the same myeloid lineage as UoC-M1 (megakaryoblastoid)[199] and (3) it shares a myeloid leukemic phenotype with UoC-M1.[199] While FA mRNA levels vary slightly between UoC-M1 and Mo7e (figure 3-6, columns 1 through 6), the RNA levels for *FANCA*, *FANCC, FANCD2, FANCE,* and *FANCG* were equivalent to or more abundant than those observed in Mo7e. An exception to this was the observed two-fold reduction in *FANCF* mRNA (figure 3-6, column 5), a reduction attributed to monosomy 11 found in UoC-M1 cells[189] because *FANCF* is located on human chromosome 11p15.[100] Levels of *FANCA, FANCC*, and *FANCG* mRNA (figure 3-6, columns 1, 2, and 6) are slightly increased relative to Mo7e. It is concluded that the MMC hypersensitivity of UoC-M1 cells does not derive from reduced transcription of Fanconi genes.

3-8: Nuclear FA proteins are markedly reduced

As shown in figure 3-7, compared to the human, cervical carcinoma cell line HeLa (lane 1) and the factor-dependent, myeloid cell line Mo7e (lane 2), UoC-M1 (lane 3) had a mild reduction in the levels of cytoplasmic FANCA, FANCC, and FANCG proteins (rows 1, 4, and 5 respectively). However, nuclear fractions (lanes 4, 5, and 6) demonstrated a reduction of FANCC in UoC-M1 (row 4, lane 6) and a near absence of nuclear FANCA (row 1, lane 6) and FANCG proteins (row 5, lane 6). This observed reduction confirmed earlier experiments

performed with whole-cell extracts (data not shown) and further indicated that the most substantial reductions in UoC-M1 cells were due to losses in the nuclear compartment, the compartment in which Fanconi protein complexes function to effect MMC resistance.[200] Purity of subcellular fractions was confirmed by reprobing stripped filters with antisera against beta-tubulin (Roche Diagnostics Corp., Indianapolis, IN) (rows 2 and 6) and topoisomerase II (NeoMarkers, Inc., Fremont, CA) (rows 3 and 7). To investigate the significance of nuclear FA protein loss in UoC-M1, a downstream component of the FA protein pathway (FANCD2-L) was assayed via immunoblot analysis.

3-9: FANCD2 is not mono-ubiquitinylated in UoC-M1 cells

A nuclear multimeric complex of the FANCA, FANCC, FANCF, and FANCG proteins is required for the mono-ubiquitinylation of FANCD2 (FANCD2-L for "long form"), a post-translational change enhanced by DNA damage.[200] This post-translational modification of FANCD2 is thought to be required for normal cellular responses to certain types of genotoxic stress.[200] If the reduction of nuclear FANCA and FANCG in UoCM1 cells is the cause of MMC hypersensitivity, then FANCD2-L should be undetectable. As shown in figure 3-8, UoC-M1 (lane 2) does not demonstrate the mono-ubiquitinylated form of FANCD2. FANCD2-L is also absent in lane 3 containing an extract from HSC536N, an EBV-transformed lymphoblastoid line from an FA-C patient, but is present in the *FANCC* complemented FA-C cells (lane 4) and in a lymphoblastoid

cell line (4081; lane 1) from the same patient as UoC-M1. To further establish a linkage between MMC sensitivity in UoC-M1 cells and the absence of nuclear FA proteins and absence of FANCD2-L, UoC-M1 cells were transduced with each of four retroviral vectors encoding *FANCA, FANCC*, *FANCF*, and *FANCG*.

3-10: Functional complementation with a FANCA cDNA

UoC-M1 cells were retrovirally transduced with the coding sequences for the *FANCA*, *FANCC, FANCF*, or *FANCG* cDNA and were positively selected with puromycin. MMC sensitivity was determined in each resulting cell line (UoC-M1/FA, UoC-M1/FC, UOC-M1/FF, and UoC-M1/FG). As shown in figure 3-9, only transduction with the cDNA encoding *FANCA* (cell line UoC-M1/FA) resulted in a significant reduction in MMC sensitivity in UoC-M1 (chi-square value of 10.058 with 1 degree of freedom, p=0.002). Transduction with constructs encoding *FANCC* (UoC-M1/FC) and *FANCG* (UoC-M1/FG) failed to significantly reduce MMC sensitivity (p=0.059, and p=0.064, respectively). In two consecutive experiments, transduction of UoC-M1 with the *FANCF* vector has not influenced MMC sensitivity (average radial formation of 32% over 100 total cells). These data support the notion that aberrant *FANCA* function is directly related to MMC sensitivity in UoC-M1. To assure that transgenes were highly expressed in transduced cells, immunoblot analysis was performed (Fig. 3-10).

3-11: Expression of *FANCA* Stabilizes FANCG

Transduction with all retroviral vectors increased levels of the proper FA protein in UoC-M1 cells (Fig. 3-10A). Transduction with *FANCC* (UoC-M1/FC) had no impact on levels of FANCA or FANCG. However, transduction with *FANCA* (UoC-M1/FA) not only resulted in an increase in cellular levels of FANCA (row 1, lanes 3 and 4) but also of FANCG (row 3, lanes 3 and 4). Conversely, transduction with *FANCG* (UoC-M1/FG) though greatly increasing cellular levels of FANCG (row 3, lanes 7 and 8), did not result in an increase in FANCA (row 1, lanes 7 and 8). Because these proteins are known to stabilize each other [201,202] the results point to FANCA as the dysfunctional FA protein in UoC-M1 cells. Row four depicts a constant, non-specific fragment detected by the FANCG antibody and demonstrated consistent loading across all lanes.

Additional support for FANCA dysfunction evolved from studies on nuclear localization of FANCA. As shown in figure 3-10B, transduction of UoC-M1 with *FANCA* resulted in FANCA accumulation in the nucleus of UoC-M1/FA (row 1, lane 3) while transduction with *FANCC* (UoC-M1/FC) (row 1, lane 4) and *FANCG* (UoC-M1/FG) (row 1, lane 5) did not increase nuclear levels of FANCA. Transduction with *FANCG,* a widely recognized FANCA interacting protein, failed to increase nuclear levels of this protein in any of the three lines (row 2, lanes 3-5). Taken together, these results suggest that a defect largely involving FANCA function was present in UoC-M1.

3-12: The FANCA gene harbors no mutations

DNA from UoC-M1 was subjected to quantitative, multiplex-PCR based dosage analysis and sequencing. As shown in table 3-1, relative to the myelin protein zero *(MPZ)* gene on chromosome (1q22)[203] or the *NCX1* sodium channel exchanger gene on chromosome (2p22-p23),[204] six exons of *FANCA* (exons 5, 11, 17, 19/20, and 31) and two exons of *FANCG* (exons 1 and 14) did not show significant dosage disparities. Therefore, the genomic regions covered by these intervals, are intact and contain a proper copy number of FANCA and FANCG.

The *FANCA* and *FANCG* genes were further analyzed by performing DNA sequencing of all exons, approximately 30 bp of sequence upstream and downstream of each exon, and all known splice sites. Sequence analysis of *FANCG* in UoC-M1 was normal with no anomalies noted. A heterozygous, silent transition polymorphism (G1290A) was noted in exon 14 of *FANCA*. *FANCA* was otherwise unremarkable including the two putative bipartite nuclear localization signals (NLS)[97], which were intact (data not shown).

3-13: Genetic analysis of a lymphoblastoid cell line from the same individual

QRT-PCR in 4081 (standardized against a lymphoblastoid cell line established from a normal individual: "JY") indicated that RNA for each of the six

cloned FA genes (as before) was present (data not shown). As shown in figure 3-10C, immunoblot analysis of nuclear fractions from 4081 indicates nuclear abundance of both FANCA and FANCG, clearly demonstrating that the function of FANCA in 4081 cells was normal.

One potential explanation for the disparities in FA protein function between the UoCM-1 and 4081 cells is that the lines are derived from different individuals. To rule this out, haplotypes were constructed using highly variable di-nucleotide repeats from chromosomes 7 and 8. As shown in table 3-2, UoC-M1 and 4081 genetically agree at 12 out of 12 markers (11 markers from chromosome 7 and 1 marker from chromosome 8). Based upon the frequencies reported for each of these alleles in the CEPH database (http://www.cephb.fr/cephdb/) the overall frequency of the indicated haplotype is conservatively estimated to be $5x10^{-17}$ confirming a common genetic identity between UoC-M1 and 4081. Of note, only a single allele for chromosome 7 markers in UoC-M1 is indicated because UoC-M1 cells are functionally monosomic for chromosome 7 (*i.e.* the cells have one "intact" chromosome 7 and another, likely endoreduplicated pseudohomolog, that is translocated to other autosomes).[189]

3-14: Inhibitory cytokine resistance in UoC-M1

As outlined in chapter one, we predicted that hematopoietic stem cells evolving to malignancy in FA-associated MDS/AML would be resistant to IFNγ. To evaluate the sensitivity of UoC-M1 to inhibitory cytokines, methylcellulose assays containing IFNγ or TNFα were performed. UoC-M1 is capable of forming colonies in methylcellulose though they are small and contain few cells (about 10-30 per colony; data not shown). Of note, this type of colony growth (*i.e.* clusters of 2 to 39 cells) is consistent with reports on the colony growth characteristics of myelodysplastic stem cells.[205] As shown in figure 3-11A, UoC-M1 is resistant to inhibition by IFNγ, at even the highest dose of 100 ng/ml, a concentration that routinely yields greater than 90% inhibition in normal CD34+ cells.[206] However, the cell line remains sensitive to inhibition by TNFα with an ID_{50} of between 0.1 and 1.0 ng/ml. Interestingly, neither TNFα nor IFNγ inhibited cellular proliferation in suspension cultures (data not shown). Next, flow cytometry was executed against activated caspase-3 in cells treated with IFNγ, TNFα, or both as a measure of apoptosis.

As shown in figure 3-11B, TNFα promotes the activation of apoptotic pathways as evidenced by positive staining for activated caspase-3 (41.5% of treated cells). However, a single high dose of IFNγ (100 ng/ml) did not result in positive staining for activated caspase-3 in UoC-M1 (4.4% of cells) (figure 8D). That TNFα was capable of activating capsase-3 in UoC-M1 suspension cultures, but incapable of inhibiting proliferation in these same cultures may speak to the

short half-life of inhibitors in liquid cultures (though this is clearly not a factor in colony-forming assays). Of note, suspension cultures contain several millions of cells while methlycellulose assays contain but a few thousand cells. In light of this, differential cytokine responses may reflect a situation wherein not all UoC-M1 cells are cytokine sensitive at any given time. Thus, the presence of a small number of resistant cells in suspension cultures may quickly lead to repopulation of the entire flask in a short period of time while this is not possible under semi-solid cell culture conditions. Panel 3 is a positive control for apoptosis and represents UoC-M1 treated with campothecin (62.6% of cells). The failure to respond to IFN can be global, as might occur in cells that had dysfunctional or absent IFN receptors, or selective, in which certain but not all genetic responses to IFN might be interdicted. To distinguish between these two general mechanisms, we sought to develop support for the hypothesis that the lack of IFN-related apoptosis was selective; that is, that certain IFN-induced responses remained intact. Immunoblots prepared from UoC-M1 whole cell extracts after treating the cells with IFNγ (10 ng/ml), TNFα (10 ng/ml), or both for 20 hours, were probed for the interferon-gamma responsive factor, IRF-1.[152,207] As shown in figure 3-11C, unstimulated UoC-M1 cells have low, constitutive levels of IRF-1 protein (row 1, lane 1) that are substantially upregulated after exposure to IFNγ (row 1, lane 2). TNFα exposure alone did not impact IRF-1 levels (row 1, lane 3) and TNFα plus IFNγ was no different than IFNγ alone (row 1, lane 4). Figure 3-11C, row 2 shows the same filter stripped and reprobed with an antibody to the

abundant, nucleolar ribosomal RNA-transport protein, nucleophosmin (NPM) which demonstrates equal loading across lanes. These data demonstrate that UoC-M1 remains responsive to IFNγ, though the cells are no longer sensitive to the induction of apoptosis by interferon.

3-15: Discussion

The Function of FA proteins

Fanconi anemia (FA) is a rare, autosomal-recessive disorder characterized by bone marrow failure and various developmental defects including skeletal, skin pigmentation, and visceral anomalies.[88,170] The disease demonstrates genetic heterogeneity. Eight complementation groups have been formally defined by somatic cell fusion studies (FA-A through FA-G including FA-D1 and FA-D2) with each complementation group corresponding to a distinct gene.[93,94] The majority of all patients (60-65%) fall within group FA-A. Six of the FA genes have been cloned though loci for *FANCB* and *FANCD1* remain to be identified[94,96-101] and there is anecdotal evidence from somatic fusion experiments in support of three additional groups ("*FANCI, FANCJ, FANCK*").[95] The cloned sequences bear homologies neither to other known genes nor to one another. The FA genes clearly protect cells from genotoxic stress. Mutant cells exhibit spontaneous chromosomal breakage[102] and increased sensitivity to the

development of cytogenetic anomalies upon *in-vitro* treatment with cross-linking agents[103] such as mitomycin C (MMC)[104] and diepoxybutane (DEB).[105]

Multiple additional functions for FA proteins have also been described including modulation of cytokine-mediated signaling,[134,136,147,153,164,165,167,208,209] responsiveness to oxidative stress,[210-216] and chromatin remodeling.[217] Moreover, at least two members of the FA genes *(FANCC* and *FANCD2)* are multifunctional. For example, selective mutations of *FANCC* can impair some functions of FANCC but not others.[164] The same is true for FANCD2 which plays a dual-role in mediating resistance to chemical crosslinking agents (for which FANCD2 monoubiquitinylation is required) and ionizing radiation (for which FANCD2 monoubiquitinylation and serine phosphorylation are required).[218] However, while distinct FA proteins likely have individual cellular activities, the universal phenotypic feature of bone marrow failure across all complementation groups suggests that these proteins share important hematopoietic activities by acting together in either a multimeric complex, a sequential linear pathway, or both.

Garcia-Higuera *et al* [200] suggest that FA proteins function in a linear pathway to effect protection from or repair of DNA damage. Indeed, there exists an "upstream" multimeric complex made up of at least the FANCA, FANCC, FANCE, FANCF, and FANCG proteins.[201,202,219-224] This complex is required to permit a post-translational modification (monoubiquitinylation) of the *FANCD2*

gene product (figure 3-12).[200] The FANCD2 protein has been shown to co-immunoprecipitate with BRCA1 and further, to co-localize with BRCA1 to damage-induced nuclear foci.[200] The ability to form such foci is absent in cell lines from multiple FA complementation groups but is restored upon correction of these lines with appropriate cDNA's.[200]

Leukemia in FA

The hematopoietic defect in FA involves initial bone marrow failure followed by myelodysplasia and AML.[106] Hematopoietic defects account for the majority of deaths in patients with FA. Only rare cases have been reported into their fourth decades. A single set of siblings diagnosed in their fifties has been reported; one with anemia and the other hematologically asymptomatic (though MMC sensitive).[225] Furthermore, no case presenting initially as MDS or AML without BMF has been reported in a patient older than 28 years of age.[226]

Involvement of FA Proteins in Sporadic Leukemia

Joenje's group has recently suggested, from co-immunoprecipitation studies, that AML cells from non-FA patients may exhibit abnormal FA protein complex formation,[227] but the functional significance of the observations were unclear. Specifically, MMC sensitivity was not reported in all cells tested, FA genes were not sequenced, complementation studies were not performed,

FANCD2-S/L status was not examined (the identification of FANCD2 was reported after the publication of the report by Xie *et al* [227]), and non-leukemic cells from the patients with AML were not analyzed. In the studies outlined herein, all of these standards were met and the results demonstrate that dysfunction of FANCA can indeed occur as an acquired defect in AML cells and that it has unambiguous functional significance.

First, the UoC-M1 myeloid cell line is hypersensitive to MMC and DEB but lymphoblasts from the same patient (4081) are not. Second, nuclear FANCA and FANCG protein levels were undetectable in UoC-M1 cells but were abundant in 4081 cells. Third, 4081 cells contained FANCD2-L and S but UoC-M1 cells contained only FANCD2-S. Fourth, transduction of UoC-M1 cells with a *FANCA* (but not *FANCG, FANCF, or FANCC*) cDNA increased both FANCA and FANCG levels, and induced MMC resistance. The capacity of FANCA to increase FANCG levels reflects the FANCG-stabilizing activity of FANCA.[201,202] That FANCG did not influence FANCA points to FANCA dysfunction in UoC-M1 cells.

Because mRNA encoded by five cloned FA genes (*FANCA, FANCC, FANCD2, FANCE*, and *FANCG*) was present in UoC-M1 at equal or greater levels than in Mo7e controls and because the sequences of all exons of *FANCA* and *FANCG* were normal, we propose that co-factors for nuclear localization of FANCA or for FANCG binding of FANCA are dysfunctional in UoC-M1 cells, a deficiency that could be overcome by overexpression of *FANCA* but not by

FANCG or *FANCC*. We have sought to identify abnormalities in FANCA structure and/or expression using genomic sequencing, western blotting, and quantitative RT-PCR but have not found such evidence. We have therefore not identified the molecular cause of FANCA dysfunction in UoC-M1 cells. Because of the capacity of FA proteins to bind to cellular chaperones,[165,228] as well as to each other[200-202,219-224,229-233] it is possible that factors governing intracellular (*i.e.* nuclear) localization may be dysfunctional in these cells. It has been reported that abnormalities in both complementation groups FA-B and FA-C lead to reductions of the FANCA/FANCG complex.[201] However, as retroviral transduction with a *FANCC* construct does not significantly reduce the MMC sensitivity of UoC-M1 it is unlikely that a defect of *FANCC* exists in these cells. It is therefore possible that the apparent abnormality of FANCA function derives from a defect in *FANCB*, a gene not yet cloned (or possibly the putative genes for complementation groups I, J, or K). Cloning of the *FANCB* gene will no doubt contribute to an understanding of whether or not a defect of *FANCB* plays a role in these cells. Should a FANCB defect be found in UoC-M1 cells, the 4081 cells might be either heterozygous or wild-type for *FANCB*. It is also possible that further investigation into the observed FANCA defect in UoC-M1 may reveal dysfunction of a novel protein corresponding to no known FA complementation group. There are two ways this might occur.

First, we have recently demonstrated that the FANCC gene encodes a multifunctional protein and that mutations in domains that subserve one function

do not necessarily impair the capacity of FANCC protein to protect cells against MMC or DEB.[164] Consequently, such mutations in nature could lead to bone marrow failure but not be ascertained as Fanconi anemia because the MMC test would be negative (and therefore, by current standards, the patient would not have classical "FA"). A logical extension of this principle is that there may be unknown complementation groups that might well cause bone marrow failure but not abnormal MMC tests. If such proteins interacted functionally with classical FA proteins and inactivating mutants caused selective dysfunction of classical FA proteins, arguments on whether the new proteins are "FA" proteins would be largely semantic.

A second mechanism by which a protein that facilitated FANCA function might not encode a known FA complementation group protein would be if inactivating mutations of such facilitators were embryonic lethal. Ascertainment of a role in disease pathogenesis could be achieved only by observing somatic dysfunction through loss of both genes in non-carriers or by loss of heterozygosity (LOH) in carriers. Such has clearly been the case with other disorders.[234-236]

Alternative Explanations for Our Results

These studies support a model of clonal evolution in this patient in which acquired FANCA dysfunction occurred in an already initiated clone that ultimately

gave rise to the cytogenetically unstable UoC-M1 cells but did not give rise to the 4081 lymphoblasts (figure 3-13). There are two alternative explanations for these results. First, the cells studied acquired the FANCA defect during propagation of this immortalized line. However, because the cytogenetic defects described in the original patient material were highly complex and were the same as they are currently in the cells of this line, indicating no new cytogenetic drift,[189] we view this as unlikely. Secondly, this patient could, theoretically, represent the oldest ever reported case of Fanconi anemia. One obligatory component of this model would be mosaicism (*i.e.* the existence of a mixed population of stem cells wherein some remain FA-mutant while others have become genetically reverted to "wild-type" FA status) in the lymphoid lineage. Lymphoid mosaicism has been described in a young adult. In this instance, leukemia occurred in a patient with high-level mosaicism (arising via mitotic recombination between alleles in a compound heterozygote).[163] The leukemic clone derived from the non-reverted stem cell population.[163] If such a circumstance is also considered in the patient reported here, mosaicism might have occurred early in life leading to subsequent correction of a hematopoietic defect with late-arising clonal evolution in a preserved, non-revertant stem cell. This is unlikely in view of his age, clinical stability, absence of other stigmata of FA in the patient or any family member, and absence of an *FANCA* sequence abnormality in either *FANCA* allele. It is also unusual for patients with mosaicism to be fully hematologically normal, as this patient was known to have been prior to the onset of AML. Consequently, the most likely interpretation of our results is that some adult patients with AML,

likely those with complex cytogenetic defects, may have acquired FA protein dysfunction serving as a progression factor for clonal evolution.

Sporadic Leukemia: Timing of FA dysfunction in clonal evolution

The function of the FA proteins also speaks to the timing of somatic mutations affecting FA protein function. Loss of FA protein function is likely a progression factor and not an initiation factor for new clones (figure 3-14). This notion derives from recent work from our lab and the labs of others[134-136,147,164-167] demonstrating that FA gene dysfunction results in an apoptotic phenotype in hematopoietic cells, hardly a selectable attribute unless an initiating mutation predates the dysfunction. This would also suppose that the initiating somatic mutations rendered the cells resistant to the very apoptotic forces that cause excessive death in the wild-type FA mutant cells. The proposition that FA mutations are not selectable, absent an initiating mutation, is supported by the finding that obligate FA heterozygotes do not appear to be at increased risk of AML, MDS, or cancer.[237]

Epidemiologically, the process of clonal evolution is expedited in the context of FA. In molecular terms there are two reasons for this. First, the homogygote cells are genetically unstable. Second, in the context of high-level mutations the apoptotic phenotype puts selective pressure on progenitors.[134,137] *Adaptive mutations* would represent a perfect initiating factor, protecting the cells from the apoptotic consequences of FA protein dysfunction but permitting

subsequent FA gene mutations to cause genomic instability and MMC hypersensitivity.[137] As such, loss of FA protein function may serve as an important progression factor for leukemic clones, especially the types bearing multiple, non-random cytogenetic defects. A comprehensive examination of FA protein function in primary and secondary MDS/AML cells should shed further light on these questions and is clearly warranted at this time.

Experimental Evidence for Adaptive Mutations

Finally, the UoC-M1 cells were shown to be resistant to IFN-mediated apoptosis in colony-forming assays though they remained sensitive to inhibition by TNFα. TNFα induced apoptosis through caspase 3 activation. High doses of IFNγ did not result in an increase in caspase-3. Interferon signaling was shown to be intact (at least between the binding of ligand and gene induction) as IFNγ exposure resulted in an increase in protein levels for the interferon-responsive transcription factor, IRF-1.[152,207] These results demonstrate that resistance to IFNγ cannot be explained by an inactive cytokine receptor. They also suggest that resistance occurs distal to the induction of *IRF-1*, and therefore distal to the activation of STAT molecules by the interferon receptor.[238] That the inhibitory capacity of TNFα is preserved in UoC-M1 points to experiments that might discern the specific apoptotic pathway that is abnormal in this system. Such a pathway would by necessity be inducible by TNFα but not inducible by IFNγ.

Additional experiments making use of apoptosis inhibitors unique and common to each cytokine (such as procaspase-cleavage blocking polypeptides)[146] should indicate at which point this process is specifically blunted.

Figure 3-1

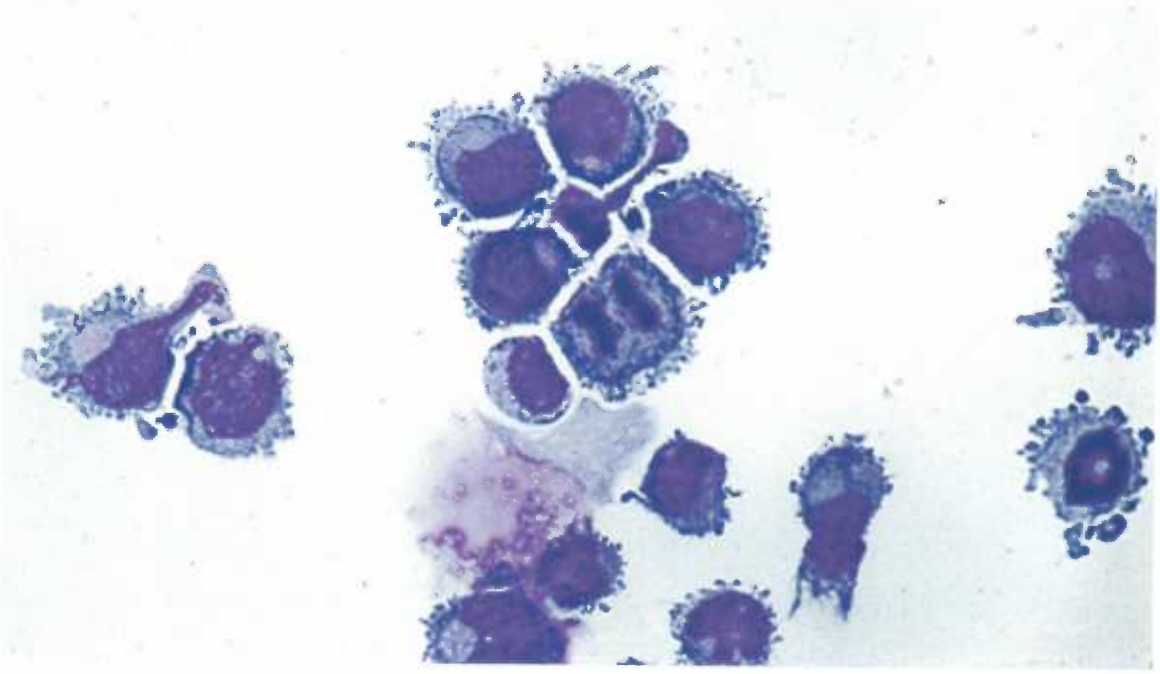


Figure 3-1 Legend: Wright's/Giemsa stain of UoC-M1 megakaryoblastoid cell line. As shown here, UoC-M1 has a primitive, myeloid cell appearance. This includes a high nuclear to cytoplasmic ratio. The cells are mitotically active and mitotic figures are clearly visible in this representative image.

Figure 3-2

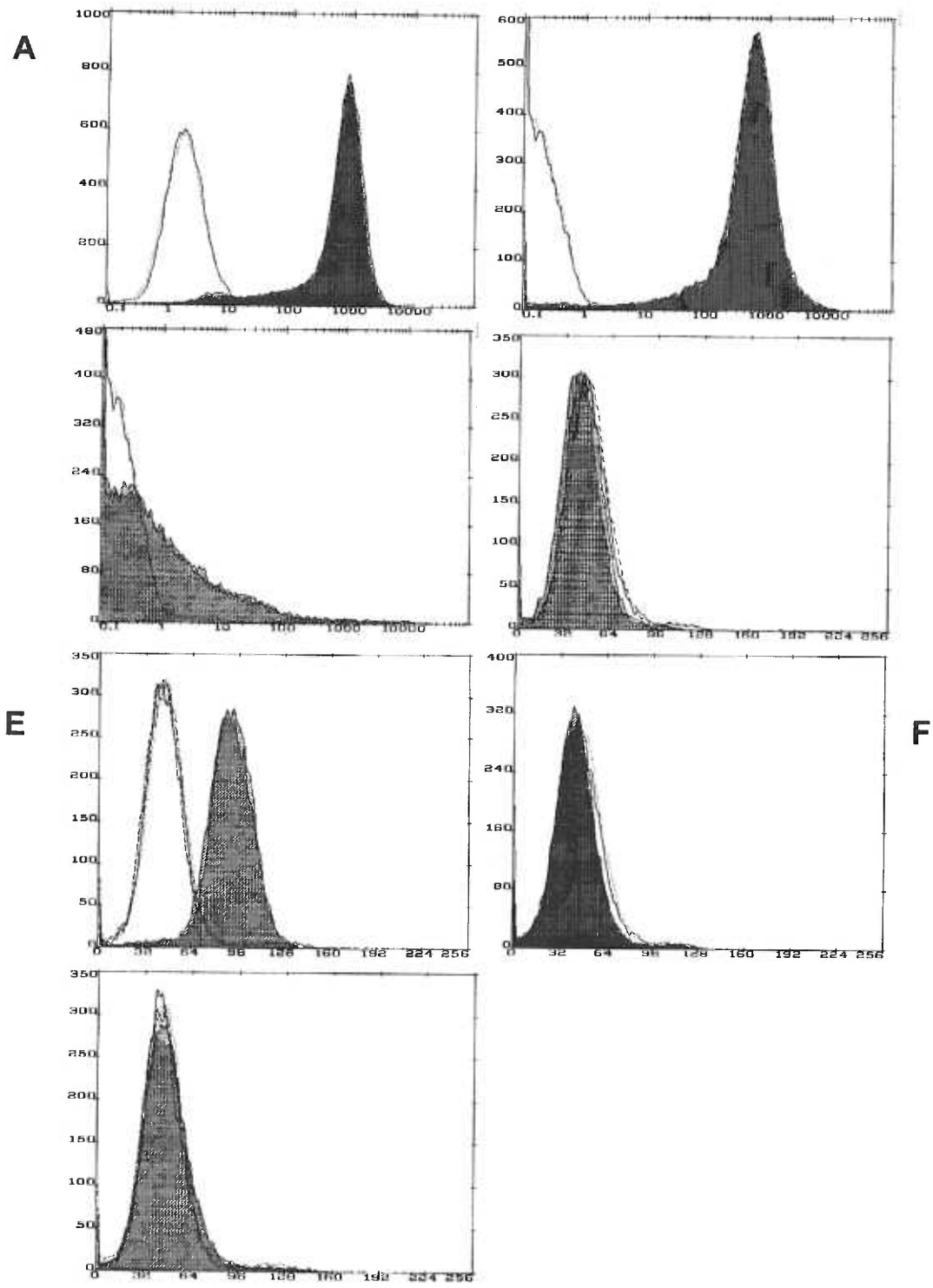


Figure 3-2 Legend: Surface antigen expression in UoC-M1. The cell line displays several markers of early, hematopoietic cell development including CD34 (panel A) and CD117 (panel C). The late stage myeloid marker CD38 is also present (panel B). These data, in conjunction with the published immunophenotype that included CD61 (platelet glycoprotein IIIa)[189] place the UoC-M1 cell line in the megakaryoblastoid lineage. The cell line also displays the interferon-gamma beta receptor (panel E), but does not demonstrate the death-domain containing receptor FAS (panel D), TNF-receptor 1 (panel F) or TNF-receptor 2 (panel G). Staining was performed on cells without exogenously added cytokines. The Y-axis in each demonstrates cell number while the X-axis indicates positive staining (closed areas) relative to antibody isotype controls (open areas).

Figure 3-3

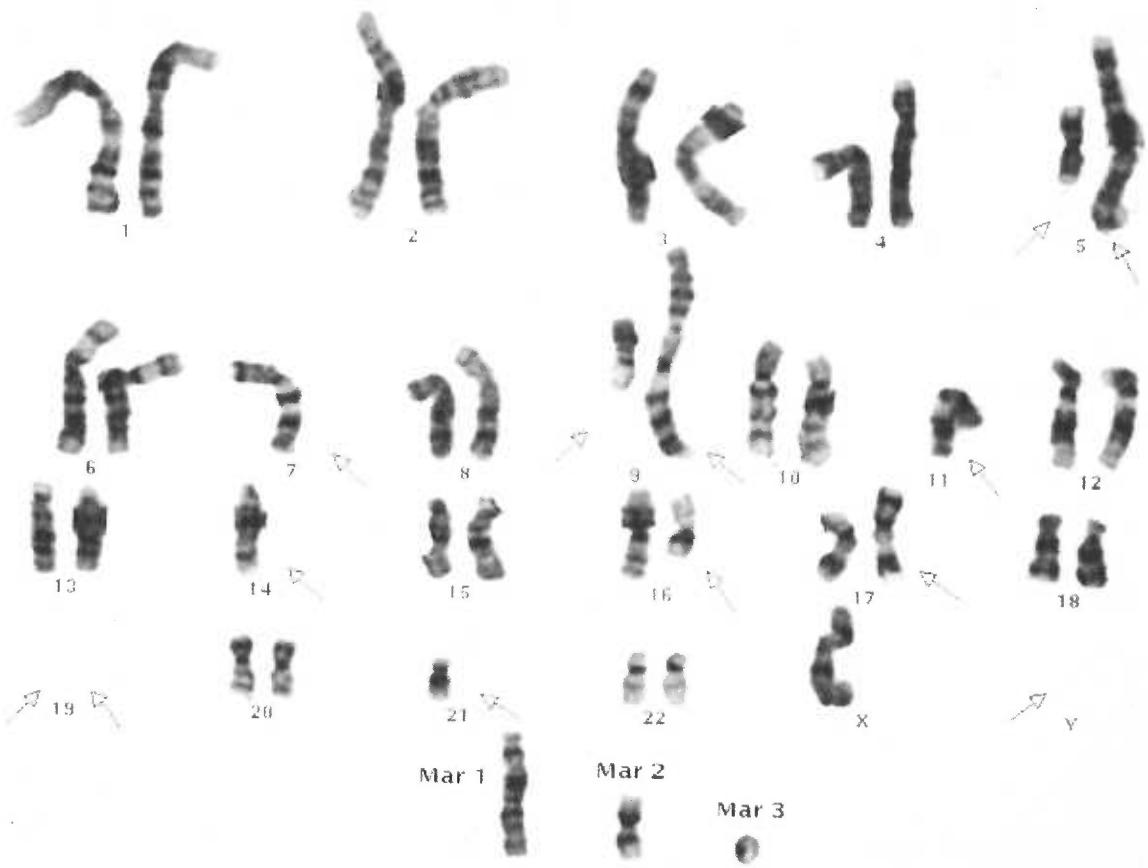


Figure 3-3 Legend: The karyotype of UoC-M1 is complex. As shown here, the cytogenetic abnormalities in the UoC-M1 karyotype are complex, including many translocations and several numerical or structural losses including 5q-, deletions of 7q, monosomy 11, nullisomy 19, monosomy 21, -Y, and marker chromosomes.[189] Such a complex karyotype is frequently seen in individuals presenting with MDS/AML derivative of prior therapy or a genetic syndrome such as seen in Fanconi anemia, though the patient's medical history is unremarkable for each of these categories of AML predisposition. G-banded karyotypes were provided by Carol Reifsteck (OHSU).

Figure 3-4

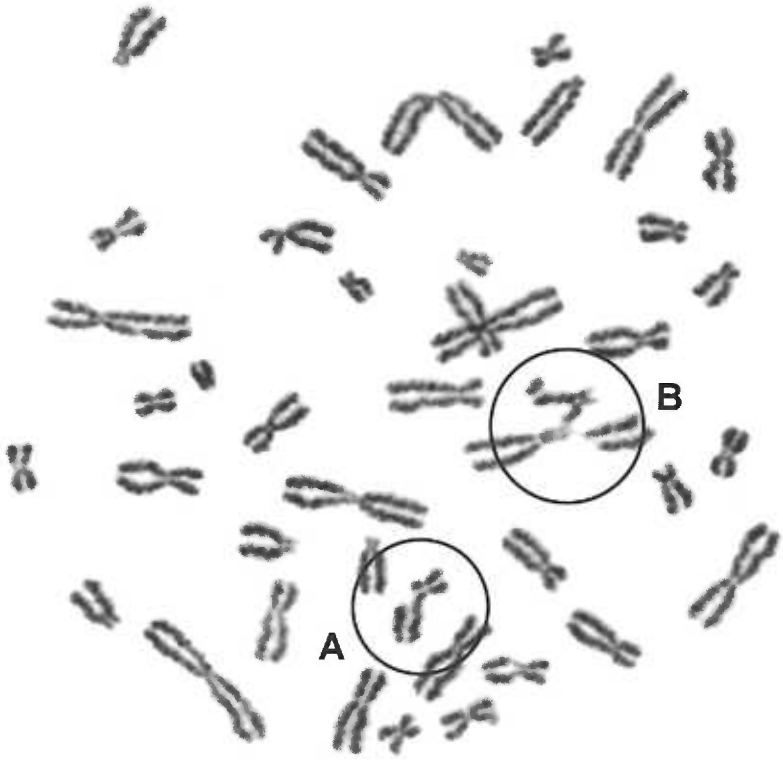


Figure 3-4 Legend: UoC-M1 is sensitive to chromosomal breakage and radial formation following mitomycin-C (MMC) treatment. The circumscribed areas indicate representative chromosomal anomalies in UoC-M1. (A) Single chromatid break, (B) Complex chromosomal rearrangement with at least two chromosomal breaks and one end-to-end fusion (radial). Such findings in clastogen-treated cells represent the clinical diagnostic laboratory standard for Fanconi anemia. UoC-M1 also demonstrated spontaneous chromosomal breakage without treatment (data not shown). Breakage analysis was performed by Carol Reifsteck (OHSU).

Figure 3-5

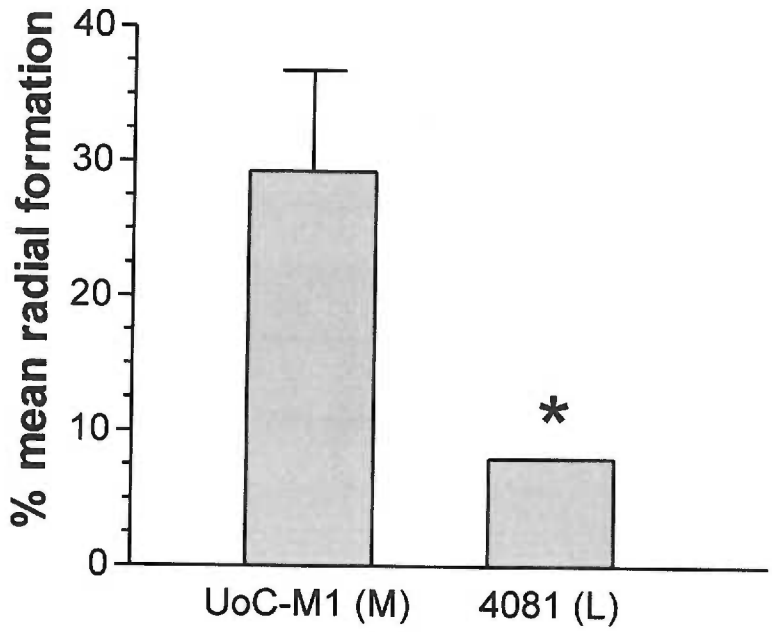


Figure 3-5 Legend: A lymphblastoid cell line (4081) from the same patient is resistant to MMC. The myeloid cell line (UoC-M1) was exposed to MMC as was a lymphoblastoid cell line (4081) derived from the same patient. While the UoC-M1 cell line continually demonstrated radial formation in excess of 20% (averaging 29%) in multiple experiments (left bar), the 4081 cell line was resistant to chromosomal breaks and radial formation after exposure to MMC (right bar). Treatment with DEB produced similar results (data not shown). Error bars represent standard deviations. * (p = 0.003) M= myeloid; L = lymphoid

Figure 3-6

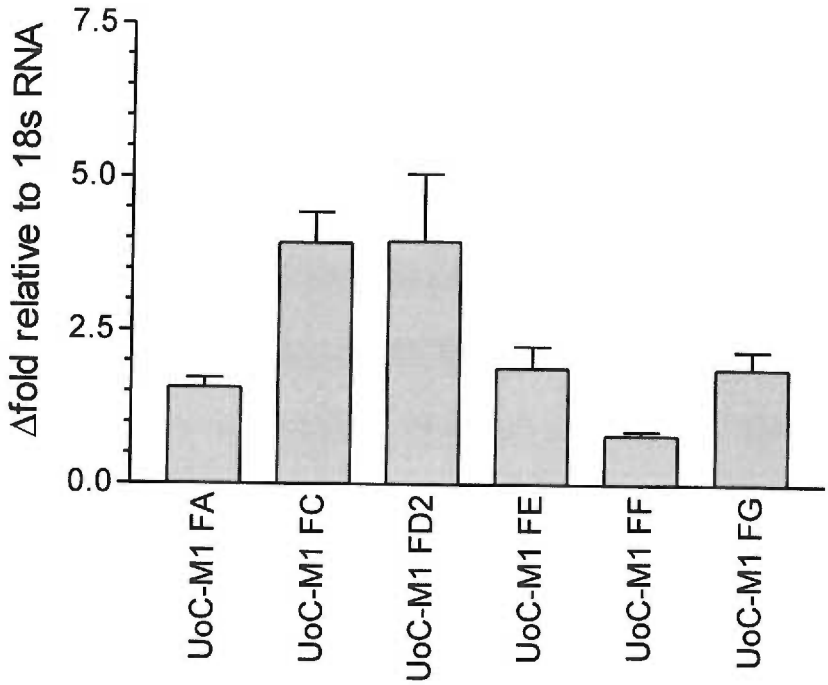

Δfold relative to 18s RNA
7.5
5.0
2.5
0.0
UoC-M1 FA
UoC-M1 FC
UoC-M1 FD2
UoC-M1 FE
UoC-M1 FF
UoC-M1 FG

Figure 3-6 Legend: Fanconi anemia gene transcripts are not reduced in UoC-M1. To determine if transcriptional repression was responsible for MMC sensitivity in UoC-M1, quantitative, real-time, reverse-transcription, polymerase chain reaction (QRT-PCR) was performed relative to the myeloid leukemic cell line Mo7e (see text) for all six cloned FA genes. Compared to Mo7e, UoC-M1 does not exhibit reduced levels of mRNA for *FANCA*, *FANCC*, *FANCD2*, *FANCE*, and *FANCG*. *FANCF* mRNA was reduced by 50%, a finding we attribute to monosomy 11 in UoC-M1[189] as *FANCF* is encoded on chromosome 11.[100] Reactions were performed in at least triplicate. Error bars represent standard deviations. Fold values were obtained by externally standardizing against identical amplifications in Mo7e and by internally standardizing against 18S RNA in each cell line.

Figure 3-7

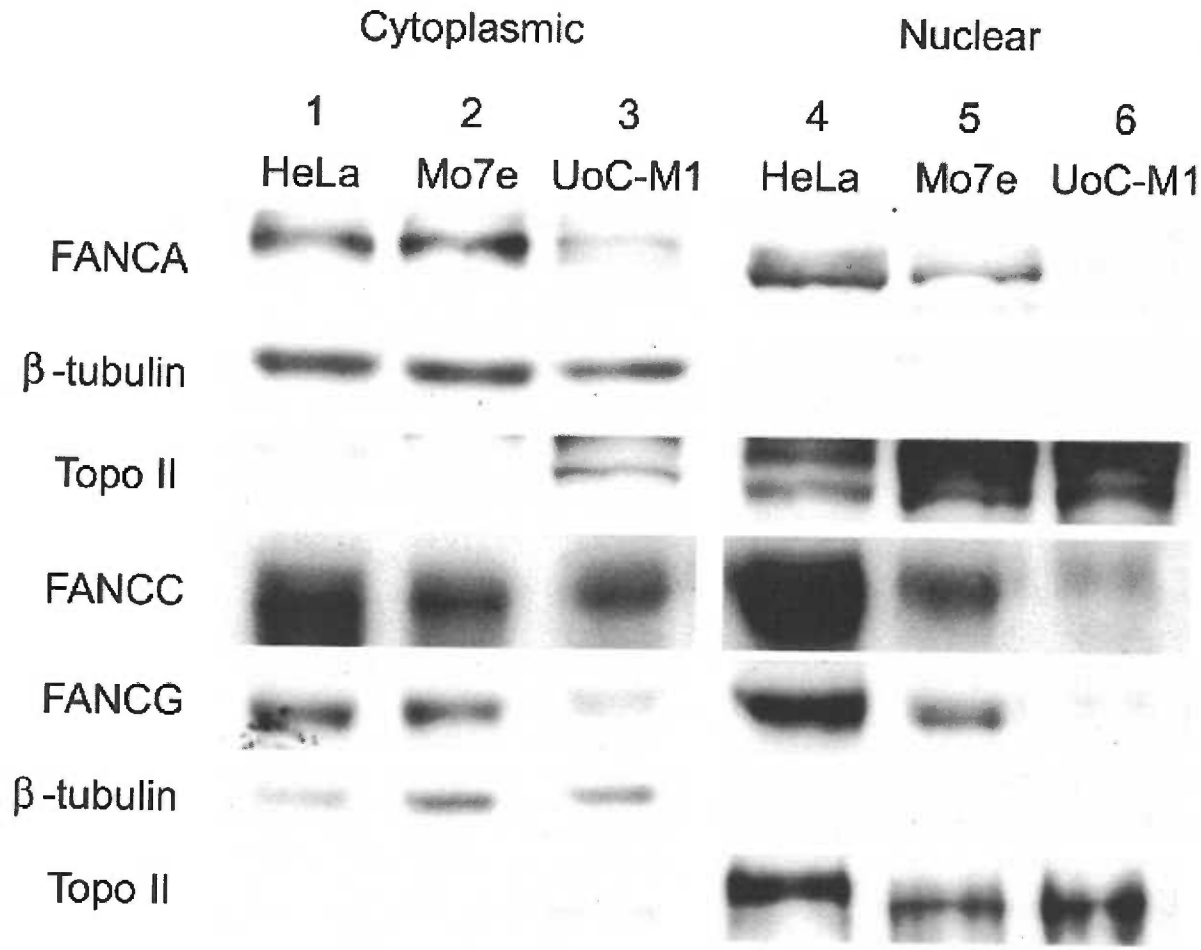

Cytoplasmic
Nuclear
1
2
3
4
5
6
HeLa
Mo7e
UoC-M1
HeLa
Mo7e
UoC-M1
FANCA
β-tubulin
Topo II
FANCC
FANCG
β-tubulin
Topo II

Figure 3-7 Legend: Nuclear FA proteins are reduced in UoC-M1 cells. To correlate FA protein levels with MMC sensitivity, UoC-M1 and control cells (HeLa and Mo7e) were fractionated into cytoplasmic and nuclear components. Immunoblots were probed with antisera for the FANCA, FANCC, and FANCG proteins. As shown here, compared to the human, cervical carcinoma cell line HeLa (lane 1) and the factor-dependent, myeloid cell line Mo7e (lane 2), UoC-M1 (lane 3) has a mild reduction in the levels of cytoplasmic FANCA, FANCC, and FANCG proteins (rows 1, 4, and 5 respectively). However, nuclear fractions (lanes 4, 5, and 6) demonstrated a reduction of FANCC in UoC-M1 (row 4, lane 6) and a nearly complete absence of nuclear FANCA (row 1, lane 6) and FANCG proteins (row 5, lane 6). Fractionation purity was confirmed by reprobing the filters with antisera against beta-tubulin (rows 2 and 6) and topoisomerase II (rows 3 and 7).

Figure 3-8

Figure 3-8 Legend: UoC-M1 does not form FANCD2-L. As it has been shown that an intact FA protein complex containing the FANCA, FANCC, FANCE, FANCF, and FANCG proteins is required for the post-translational modification of FANCD2 (*i.e.* monoubiquitinylation = FANCD2-L or "FANCD2-long form"),[200] a purified polyclonal antibody was developed to FANCD2 in order to interrogate the post-translational modification status of this protein. As shown in figure 3-8, a lymphoblastoid cell line control from an FA-C patient (HSC536N) does not have FANCD2-L while retroviral correction of the *FANCC* defect in these cells (HSC536N/FANCC) restores FANCD2-L. In keeping with its proposed FA status, the UoC-M1 myeloid cell line is incapable of forming FANCD2-L while a lymphoblastoid cell line derived from the same patient (4081) is capable of forming FANCD2-L, supporting the assignment of a lineage-restricted FA defect in the myeloid cell line. FANCD2 immunoblot analysis was performed by Tracy Christianson in our lab.

Figure 3-9

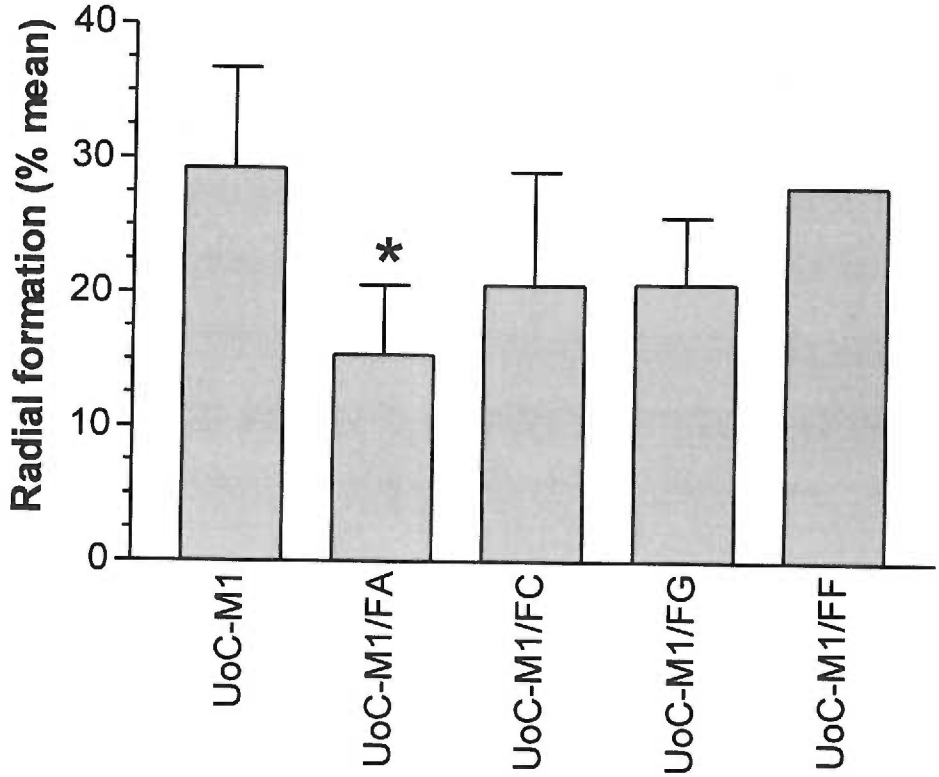

Radial formation (% mean)
40
30
20
10
0
*
UoC-M1
UoC-M1/FA
UoC-M1/FC
UoC-M1/FG
UoC-M1/FF

Figure 3-9 Legend: Introduction of a *FANCA* cDNA reduces MMC sensitivity in UoC-M1. Restoration of MMC resistance in UoC-M1 was attempted by introducing cDNA's for *FANCA*, *FANCC*, *FANCF*, or *FANCG*. Resistance was evaluated by cytogenetic analysis of radial formation in transduced lines compared to non-transduced UoC-M1 cells after treatment with MMC. As shown here, compared to UoC-M1 controls, only transduction with a *FANCA* cDNA (UoC-M1/FA) reduced MMC resistance significantly (*p = 0.002). Neither *FANCC, FANCF,* nor *FANCG* transduction (UoC-M1/FC, UoC-M1/FF, and UoC-M1/FG) significantly altered MMC sensitivity (p = 0.059 and p = 0.064 for UoC-M1/FC and UoC-M1/FG respectively). UoC-M1/FF demonstrated MMC sensitivity in two independent experiments with 38% and 26% radial formation in each.

Figure 3-10A

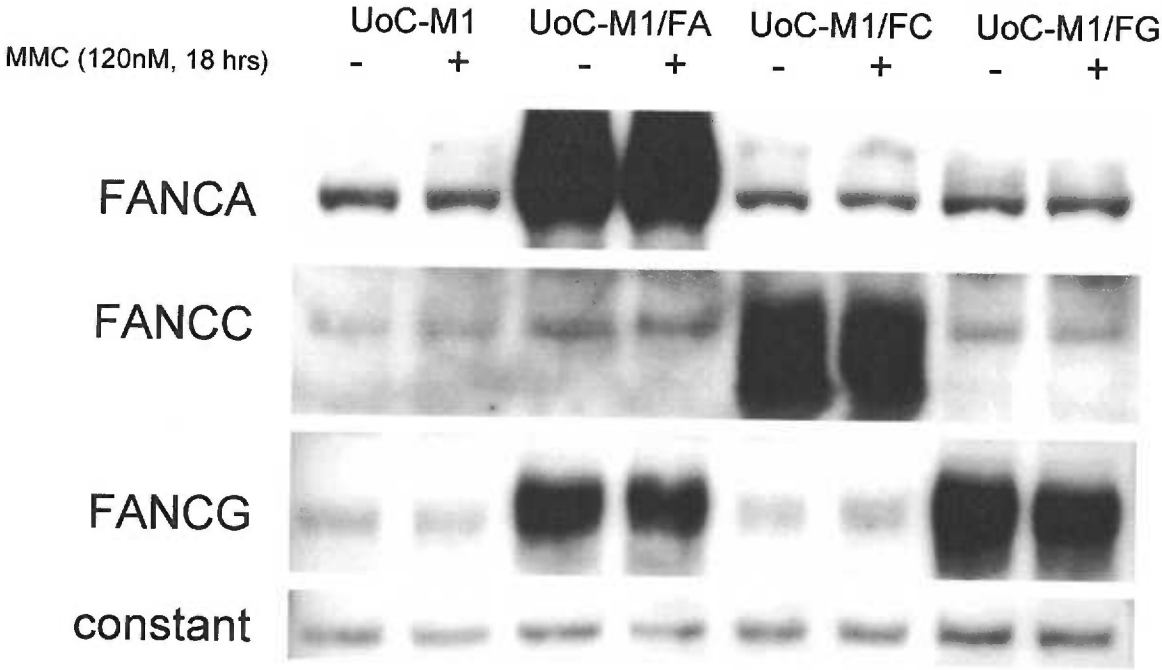


Figure 3-10B

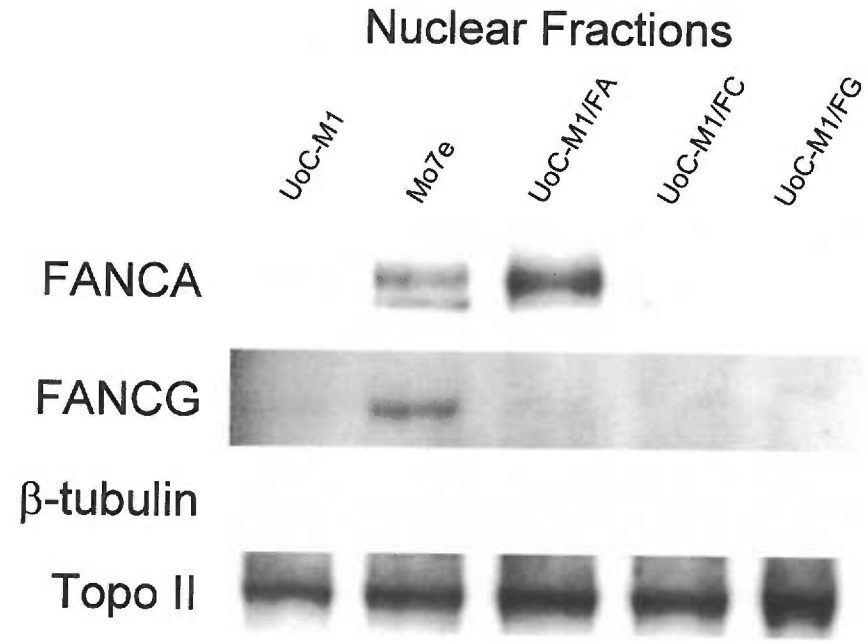


Figure 3-10C

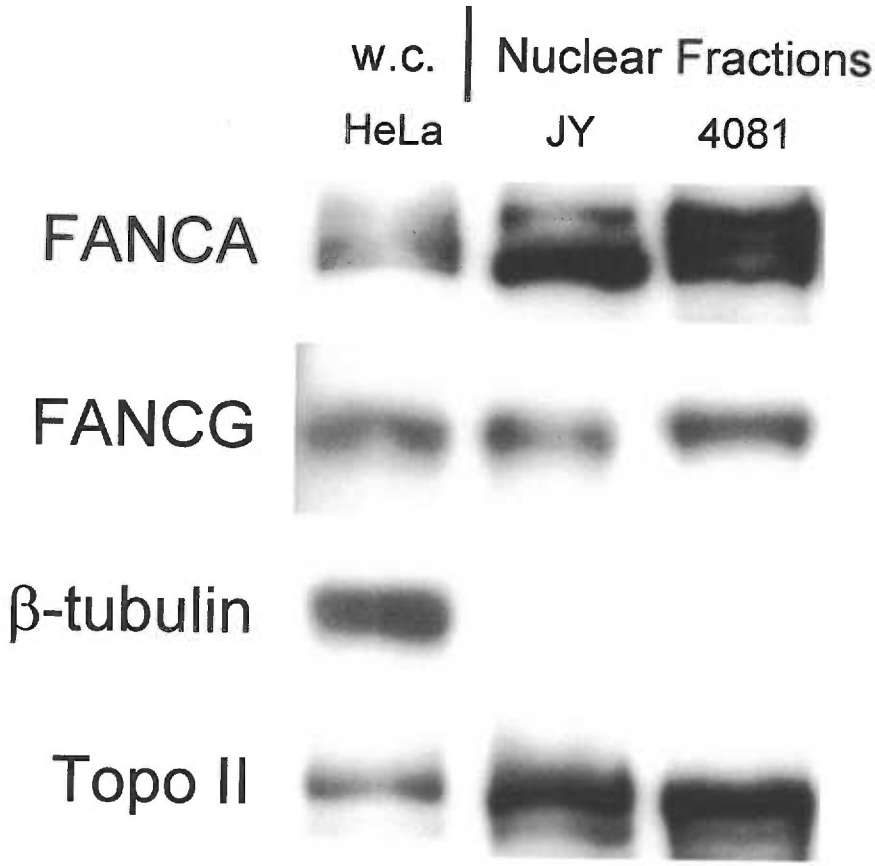

w.c.
Nuclear Fractions
HeLa
JY
4081
FANCA
FANCG
β-tubulin
Topo II

Figure 3-10A-C Legend: *FANCA* transduction enhances FA protein complex stability and restores nuclear FANCA levels. As shown in figure 3-10A, retroviral transduction with each of three FA cDNA constructs resulted in increased levels of their respective proteins. Furthermore, transduction with *FANCA* (UoC-M1/FA) also led to a dramatic increase in cellular levels of FANCG while the reverse was not observed (*i.e.* transduction with *FANCG* [UoC-M1/FG] did not increase cellular levels of FANCA). Due to reports that FANCA and FANCG form a protein complex that leads to the increased stability of each molecule,[201,202] we propose that UoC-M1 has a specific defect of either *FANCA* or a FANCA cofactor. All four rows depict data obtained from the same filter. Figure 3-10B illustrates nuclear fraction immunoblots from UoC-M1, the three transduced lines (UoC-M1/FA, UoC-M1/FC, and UoC-M1/FG), and the myeloid cell line Mo7e probed with antisera raised against FANCA (row 1) and FANCG (row 2). Transduction with *FANCA* restored nuclear levels of FANCA in UoC-M1/FA (row 1, column 2) while transduction with *FANCG* did not increase nuclear levels of FANCG (UoC-M1/FG) (row 2, column 5). 3-10C: Nuclear levels of FANCA and FANCG are not reduced in the lymphoblastoid cell line 4081. Nuclear levels of the FANCA and FANCG proteins correlate with MMC resistance in a lymphoblastoid cell line (4081) established from the same patient as UoC-M1. The cell line JY is an EBV-transformed lymphoblastoid cell line established from a normal adult volunteer and also demonstrates cellular resistance to MMC. Lane 1 is a whole cell extract from HeLa to demonstrate immunoreactivity for all antisera.

Table 3-1: Gene dosage analysis for *FANCA* and *FANCG* in UoC-M1.

FANCA

Exp.#	Control	ex 5	ex 11	ex 17	ex 19/20	ex 31
1	MPZ1	1.6	0.84	0.92	0.8	0.94
2	MPZ1	0.87	0.82			
3	NCX1	1.03	0.61			
4	NCX1		0.97			
ave. dosage quotient:		1.17	0.81	0.92	0.8	0.94

FANCG

Exp.#	Control	ex 1	ex 14
1	MPZ1	1.26	1.7
2	NCX1	1.13	1.52
ave. dosage quotient:		1.2	1.61

Gene dosage and sequencing analysis was performed by Marc Tischkowitz, King's College, London.

Table 3-2: Haplotype analysis in UoC-M1 and 4081 indicates they are derived from the same individual.

Marker	UoC-M1 alleles	4081 alleles	band
D7S531	295	295, 280	7p22.3-7p22.2
D7S493	206	206, 229	7p21.3-7p15.2
D7S484	114	114, 114	7p22.1-7p14.2
D7S519	267	267, 271	7p13-7p12.3
D7S502	300	300, 304	7q11.21-7q11.22
D7S669	190	190, 181	7q11.22-7q21.11
D7S486	221	221, 231	7q31.31
D7S640	134	134, 120	7q23.3-7q33
D7S661	313	313, 319	7q34-7q35
D7S798	71	71, 76	7q35
D7S2465	333	333, 335	7q35
D8S550	193, 204	193, 204	8pter-8qter

Note: Only a single allele for chromosome 7 makers in UoC-M1 is indicated as UoC-M1 has complex cytogenetics including functional monosomy 7 (see text).[189] Haplotype analysis was performed in conjunction with Petra Jakobs (OHSU).

Figure 3-11A

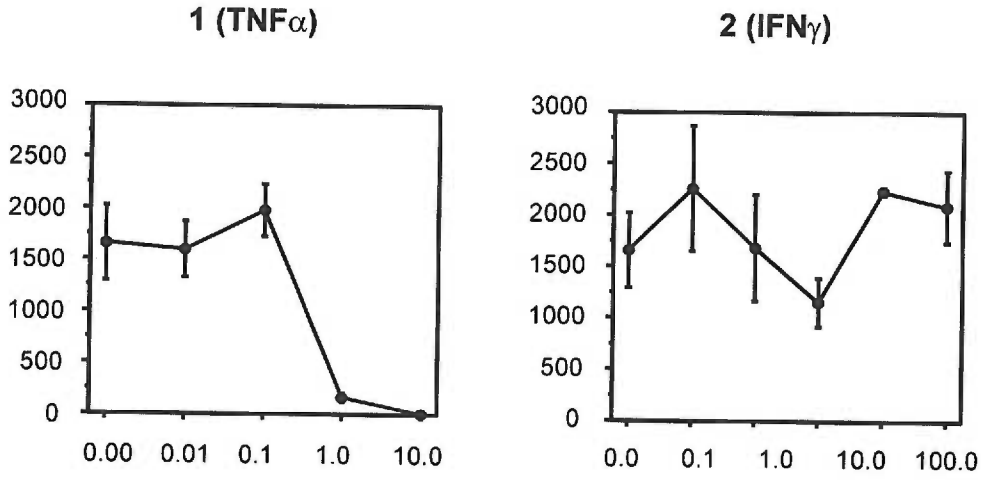


Figure 3-11B

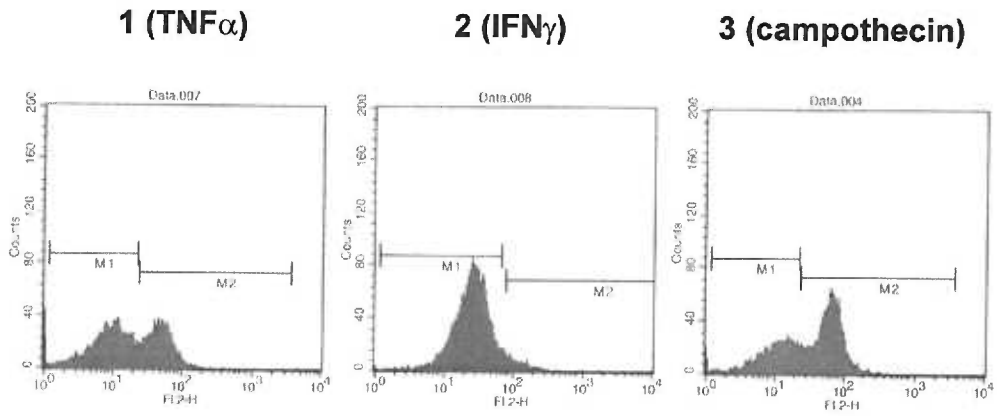


Figure 3-11C

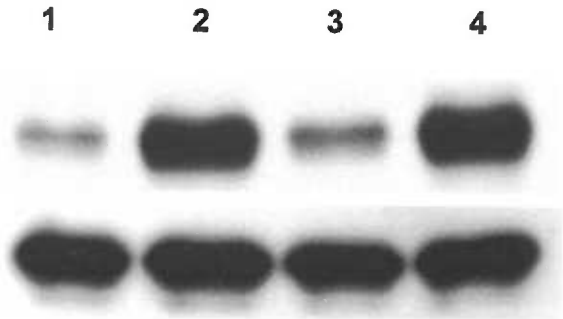


Figure 3-11A-C Legend: UoC-M1 is resistant to IFNγ but sensitive to TNFα mediated apoptosis. In figure 3-11A, the X-axis denotes increasing concentrations of cytokine (ng/ml) while the Y-axis shows absolute colony numbers per plate (with standard deviations). Colony forming assays with UoC-M1 and TNFα (panel 1) or IFNγ (panel 2) demonstrate differential cytokine responsiveness with sensitivity to TNFα and resistance to IFNγ. The nature of TNFα inhibition is apoptotic as shown in figure 3-11B. Panel 1 indicates the result of flow cytometry against activated caspase-3 after cellular treatment with a single high dose of TNFα (10 ng/ml) while panel number 2 reflects the result of treatment with a single high dose (100 ng/ml, the highest concentration routinely used in colony forming assays in our lab)[206] of IFNγ. Panel 3 is a campothecin positive control. To determine whether or not IFN-resistance was derivative of an inactive cytokine receptor, cells were either untreated (panel 1), treated with IFNγ (panel 2), TNFα (panel 3), or both (panel 4) and the ability to increase levels of an IFN-responsive protein (IRF-1) was determined via immunoblot analysis of whole-cell lysates. As shown in figure 3-11C (top portion), probing with antiserum to IRF-1 indicated an increase with IFNγ treatment, clarifying that IFN-resistance could not be explained by a receptor complex that was enzymatically inactive. The bottom portion of 3-11C demonstrates equal loading by reprobing with the nucleolar, ribosomal RNA-transport protein, nucleophosmin (NPM). Caspase 3 flow cytometry was performed by Michael O' Dwyer in our lab.

Figure 3-12

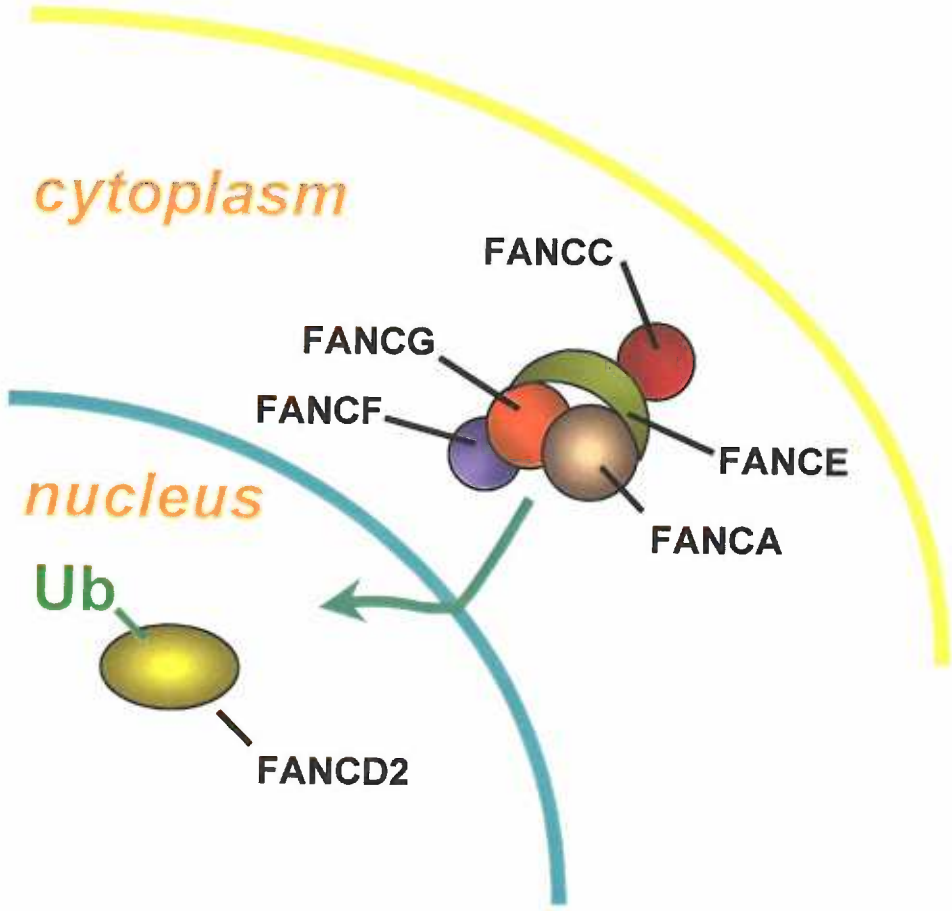


Figure 3-12 Legend: At least five FA proteins act in a multimeric complex upstream of a sixth FA protein. There exists an "upstream" multimeric complex made up of at least the FANCA, FANCC, FANCE, FANCF, and FANCG proteins.[201,202,219-224] This complex is required to permit a post-translational modification (monoubiquitinylation) of the *FANCD2* gene product.[200] Regarding these observations, two important aspects remain unknown: how the FA multimeric protein complex enters the nucleus and how this complex leads to the addition of a single ubiquitin molecule (Ub) to FANCD2.

Figure 3-13

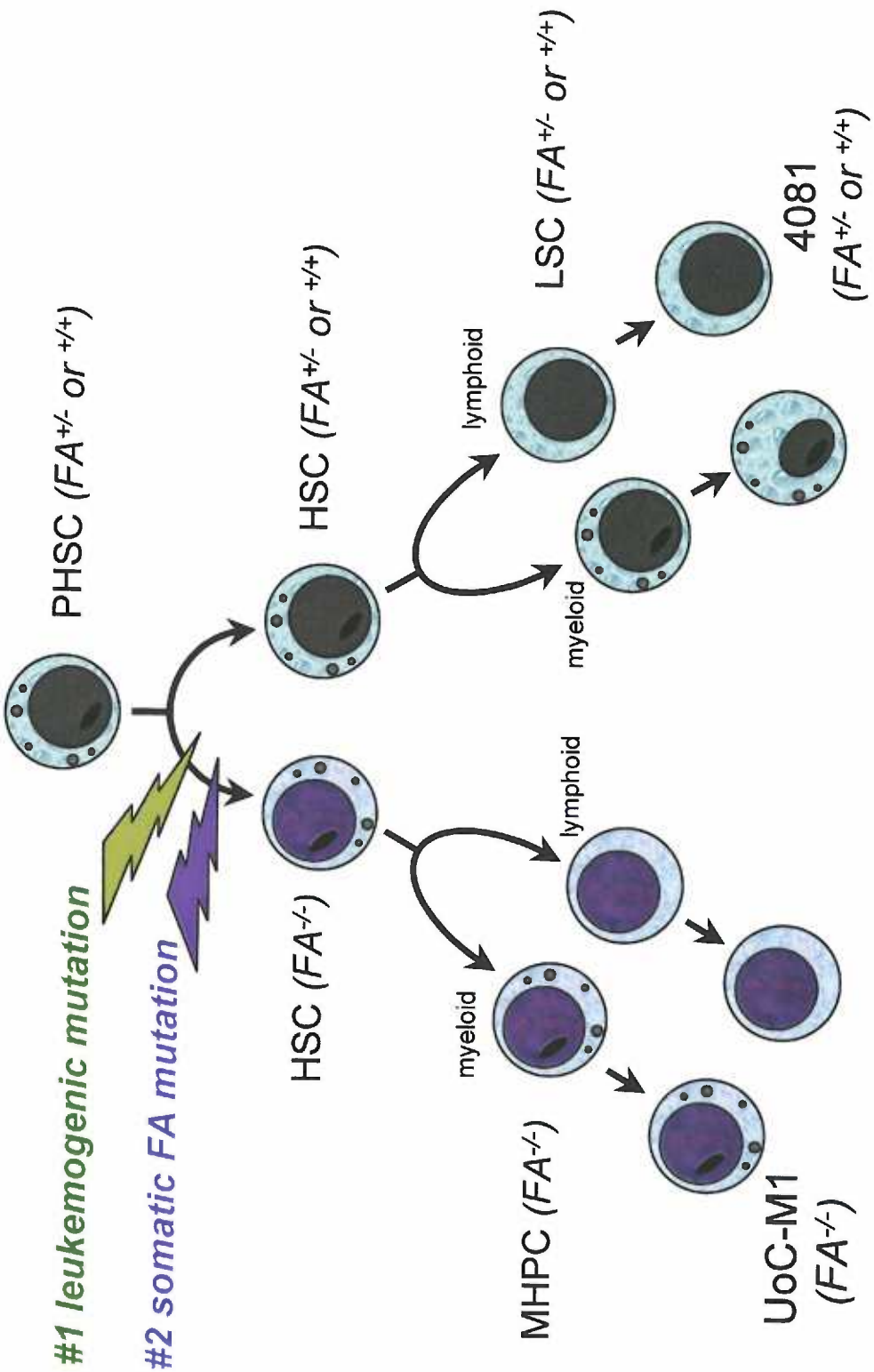

PHSC (FA+/- or +/+)
#1 leukemogenic mutation
#2 somatic FA mutation
HSC (FA+/- or +/+)
HSC (FA-/-)
lymphoid
myeloid
lymphoid
myeloid
LSC (FA+/- or +/+)
4081
(FA+/- or +/+)
MHPC (FA-/-)
UoC-M1
(FA-/-)

Figure 3-13 Legend: Schematic model for lineage-restricted anomalies of FA protein function in UoC-M1 and 4081. A FANCA correctable defect of FA protein dysfunction exists in leukemic cells (UoC-M1) of a patient but not in lymphoblasts (4081) from the same individual. We propose that a lineage-restricted FA defect occurred during the evolution of leukemia in the UoC-M1 patient. As shown here diagrammatically, an acquired defect of FANCA protein function could have occurred during hematopoietic ontogeny at a point coincident to other observed genetic defects that contribute to leukemogenesis.[11] We propose that this resulted in complex cytogenetics, a more severe clinical course, and in essence, a presentation in this patient of AML cytogenetically identical to the type of AML seen in children and adults with Fanconi anemia. PHSC = pluripotential hematopoietic stem cell; HSC = hematopoietic stem cell; MHPC = multipotential hematopoietic progenitor cell; LSC = lymphoid stem cell; FA = any FA protein or factor that enhances FA protein function

Figure 3-14

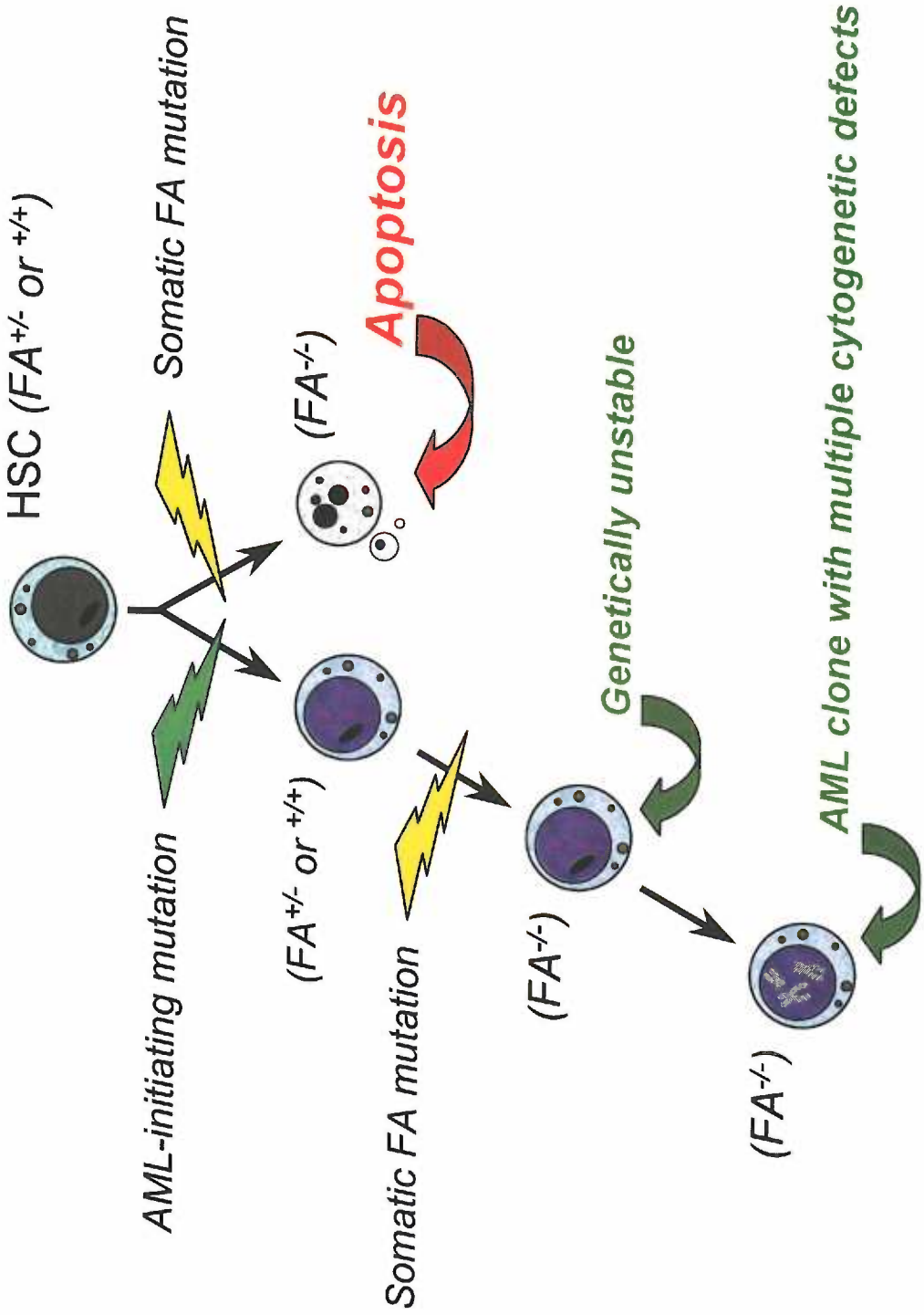

HSC (FA+/- or +/+)
Somatic FA mutation
AML-initiating mutation
(FA-/-)
Apoptosis
(FA+/- or +/+)
Somatic FA mutation
(FA-/-)
Genetically unstable
(FA-/-)
AML clone with multiple cytogenetic defects

Figure 3-14 Legend: Acquired dysfunction of FA proteins; a potential progression factor in secondary AML/MDS. We propose that FANCA dysfunction was acquired by an evolving clone of initiated hematopoietic stem cells.[11] As shown on the right, an early FA inactivating event would render the cell hypersensitive to apoptotic stimuli, resulting in apoptosis. Therefore, unless some adaptive mutation occurred rapidly in progeny of the transformed clone, the clonal progeny, being highly apoptotic, would not survive. However, as shown on the left, if a similar event occurred in an already initiated cell, the cell could resist apoptotic cues. The FA defect would then contribute to genetic instability and lead to multiple cytogenetic defects in the evolving clone. HSC = hematopoietic stem cell; FA = any FA protein or factor that enhances FA protein function

Chapter Four:

Factors that contribute to the survival of the leukemic blasts

4-1: Introduction

The malignant process occurs due to the disruption of normal mechanisms of cellular homeostasis. Such abnormalities may arise in many forms and most avenues controlling cellular growth, differentiation, and/or senescence have been associated with either the onset or progression of cancer. In the preceding chapters, hypotheses were made and experiments detailed investigating the proximal end of the leukemogenic process, namely the role of selective pressure in MDS/AML in Fanconi anemia and in non-FA, secondary MDS/AML

In chapter one, it was proposed that acquired resistance to inhibitory cytokines would allow evolving MDS/AML clones to evade the hyperapoptotic ground state extant in the FA bone marrow.[137] In chapter two, proof-of-principle for this adaptive evolution hypothesis was established by means of demonstrating acquired inhibitory cytokine resistance in individuals and multiplex families progressing to MDS/AML from an initial state of bone marrow failure in FA. Such cytokine insensitivity was also shown to occur in non-FA, secondary MDS/AML and in an index case of somatic Fanconi anemia (chapter three). In chapter 3 I illustrated the potential contribution of genetic instability (via acquired

FA protein dysfunction) to leukemia progression in patients with sporadic leukemia (with no evidence of inherited FA) lending greater support to the premise that FA-MDS/AML and non-FA, secondary MDS/AML are closely related diseases.

However, while the development of apoptotic resistance prolongs cell survival, and while the acquisition of genetic instability endows them with the flexibility to rapidly overcome other regulatory hurdles by adaptive mutations, these events do not in themselves define malignancy. Rather, they set the stage (by simply preventing apoptosis) for the decay of other pathways such as those that control mitosis, growth factor receptor signaling, cellular adhesion, and differentiation. One attribute of UoC-M1 cells that remained unexplained was its capacity to proliferate in culture without requiring the addition of exogenous hematopoietic growth factors.

In an effort to better understand the molecular circumstances that contributed to this autonomous growth, a series of *in vivo* and *in vitro* experiments were performed with the spontaneously-immortalized, myeloid leukemic cell line UoC-M1.[189] This chapter will illustrate results of these studies and indicate that:

(a) UoC-M1 cells form highly vascularized solid tumors in immunodeficient (NOD/SCID)[239] mice; a process likely derivative of the cell's

constitutive production of the key angiogenic cytokine, vascular endothelial growth factor (VEGF),[240]

(b) other cytokines important for the growth and maintenance of hematopoietic cells (including VEGF-modulators) were absent from cell culture supernatants,

(c) UoC-M1 is capable of responding to VEGF in a growth-stimulatory, autocrine manner; a situation congruent with recent observations from other investigators that a proangiogenic phenotype is common in MDS and AML cells,[241-250]

(d) that constitutive phosphorylation of STAT (signal transducer and activator of transcription) molecules is present in UoC-M1, a situation that probably derives from VEGF signaling, signaling through an as yet unidentified cytokine receptor or intracellular aberrations that result in ground state activation of STAT molecules absent a ligand-receptor interaction. In fact, that a VEGF receptor-specific tyrosine kinase inhibitor was marginally capable of retarding UoC-M1 cell growth in colony-forming unit assays (CFU-A), while a VEGF-neutralizing antibody was capable of only transiently impairing STAT phosphorylation suggests that more than one mechanism may underlie STAT activation, and

(e) a large number of experiments (19 in all), seeking to test the role of genes located on chromosome 7 in UoC-M1 cells, failed. I attempted to accomplish this objective by reintroducing human chromosome 7 via whole cell fusion and microcell-mediated chromosome transfer.

4-2: Materials and methods

Tumor formation - UoC-M1 cells were cultured in suspension to sub-confluency in RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum (Hyclone Labs, Logan, UT). Cells were counted, rinsed three times in sterile PBS, and resuspended to a final concentration of $5x10^7$ cells per ml. 100 µl of the cell suspension ($5x10^6$ cells) was injected sub-cutaneously (left, epi-scapular pad) into each of three anesthetized, 9-week old, female, non-obese diabetic severe combined immunodeficient (NOD-*scid/scid*) mice[239] and allowed to incubate for 30 days *in vivo*. The specific strain of NOD/SCID mice used was (NOD/LtSz-*Prkdc*^{scid}/J) (Jackson Laboratories, Bar Harbor, ME).

Tumor harvest - When the diameter of masses at the injection site was estimated to be 2 cm, experimental animals were euthanized and subjected to general necropsy. Multiple tissues were harvested including tumor for serial injections into secondary recipients or karyotype analysis, and preparation of paraffin-embedded sections of tumor, liver, spleen, and whole femurs. Primary tumors were excised from freshly euthanized animals in a sterile field, weighed, and a single cell suspension obtained by grinding the tissue through a grade 100 stainless steel mesh (Sigma, St. Louis, MO) followed by repeated aspirations through a series of progressively-higher gauge needles (20 ga., 23 ga., and 25 ga.) in a suspension of sterile PBS. $5x10^6$ cells were prepared and injected into

secondary NOD-*scid/scid* recipients as before. Cytopreps were obtained by resuspending ground tumor tissue to a concentration of $1x10^6$ cells/ml in PBS + 5% FCS and centrifugation in a cytopreparation centrifuge loaded with glass microscope slides. Slides were stained with Wright's/Giemsa after fixing in 100% methanol and visualized via oil immersion microscopy.

Paraffin Sections - Tissue sections were obtained by dehydrating specimens in graded alcohol and xylenes before being placed in paraffin. Mounted tissues were sectioned at 5 μm on a standard, rotary microtome and fixed to glass slides. Slides were baked at 60°C for I hour, deparaffinized, stained with haematoxylin and eosin (H/E), coverslipped, and visualized by light microscopy.

ELISA - To determine if UoC-M1 cells were secreting factors that could account for their factor-independent growth and/or angiogenic phenotype, enzyme-linked immunosorbent assays (ELISAs) were performed on supernatants from cells cultured *in vitro*. Cells were seeded at a density of $1x10^6$ cells/ml in RPMI 1640 (Gibco, Gaithersburgh, MD) and 10% FCS (Hyclone Labs, Logan, UT) and allowed to incubate for 5 days in a humidified, CO_2-buffered (5%) chamber at 37°C. At the end of the incubation period, 5 ml of conditioned media was removed from each flask and passed through an 0.22 μm cellulose acetate filter to exclude whole cells. The filtered eluate was then aliquoted into 1.5 ml Eppendorf micro-centrifuge tubes and spun at 750 x g in a bench-top

centrifuge for 5 minutes, 750 ml of supernatant transferred to a new tube, and centrifuged once again as before. Supernatants prepared in this manner were then stored at –20°C prior to analysis. ELISA was performed using commercially available kits (R&D Systems, Inc., Minneapolis, MN) for the following cytokines: fms-like tyrosine kinase 3 ligand (FLT-3l), granulocyte-macrophage colony stimulating factor (GM-CSF), interleukin-1 beta (IL-1β), IL-4, IL-6, stem cell factor (SCF), tumor necrosis factor alpha (TNFα), thrombopoietin (TPO), and vascular endothelial growth factor (VEGF) according to the manufacturer's instructions.

CD34 cell isolation - CD34+ bone marrow cells were purified from normal volunteers under a VAMC/IRB-approved protocol of informed consent. Heparinized bone marrow aspirates were obtained by iliac crest puncture in unsedated donors. Low density bone marrow cells (LDBMC's) were obtained via density centrifugation by underlaying 10 ml of polysucrose (Ficoll) (Sigma, St. Louis, MO) to 10 ml of heparinized bone marrow mixed with 10 ml of warm RPMI-1640. Gradients were centrifuged at 1,200 X g for 20 minutes at 20°C with no brake. LDBMC's were rinsed three times in room temperature PBS before purification of the CD34+ cell fraction via antibody-conjugated magnetic particles using a commercial kit per the manufacturer's instructions (Miltenyi Biotech, Auburn, CA). CD34+ cells were seeded at a density of $1x10^6$ cells/ml in RPMI 1640 + 10% FCS and a cocktail (termed SIE) of human stem cell factor (hSCF, 50 ng/ml), interleukin-3 (IL-3, 10 ng/ml), and erythropoietin (EPO, 2U ng/ml) to foster primitive hematopoietic elements.

Flow cytometry - Flow cytometry analysis was performed to verify and add to reported cell surface markers in the UoC-M1 cell line.[189] Cells were cultured and washed as before, then fixed and permeabilized by the Cytofix/Cytoperm kit (Pharmingen, San Diego, CA) according to the manufacturer's instructions. $5x10^5$ cells were stained (in duplicate tubes) using rabbit anti-human primary antibodies that recognize the carboxy terminal portions of FLT-1 (*sc-316*, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and KDR (*sc-6251*, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) followed by FITC-conjugated, goat anti-rabbit or goat anti-mouse secondary antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for FLT-1 and KDR respectively. FITC-conjugated, goat anti-rabbit or anti-mouse immunoglobulin (Ig) isotype controls were also incubated and analyzed as staining specificity controls. 10,000 cells were analyzed per condition by flow cytometry using a FACSCaliber machine (Becton-Dickinson, Mansfield, MA) and plots obtained via Cell Quest software (v. 3.3, Becton Dickinson, Mansfield, MA).

RT-PCR - Reverse transcription, polymerase chain reaction (RT-PCR) was performed to qualitatively assess the transcriptional state of VEGF-related factors. $1x10^6$ UoC-M1 cells were rinsed three times in PBS and plated into each well of a 12-well polystyrene tissue culture dish and allowed to incubate for 48 hours at 37°C. After incubation, the cell suspension (1 ml) was aspirated and pelleted at 300 x g in a microcentrifuge tube for 5 minutes. 750 μl of the cell

culture supernatant was then removed and replaced with 750 μl of Tri-Reagent (Molecular Research Center, Cincinnati, OH) and 200 μl of chloroform, vortexed, and centrifuged at 12,000 x g for 15 minutes at 4°C to separate aqueous (RNA-containing) and organic (genomic DNA and protein containing) phases. The aqueous phase was aspirated and added to 650 μl of 100% iso-propanol and stored at –20°C overnight to precipitate RNA. Following RNA precipitation, tubes were spun at 12,000 x g for 15 minutes at 4°C, rinsed once with cold 70% EtOH, and recentrifuged. Alcohol was aspirated and the RNA pellets were air-dried before being resuspended in 100 μl of sterile, DEPC-treated water and quantitated spectrophotometrically. Total RNA was used to create first-strand cDNA as follows: 1 μg of total RNA was combined with 200 ng random hexamers and water then heated to 70°C for 5 minutes. To this mixture was added dNTP's (final concentration 2 mM), first strand buffer (to 1X), DTT (final concentration 10 mM), 200 units of Superscript II reverse transcriptase, and water to a final volume of 20 μl (all reagents: Life Technologies, Gaithersburgh, MD). The first-strand reaction was performed in an automated thermocycler as follows: 10 minutes at 25°C, 50 minutes at 42°C, 15 minutes at 70°C, and hold at 15°C. 0.1 μl of the subsequent cDNA reactions were used per RT-PCR amplification (7 minute denaturation at 94°C followed by 35 cycles of 94°C for 1 minute, 55°C for 1 minute, 72°C for 2 minutes with a final extension cycle at 72°C for 10 minutes). Primer sequences were designed online using Primer 3 software (Whitehead Institute/MIT Center for Genome Research, Steve Rozen, Helen J. Skaletsky

(1998); Primer3 Code available at http://www-genome.wi.mit.edu/genome_software/other/primer3.html). The sequences are as follows (F = forward primer; R = reverse primer): CUL-2/F-CTTACTCCGTGCTGTGTCCA; CUL-2/R-GCCTTATCCAACGCACTCAT; elongin-B/F-TCTTGGATGATGGCAAGACA; elongin-B/R-TTGTTCATTGGCACTGCTTC; elongin-C/F-GGCACGATAAAAGCCATGTT; elongin-C/R-TAAGAAGTTCGCAGCCATCA; HIF-1a/F-CCTTCGATCAGTTGTCACCA; HIF-1a/R-TGGGTAGGAGATGGAGATGC; HIF-1b/F-CAAGCCCCTTGAGAAGTCAG; HIF-1b/R-GAGGGGCTAGGCCACTATTC; SP1/F-CACCACTCTCACACCCATTG; SP1/R-ACCAAGCTGAGCTCCATGAT; VHL/F-ATGCCCCGGAGGGCGGAGAA; VHL/R-TCAATCTCCCATCCGTTGAT.

Western blots and immunoprecipitation- $5x10^6$ UoC-M1 cells were rinsed three times in PBS and lysed in RIPA buffer with protease inhibitors (RIPA: 10 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulfate [SDS], 1 μg/ml leupeptin, 1 mM pepstatin A, 1 mM phenylmethylsulfonylfluoride (PMSF), 1% aprotinin, and 2 mM sodium orthovanadate [Na_3VO_4]). Lysates were incubated on ice for 5 minutes and then centrifuged at 12,000 x g for 15 minutes at 4°C. Protein concentrations were determined via the method of Bradford[251] (Bio-Rad, Hercules, CA) and diluted with 2x SDS sample buffer (0.1 M Tris-HCl, pH 6.8, 4.0% SDS, 0.2% bromophenol blue, 20.0% glycerol, and 1 M DTT). Lysates were heated at 94°C for 5 minutes, subjected to SDS-polyacrylamide gel electrophoresis (7.5% for c-

KIT and STAT analysis and 12% for VHL) and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA) using the method described by Towbin *et al.*[191] Filters were blocked for one hour in a suspension of 5% (w/v) non-fat powdered milk (Nestle, Solon, OH) in TBS (10 mM Tris-base, 150 mM NaCl, pH 8.0).

The von Hippel-Lindau protein (VHL) was detected by incubating blots with a mouse monoclonal anti-human VHL antibody (Pharmingen, San Diego, CA) at 1:1000 in 5% (w/v) non-fat powdered milk in TBS overnight at 4°C. The following day, filters were washed 6 times in a solution of 50 μl TWEEN-20 (BioRad, Hercules, CA) per liter of TBS. Goat anti-mouse horseradish peroxidase (HRP) secondary antibodies (BioRad, Hercules, CA) were then added in a 5% solution of powdered milk in TBS with rocking for one hour at room temperature. Filters were washed as before and chemiluminescence was developed using a commercial kit (ECL, Amersham-Pharmacia, Piscataway, NJ). Treated filters were exposed to Kodak X-OMAT film (Kodak, Rochester, NY). Rabbit anti-human polyclonal antibodies raised against the phosphorylated species of STAT-1, STAT-3, and STAT-5 as well as their unphosphorylated, forms (Transduction Laboratories, Lexington, KY) were used as described above at 1:1000 dilutions each.

Immunoprecipitation (I/P) for c-KIT was performed by rinsing and lysing (in RIPA buffer) $5x10^6$ UoC-M1 cells as before. Lysates were precleared with 5 μl of

rabbit Ig (*sc-2345*, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 1 hour at 4°C before the addition of 5 μl of agarose-conjugated rabbit anti-human c-KIT polyclonal antibody (sc-168AC, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and overnight incubation at 4°C on a rotating platform. The following day, I/P's were centrifuged at 12,000 x g for 15 minutes and the pellets rinsed twice with PBS containing protease inhibitors. Laemli sample buffer was added, the tubes heated at 94°C for 10 minutes, chilled on ice for 1 minute, and briefly centrifuged to pull down condensation before being stored at –70°C prior to use. Immunoblots were prepared as stated above and interrogated with rabbit, anti-human c-KIT polyclonal antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Filters were stripped by placing them in the appropriate buffer (62.5 mM Tris-HCl - pH 6.7, 100 mM β-mercaptoethanol, and 2% SDS) at 50°C for 30 minutes, rinsing 6 times in 20 ml of 50 μl TWEEN-20 per liter of TBS, and blocking for 1 hour in 5% powdered milk in TBS prior to reprobing with a rabbit, anti-human polyclonal antibody (PY20) to phosphotyrosine (Transduction Laboratories, Lexington, KY). Control preparations of c-KIT I/P's from CHO-D816V and whole-cell extracts from HMC-1 and hSCF-stimulated Mo7e were generously provided by Michael Heinrich (OHSU, Portland, OR).

Whole-cell fusion - UoC-M1 is a spontaneously immortalized, factor-independent, megakaryoblastoid cell line[189] and is maintained in suspension in RPMI-1640, 10% heat-inactivated, fetal calf serum (FCS) (Hyclone Labs, Logan, UT), penicillin, and streptomycin. A9-7 (generously contributed by Markus

Grompe, Oregon Health & Science University, Portland, OR) is a murine, *Hprt*$^{-/-}$, fibroblast cell line that contains a single copy of a retrovirally-tagged (resistance to hygromycin B and sensitivity to ganciclovir via HSV-thymidine kinase [Hy/Tk])[252,253] human chromosome 7 (isolated from foreskin fibroblasts)[254,255] and is cultivated in alpha-DMEM + 10% FCS, penicillin, streptomycin, and 400 μg/ml hygromycin B (Roche, Indianapolis, IN). $5x10^6$ UoC-M1 cells were counted, pelleted at 300 x g for 5 minutes, and rinsed three times in sterile PBS. A9-7 cells were rinsed two times on the plate before being subjected to trypsinization for 1 minute at room temperature. A9-7 cells were then rinsed in alpha-DMEM containing 10% FCS to inactivate the trypsin, counted, and pelleted at 300 x g for 5 minutes. $5x10^6$ A9-7 cells were combined with an equivalent number of UoC-M1 cells in a 15 ml polypropylene centrifuge tube and co-sedimented at 300 x g for 5 minutes and the media aspirated. The A9-7/UoC-M1 pellet was then loosened by gentle agitation. 1 ml of 37°C, 50% (v/v in PBS) polyethylene glycol (PEG; ave. mol. weight 1450)(Sigma, St. Louis, MO) was added drop-wise over 1 minute with gentle agitation. 30 ml of warm RPMI-1640 (supplemented with 10% FCS, penicillin and streptomycin) was then added immediately and the resulting cellular suspension aliquoted equally into 15 tissue culture flasks (T-25) and allowed to recover 24 hours before being placed under dual selection in 1X HAT medium (Life Technologies, Gaithersburg, MD) and 400 μg/ml hygromycin B (Roche, Indianapolis, IN). Selection was maintained for at least two weeks before flasks were visually scored for positive fusions.

Alternatively, two other "on-plate" fusion methods were devised based upon the method outlined above with exceptions as follows:

(1) Instead of co-sedimenting A9-7 and UoC-M1 cells, the A9-7 plates were rinsed as before but not trypsinized. A 50 μg/ml solution of phytohemmaglutinin-P (PHA-P) (Sigma, St. Louis, MO) (from a stock of 500 μg/ml) in RPMI-1640 was added to the UoC-M1 cell suspension to a final concentration of 50 μg/ml. This UoC-M1/PHA-P suspension was then added to the rinsed A9-7 cell plate, and incubated at 37°C for 30 minutes to allow UoC-M1 cells to adhere to A9-7 targets. Excess PHA-P solution was then carefully aspirated before 2 ml of 50% PEG-1450 was added dropwise to the plate and mixed with gentle agitation. After allowing fusion to ensue for 1 minute, the plates were carefully rinsed two times with warm RPMI-1640 before being placed in RPMI-1640 + 10% FCS and subjected to the same recovery and selection scheme as before.

(2) Though UoC-M1 grows in suspension, a minor fraction of cells have been observed to loosely adhere to the bottom of tissue culture flasks. Additional cellular adherence can be induced under certain circumstances including reduced serum content (5% FCS) or cultivation on Primaria surfaces (Becton Dickinson Labware, Franklin Lakes, NJ) to confluency and then daily media replacement to maximize opportunities for cells to settle via gravity and attatch.[256] These observations were exploited and UoC-M1 cells were used as adherent targets (as outlined above for "on-plate" A9-7 cells). A9-7 cells were trypsinized (as before), resuspended in a warm solution of PHA-P, added to

flasks of adherent UoC-M1 cells, and incubated for 30 minutes at 37°C. Excess PHA-P solution was aspirated, fusions performed, and resulting flasks put under selection as before.

Microcell-mediated chromosome transfer - To minimize the contribution of murine genetic material from the A9-7 somatic cell hybrid line, microcell-mediated chromosome transfer (microcell fusion) was employed after the technique of Fournier[257,258] as follows. Polystyrene "bullets" were cut from T-75 tissue culture flasks and shaped so as to fit into 50 ml polycarbonate centrifuge tubes (Nalge Company, Rochester, NY). Bullets were sterilized by immersion in 95% ethanol for 24 hours, air-dried in a laminar flow hood for 45 minutes under U.V. light, and placed into 150 mm tissue culture dishes (four bullets per dish, 12 total bullets per experiment). A9-7 cells were seeded onto bullets (and also to a separate dish containing a flame-sterilized glass microscope slide) in appropriate culture media and allowed to incubate at 37°C for 4 hours to allow attachment of cells. Colcemid (Sigma, St. Louis, MO) was then added to each plate to a final concentration of 0.08 μg/ml (from a 100 μg/ml stock in PBS) and allowed to incubate for 48 hours. When 24 hours remained in the A9-7/colcemid incubation, 10 million UoC-M1 cells were seeded into a T-75 Primaria (Becton Dickinson Labware, Franklin Lakes, NJ) flask in 10 ml of RPMI-1640 + 10% FCS (Hyclone Labs, Logan, UT) and allowed to incubate for 24 hours at 37°C. At the end of the 48 hour A9-7/colcemid incubation, micronucleation efficiency was determined by staining the A9-7 cells grown on

the glass microscope slide with Wright's/Giemsa and analysis under oil immersion microscopy (see figure 4-11B). The high-speed centrifuge rotor was prewarmed to 33°C. Bullets were removed from culture dishes and two were placed back to back per 50 ml polycarbonate centrifuge tube containing 38 ml of pre-warmed (37°C) serum-free DMEM + 10 μg/ml cytochalasin B (Sigma, St. Louis, MO) (from a 1 mg/ml stock in DMSO [Sigma, St. Louis, MO]), capped, sealed with teflon film and centrifuged (with the plane of the bullets parallel to the rotor radius) at 27,000 x g for 70 minutes at 33°C. At the end of centrifugation, bullets were removed and placed into 3% hypochlorite for reuse. The media was vacuum aspirated from each centrifuge tube, replaced with 2 ml of warm, serum-free DMEM, and the microcells gently resuspended before being pooled to a single tube. Size exclusion was achieved via serial filtration (8, 5, and finally 3 μm) with polycarbonate filter disks in Swinex Lok filter holders (Corning, Ithaca, NY) and verified microscopically. Filtered microcells were pelleted at 2000 x g for 15 minutes at 37°C and resuspended in 6 ml of warm (37°C) 50 μg/ml PHA-P (in serum-free DMEM). The medium was aspirated from the UoC-M1 flask and replaced with the microcell/PHA-P solution and incubated at 37°C for 20 minutes. Microcell adherence to UoC-M1 was verified microscopically. The PHA-P solution was vacuum aspirated from the UoC-M1 flask and 1.5 ml of warm (37°C) 50% PEG-1450 solution (in PBS) was added to the flask drop-wise over 1 minute with gentle rocking. 10 ml of warm, serum-free DMEM was then added, aspirated, and the rinse repeated a total of three times. After rinsing, 15 ml of RPMI-1640 + 15% FCS was added and the cells were allowed to recover for 48

hours before being placed under selection in 400 μg/ml of hygromycin B (Roche, Indianapolis, IN). Cells were maintained under selection in the T-75 flask for 48 hours before being re-plated via one of the following methods (though experiments were also performed where post-fusion cells remained in the original T-75 for at least two weeks): seeding over 10, 96-well U-bottom dishes at 100 μl per well (plus 100 μl of 50% conditioned media from UoC-M1 cells) or splitting into 10 T-25 tissue culture flasks with 50% conditioned media.

Microcell fusions were also attempted wherein filtered microcells were co-sedimented with UoC-M1 cells (similar to that previously described in the section on whole-cell fusions) and fused via dropwise addition of warm 50% PEG-1450. Cells were then either rinsed by repeated addition of warm media and centrifugation or by dilution of the post-fusion material with RPMI-1640 + 15% FCS and direct transfer to multiple T-25 flasks. Selection was performed as described above. In all cases, positive fusion products were scored at two weeks post-fusion.

Results

4-3: UoC-M1 cells form highly-vascularized, solid tumors in NOD/SCID mice

As shown in figure 4-1, sub-cutaneous injection of $5x10^6$ UoC-M1 cells into NOD/SCID mice produced solid tumors. NOD/SCID mice[239] were used as

they represent a reliable means of amplifying human hematopoietic tissues without eliciting an overt immune response (and subsequent graft rejection) in the host.[259-264] All three animals injected demonstrated the formation of large, externally visible masses at or near the injection site within 20 days (data not shown). Observation of live animals revealed no other signs of morbidity and the animals maintained an active habitus, good appetite, and adequate reflexes. Necropsy revealed large, sub-cutaneous masses that weighed in excess of 1 gram in each mouse (data not shown). Further resection of each tumor resulted in abundant bleeding, suggestive of a particularly high degree of microvascularity. To determine the degree of vascularity present in the tumors, H/E stained paraffin sections were obtained. As shown in figure 4-2, microvascular density was pronounced. The high degree of vascularity correlated with the additional finding that the tumor was minimally necrotic (it is widely accepted that vascularity and necrosis are inversely related).

Necropsy also revealed mild to moderate hepato-splenomegaly. H/E stained sections of liver, spleen, and de-calcified femur revealed spread of leukemic blasts in all mice (data not shown). Microscopically, the tumor derived cells were identical to the parent UoC-M1 cells and demonstrated high nuclear to cytoplasm ratio, frequent mitotic figures, and the appearance of an hematopoietic blast (data not shown). Cytogenetic analysis was performed on the tumor material from two of three mice and each resulted in a karyotype identical to UoC-M1 (figure 4-3).

Serial tumor passage to secondary and tertiary hosts demonstrated maintenance of the tumor forming potential of the primary tissue (data not shown). Furthermore, these tumors arose within the same time frame as previously noted, demonstrated cellular morphology identical to that seen in the primary tumor tissue, and also contained a high degree of microvasculature formation (data not shown). As the presence of abundant blood vessels within areas of malignancy is positively associated with cancer progression in not only solid tumors[265-269] but liquid tumors (hematological malignancies) as well,[241-250] the secretion of factors capable of remodeling/redirecting existing vasculature (a process known as angiogenesis) was investigated in UoC-M1.

4-4: UoC-M1 cells secrete the angiogenic mitogen VEGF

As the tumors formed by UoC-M1 in NOD/SCID mice were microvascularly dense, it was hypothesized that such a phenotype was perhaps due to the secretion of angiogenic proteins by the UoC-M1 cells *in vivo*. To evaluate this notion, enzyme linked immunosorbent assays (ELISA's) were performed on supernatants from UoC-M1 cells cultured *in vitro*. As shown in table 4-1, the pro-angiogenic cytokine vascular endothelial growth factor (VEGF; also known as vascular permeability factor or VPF)[270] was found to be detectable at concentrations in excess of 2,600 pg/ml in supernatants from confluent culture flasks. In contrast, cultured peripheral blood mononuclear leukocytes (PBML)

stimulated for 5-days with 10 μg/ml phytohemmaglutinin (PHA) are reported to averaged 1,440 pg/ml while unstimulated PBML's produce quantities at the lower end of detectability.[271] ELISA's for other factors known to be involved in the regulation of VEGF and/or angiogenesis were either negative or found to be present at very low levels (table 4-1). These included GM-CSF,[243,272] IL-1β,[273,274] IL-6,[244,275,276], SCF,[277] TNFα,[278,279] and TPO.[280,281]

The observation of high levels of VEGF production in unstimulated UoC-M1 cells, while impressive compared to even stimulated PBML's, was difficult to evaluate as comparison data for primitive hematopoietic cells was unavailable. To determine the levels of *in vitro* UoC-M1 VEGF production relative to a normal hematopoietic cell population, primitive hematopoietic cells (CD34+)[13] were obtained from a normal donor and used in a comparative, 72-hour VEGF-production assay. CD34+ cells were chosen as UoC-M1 also maintains the primitive hematopoietic cell surface marker CD34+.[189] As shown in figure 4-4, CD34+ cells cultured in the presence of SIE (h<u>S</u>CF, <u>I</u>L-3, and <u>E</u>PO) produced a minimal amount of VEGF (4.1 pg/ml per million cells). The amount of VEGF production could be increased roughly ten-fold (56.6 pg/ml per million cells) with the addition of the stimulatory cytokine GM-CSF, a factor known to influence VEGF production in hematopoietic cells.[243] However, unstimulated UoC-M1 cells produced over 700 pg/ml per million cells. Compared to both stimulated and unstimulated CD34+ cells, VEGF production by UoC-M1 is clearly excessive. While VEGF's ability to regulate the normal and solid tumor-promoting

angiogenic process has been well-documented[240,270,282-284] its role as a factor in the autocrine and paracrine stimulation of myeloid cells, and thus as a potential element contributing to leukemogenesis, has only recently been explored.[241-250] As a first step toward the determination if VEGF could be acting as an autocrine stimulatory factor by UoC-M1, flow cytometry was employed to demonstrate the presence of high-affinity receptor tyrosine kinases for VEGF.

4-5: UoC-M1 has two high-affinity, VEGF receptor tyrosine kinases

As shown in figure 4-5, UoC-M1 demonstrates robust staining for both VEGF receptor 1 (VEGFR1, otherwise known as fms-like tyrosine kinase receptor or FLT-1)[285] and VEGF receptor 2 (VEGFR2, otherwise known as kinase insert domain-containing receptor or KDR).[286] The presence of two high-affinity VEGF receptors in conjunction with constitutive secretion of VEGF suggests that UoC-M1 may be utilizing the cytokine as an autocrine, growth stimulatory factor. To determine which VEGF-regulatory molecules might be responsible for constitutive VEGF production, reverse-transcription, polymerase chain reaction (RT-PCR) was used to interrogate effectors of *VEGF* transcription.

4-6: Effectors and inhibitors of *VEGF* transcription are present

VEGF is primarily regulated at the transcriptional level by hypoxia-inducible transcription factors.[287-294] Other post-transcriptional mechanisms

related to increased mRNA stability [288,295] and alternative ribosomal entry sites[296] have also been described. As VEGF is constitutively produced in UoC-M1 cells, it was expected that effectors of *VEGF* transcription would be expressed. As shown in figure 4-6A, mRNA for key *VEGF* transcriptional effectors are present in UoC-M1 cells. These include the transcription factors Sp1[297] and hypoxia-inducible factors 1-alpha and 1-beta (HIF1-α/HIF1-β).[288-290,292-294,298] Also present however, was mRNA for four known repressors of *VEGF* transcription: elongin B, elongin C,[299-305] cullin-2 (CUL-2),[304-308] and von Hippel-Lindau protein (VHL)(not shown).[298,299,301-304,306-311] As VHL is the major repressor of *VEGF* transcription (at least in endothelial cells), working by forming a multimeric complex with *VEGF* transcriptional effectors, elongin B/C, and CUL-2 immunoblots were prepared and probed with VHL antiserum (figure 4-6B). While the factor-dependent myeloid leukemia cell line Mo7e[199] and the human embryonic kidney cell line 293[312] both exhibit comparable levels of VHL protein, UoC-M1 has a reduced amount. Such a reduction in VHL protein may account for constitutive VEGF production. In an attempt to correlate reductions in VHL protein levels with *VHL* gene mutation, the *VHL* cDNA was sequenced in UoC-M1.

4-7: The *VHL* gene sequence is wild-type

VHL is encoded by only three exons and exists as two distinct mRNA species of 6.0 and 6.5 kb depending on whether or not exon 2 is included.[313]

The relative significance of these two mRNA forms is unknown. UoC-M1 expresses both forms of *VHL* as evidenced by RT-PCR analysis (data not shown). Bi-directional sequencing of multiple colonies containing cloned *VHL* cDNA's (both forms) demonstrated no mutations (data not shown). While an unidentified mechanism of VHL protein reduction may account for VEGF secretion in UoC-M1, a more direct connection between VEGF levels and cellular proliferation was sought by making use of VEGF inhibitors.

4-8: Seeking to confirm autocrine growth in UoC-M1 cells; growth inhibition using VEGF antagonists

As VEGF has been shown to be a potent endothelial cell mitogen,[270] it is possible that it may also function to foster proliferation in hematopoietic cells. Due to the importance of VEGF-mediated angiogenesis in tumor development,[240,269,282,283] VEGF and its receptors are suggestive targets for anti-tumor therapies.[314-318] One method for blocking VEGF-mediated signaling is to inhibit the receptor-ligand interaction. Theoretically, this can be achieved by either blocking the ligand binding site at the receptor, masking the receptor binding site of the ligand, or by inhibiting the formation of multimers where such complexes are required for activity. Alternatively, signal attenuation may be achieved by impairing the intracellular tyrosine kinase domains of the VEGF receptors. To directly tie VEGF to UoC-M1 proliferation, growth inhibition using VEGF signaling antagonists was attempted. Antagonists, acting by binding

VEGF and blocking its ability to activate the receptor (Bevacizumab; Genentech, San Francisco, CA) or by impairing the tyrosine kinase activity of the receptor directly (PTK787; Novartis Pharmaceuticals, Basel, Switzerland), were obtained and evaluated for their ability to hinder UoC-M1 proliferation.

The mouse monoclonal antibody A.4.6.1 has been shown to inhibit VEGF function in the sub-nanomolar range (IC_{50} ~ 0.3 nM or 50 ng/ml) by binding VEGF directly.[315] A humanized form of this antibody (Bevacizumab; Genentech, San Francisco, CA) has been created via site directed mutagenesis of a cDNA derived from the A.4.6.1 producing hybridoma and maintains a similar affinity for VEGF.[315] Bevacizumab was evaluated for its ability to impair UoC-M1 growth in both proliferation and inhibition assays.

Bevacizumab did not significantly impair the growth of UoC-M1 cells in proliferation assays (data not shown). When used in inhibition assays (colony-forming unit: CFU-A) at concentrations up to (and including) 30 nM, no effect was observed (figure 4-7A). The highest concentration of Bevacizumab used was one log greater than the reported IC_{90} in bovine capillary endothelial cells stimulated with 3,000 pg/ml of VEGF.[315] However, it is known that heparan sulfate proteoglycans are abundant on the surfaces of many cells where they facilitate ligand-receptor interactions.[319] As VEGF is known to be a heparin-binding protein,[270] it was thought that local concentrations of VEGF bound to heparan sulfate proteoglycans in the immediate vicinity of the VEGF receptors

might be very high indeed. Thus, inhibition of VEGF ligand/receptor binding may be difficult to achieve. To circumvent such a situation, the protein tyrosine kinase inhibitor PTK787 was employed in an attempt to block VEGF-mediated signaling at a point distal to ligand binding.[320]

PTK787 (Novartis Pharmaceuticals, Basel, Switzerland; kindly provided by Michael Heinrich, OHSU) has specificity for a limited set of receptor tyrosine kinases including KDR, FLT-1, and c-KIT (the receptor for stem cell factor [SCF]).[320] No inhibitory effect was observed at concentrations ranging from 0.1 to 10.0 μM in proliferation assays (data not shown). In inhibition assays, an ID_{60} was observed at the highest dose (10 μM) (figure 4-7B). It is tempting to speculate that inhibition was the result of VEGF-specific PTK787 treatment. However, the need for concentrations as high as the micromolar range also suggest that non-specific receptor interactions or toxicity may have occurred.[320]

As growth inhibition could not be reliably demonstrated with two VEGF-specific blocking agents, alternative explanations were sought to account for the factor-independent growth of the UoC-M1 cells. As PTK787 also possesses cross-specificity for c-KIT, it was felt that c-KIT activation might be at the root of UoC-M1 proliferation. Though ELISA's performed on cell culture supernatants from UoC-M1 did not demonstrate detectable levels of SCF (see table 4-1), it remained a possibility that mutations might exist within *c-KIT* that could account for constitutive receptor activation in the absence of a ligand binding event.

Recall that in earlier studies (see figure 3-2) it was demonstrated that UoC-M1 is CD117 (c-KIT) positive, albeit at a low level. To determine if activated (tyrosine phosphorylated) c-KIT was present, immunoprecipitation of c-KIT followed by anti-phosphotyrosine immunoblotting was employed in UoC-M1.

4-9: c-KIT immunoprecipitation in UoC-M1

c-KIT immunoprecipitation was performed in UoC-M1 and CHO/D816V (CHO = Chinese hamster ovary cells bearing an activating *c-KIT* mutation)[321] and run alongside UoC-M1, hSCF stimulated Mo7e, and human mast cell leukemia-1 (HMC-1; constitutively c-KIT active)[14,322] whole-cell extracts. Blots were then probed with antisera for both c-KIT and anti-phosphotyrosine. As demonstrated in figure 4-8, top panel, c-KIT was present in all extracts. Probing with anti-phosphotyrosine (figure 4-8, bottom panel) did not indicate c-KIT phosphorylation in UoC-M1 immunoprecipitation extracts, suggesting that while c-KIT was present (in agreement with previous flow cytometry results) it was not constitutively active in UoC-M1 cells. However, two prominent tyrosine-phosphorylated species were detected in UoC-M1 (whole cell extract lane). The uppermost band (> 140 kDa) is in the range of possible Janus-kinases (JAK) or cytokine receptor subunit species[323] while the lower band (ca. 95 kDa) is in the range of phosphorylated signal transducer and activator of transcription (STAT) species.[324] STAT-3 has been shown to induce *VEGF* mRNA expression in rat cardiac myocytes.[325] VEGF is known to induce phosphorylation of STAT's 1, 3

and 6 in bovine aortic endothelial cells.[326] In other work, overexpression of KDR in endothelial cells resulted in activation of STAT's 3 and 5.[327] As STAT activation has also been shown to be key in the factor-independent growth of certain hematopoietic lineages,[328,329] STAT phosphorylation was assayed in UoC-M1 by immunoblot analysis.

4-10: UoC-M1 constitutively phosphorylates STAT's 1, 3, and 5

STAT-mediated signal transduction events are important in myeloid cell development.[329] Constitutive STAT activation has previously been associated with AML.[328-334] While in some instances, STAT activation has been associated with autocrine/paracrine cytokine release/binding,[335] still others have demonstrated insensitivity to exogenous cytokines.[334] Work performed by others has suggested that constitutive activation of STAT-5 in particular correlates positively with factor independence in megakaryoblastoid cell lines.[328] To determine whether STAT molecules were activated in UoC-M1, a series of immunoblots was prepared and probed with phospho-specific antisera for STAT family members.

As shown in figure 4-9, UoC-M1 demonstrates constitutive phosphorylation of STAT-1 (top panel), STAT-3 (middle panel), and STAT-5 (bottom panel). Increased immunoreactivity could not be accounted for on the basis of unequal gel loading as evidenced by reprobing stripped filters with

antibodies that recognize non-phosphorylated forms of each molecule (figure 4-9). To determine if constitutive STAT phosphorylation correlated with VEGF-mediated signaling, STAT phosphorylation was assayed following cellular treatment with the VEGF-signaling inhibitors herbimycin-A and genistein and the VEGF-neutralizing antibody Bevacizumab.

4-11: VEGF-inhibitors suppress STAT phosphorylation

Two tyrosine kinase inhibitors, herbimycin-A and genistein were used in an attempt to impair STAT phosphorylation in UoC-M1. The global tyrosine kinase inhibitor herbimycin A has been shown to be capable of suppressing VEGF secretion in human mesangial cells.[336] An unrelated tyrosine kinase inhibitor, the soy isoflavone genistein, has also been shown to abrogate VEGF-signaling, though by a different mechanism involving blockade of events distal to VEGF-dependent activation of the non-receptor tyrosine kinase SRC.[337] The VEGF-specific neutralizing antibody Bevacizumab was also employed to determine if STAT phosphorylation in UoC-M1 could be impaired by blocking VEGF signaling. As shown in figure 4-10A, treatment of UoC-M1 with both herbimycin-A and genistein was able to completely abrogate STAT-1 phosphorylation up to at least 6 hours following exposure. Identical results were obtained for STAT-3 and STAT-5 (data not shown). However, treatment with 500 ng/ml of Bevacizumab (equivalent to an IC_{90} for VEGF-stimulated endothelial cells)[315] resulted in only transient inhibition (1 hour) of phosphorylation of STAT-3

and STAT-5 (figure 4-10B). STAT-1 phosphorylation was not impacted by Bevacizumab (figure 4-10B).

Reasoning that constitutive STAT phosphorylation is likely an important aspect of the transformed nature of the UoC-M1 cells (whether or not VEGF-mediated events are related to either STAT phosphorylation and the unrestricted growth of the cell line) additional work was conducted to establish a link between the transformed phenotype, STAT phosphorylation, and the aneuploidy present in UoC-M1. Specifically, as UoC-M1 has functional chromosome 7 monosomy (see chapter three)[189] (a non-random chromosomal anomaly associated with MDS/AML [see chapter 1]), reintroduction of chromosome 7 material into UoC-M1 was attempted as a means to impair cellular growth or to promote differentiation.

4-12: Chromosome 7 transfer into UoC-M1 via whole-cell fusion

A series of nucleotide salvage deficient (via hypoxanthine guanine phosphoribosyltransferase or *Hgprt* gene mutation) rodent/human somatic cell hybrids has been created that carry antibiotic resistance gene-marked, single human chromosomes on the mouse A9 background.[254,255] Such lines have proven useful for the mapping of loci for both dominant and recessive human conditions. In addition to a full array of murine chromosomes, the rodent/human somatic cell hybrid A9-7 contains an intact human chromosome 7 (figure 4-11A)

that has been retrovirally tagged with a hygromycin-resistance (*hygro*) gene in a bi-cistronic cassette with the herpes-simplex virus thymidine kinase (HSV-*tk*) gene.[253] The hygro-resistance gene allows for positive selection of the human chromosome 7 while *tk* allows for selection against this same chromosome with the antiviral drug ganciclovir.[252,253] Furthermore, as the mouse A9 cells are defective in the nucleotide salvage metabolic pathway (due to bi-allelic *Hgprt* mutation) it is possible to select against the mouse A9 cells using cell culture media containing hypoxanthine, aminopterin, and thymidine (HAT medium).[253-255] This is due to the presence of the antifolate aminopterin in HAT medium which inhibits *de novo* nucleotide synthesis. As the cells are incapable of making use of hypoxanthine present in the culture medium, they will die.

A variety of experiments attempting to obtain whole cell fusions between the A9-7 donor cells and UoC-M1 were unsuccessful (data not shown). No cells resistant to both hygro and HAT were obtained after the initial dual-selection period was complete (though cell-cell fusions were noted microscopically by the presence of large, multinucleated cells in the hours following completion of fusion protocols). Reasoning that dominant effects of murine chromosomes may have contributed to the inability to generate whole cell fusion products, microcell-mediated chromosome transfer was enlisted as a means to reduce the quantities of murine genomic material participating in fusions.[257,258]

4-13: Microcell-mediated chromosome 7 transfer

By use of tubulin polymerization inhibitors such as colcemid (N-Deacetyl-N-methylcolchicine), mitotic arrest can be obtained, resulting in nuclear fragmentation.[257,258] These nuclear fragments (or micronuclei) are microscopically visible in Wright's/Giemsa stained cells (figure 4-11B). Micronuclei may be isolated from treated cells, size-fractionated via filtration to less than 3 μm (and thus, contain on average fewer than four chromosomes), and fused to recipient cells to affect genetic complementation in a manner that minimizes the quantity of genomic material introduced.[257,258] Micronucleation was optimized for the A9-7 somatic cell hybrid and microcell-mediated chromosome transfer was attempted (figure 4-12). As before, hygro/HAT resistant products were unobtainable notwithstanding attempts at fusion where multiple protocol refinements were made and also despite microscopic verification of microcell adherence to target cells following fusion (data not shown).

4-14: Discussion

In 1945, Algire and Chalkley noted that tumor growth in xenotransplanted mice invariably occurred subsequent to the formation of new microvasculature.[265] Nearly 30 years later, Judah Folkman's seminal observations regarding the angiogenic nature of developing tumors[267,268] defined the potential of anti-tumor therapies directed against new blood vessel formation. Solid tumors are most

often limited in size due to sub-optimal oxygen tension in the center of the growing mass. Oxygen diffusion is only effective at a distance of less than 2.0 mm,[244] though detectable oxygen reductions occur at distances in excess of 0.1 mm (less than eight cell layers).[338] However, many tumors enlarge beyond this threshold by manifesting an ability to remodel/redirect existing vasculature (a process known as angiogenesis) at the tumor site and thereby perfuse the mass with oxygen-rich blood.[266-269] Endothelial cells chemotactically migrate toward the tumor mass along a gradient of hypoxia-inducible proteins secreted by the oxygen-starved tumor cells.[338] An important member of this group of molecules is vascular endothelial growth factor or VEGF (also known as VEGFA).[270]

VEGF

VEGF is a hydrophilic, secreted protein of approximately 45 kDa that functions as a homodimer[339] by binding to two high-affinity receptor tyrosine kinases, FLT-1[285] and KDR.[286] Members of the VEGF receptor family have seven, extracellular, immunoglobulin-like domains (binding ligand via domains two and three) and an intracellular tyrosine kinase domain.[240] That the VEGF signaling pathway is vital is supported by studies in transgenic mice. Knockouts for *VEGF*, *FLT-1*, and *KDR* are all embryonic lethal between gestational days 8.5 (for *Flt-1*$^{-/-}$ and *Kdr*$^{-/-}$ [*KDR* is known as *Flk-1* in mice]) and 10.5 (for *Vegf*$^{-/-}$: *Vegf*$^{+/-}$ mice are also embryonic lethal at day 11.5).[340] The phenotypes of these knockout mice are variable however. *Vegf* loss primarily impairs the formation of

heart and aorta along with creating faulty vessel junctions.[341,342] *Flt-1* knockouts demonstrate excessive endothelial cell proliferation and disordered vessel formation[343] while *Flk-1* knockouts have a failure of endothelial cell growth and reduced embryonic myelopoiesis.[344,345] Regarding these observations, it is clear that though *Flt-1* and *Flk-1/KDR* share a common ligand, they enact disparate phenomena within cells. The two receptors maintain an inverse relationship with regard to their interactions with VEGF in that *Flt-1* has a higher affinity but lower kinase activity than *Flk-1/KDR* with the opposite being the case for *Flk-1/KDR* (see Hiratsuka *et al*, 1998 and references therein).[346] That *Flt-1*$^{-/-}$ mice can be rescued by expression of an *Flt-1* mutant lacking the intracellular tyrosine kinase domain has suggested that Flt-1 plays a more central role as a negative regulator of endothelial cell growth, perhaps by preventing VEGF from interacting with Flk-1/KDR.[346]

VEGF Signal Transduction

VEGF production is positively regulated at the transcriptional level via hypoxia-inducible transcriptional machinery.[287-294] The mechanisms by which oxygen sensing occurs proximal to the induction of VEGF transcription are poorly understood though the finding that hypoxic conditions can be mimicked by cobalt-chloride or iron-chelator exposure suggests that heme-containing proteins are perhaps involved.[287,347,348] VEGF has been shown to be upregulated by events involving protein kinase C (PKC) and protein tyrosine kinases (PTK) in

mesangial cells responding to mechanical stretching.[336] PKC-δ and PKC-ζ have been implicated in upregulation of VEGF, both being shown to act upstream (in part) of the mitogen activated protein kinase (MAPK) signaling pathway in VEGF induction.[349] The upregulation of VEGF by the transcription factor Sp1 is specifically mediated by PKC-ζ.[297] However, the transcription factors HIF-1α (hypoxia inducible factor) and HIF-1β appear to be the major effectors of *VEGF* transcription.[288-290,292-294,298] The HIF's are basic helix-loop-helix transcription factors that are upregulated in response to hypoxia and cellular stress and act as a heterodimer to induce downstream targets including: *VEGF*, erythropoietin (*EPO*), *p53*, *p21*, and *BCL-2*.[293] Recently, a series of reports have suggested that HIF-1 itself may be the molecule that directly responds to oxygen tension. Work published by three groups suggests that HIF-1α is post-translationally modified via hydroxylation of proline residue 564, a process requiring dioxygen, the activity of an iron-dependent prolyl hydroxylase, and inhibitable by cobalt.[350-352] Due to the rapid kinetics observed for proline hydroxylation by the authors, a mechanism is suggested wherein an abundant (constitutively expressed) prolyl hydroxylase acts on HIF-1α only when molecular oxygen tension is high and can provide a substrate for hydroxyl formation.[350-352] A conserved family of prolyl-4-hydroxylases, referred to as HPH's (HIF prolyl hydroxylase), has been cloned (HPH-1, 2, and 3); each capable of hydroxylating HIF-1α in a cell free system that was inhibitable by cobalt.[353] Proline hydroxylated HIF-1α is incapable of forming a homo-dimer with HIF-1β and thus, cannot induce gene transcription.[350-

[352] However, proline hydroxylated HIF-1α does bind to the protein product of the von Hippel-Lindau gene (VHL).[350-352] HIF's are then downregulated via degradation in the proteosome (or multicatalytic protease = MCP) by a mechanism that involves VHL protein.

Regulation of VEGF gene expression

Von Hippel-Lindau disease (OMIM 193300) is a dominantly-inherited condition of benign vascular tumor formation (hemangioblastomas) and cancer-predisposition (including renal cell carcinoma and pheochromocytoma). VHL-knockout mice are embryonic lethal.[235] The dominant inheritance in VHL is in apparent disagreement with its molecular basis as lesions have loss of protein activity and thus, are either dominant-negative or recessive. The findings of disease-associated chromosomal deletions,[313] frequent protein truncations,[41] and transcriptional silencing-associated tumorigenesis,[354] along with the observation that overexpression of wild-type VHL abrogates cell growth in tumor cell lines[355] all suggest that the latter is the case. Thus, VHL disease is perhaps more appropriately termed a condition of dominantly-inherited cancer vulnerability with a high likelihood of acquired loss of heterozygosity (LOH) in carriers that then manifest tumors. As such, in keeping with the Knudson model,[32] VHL disease is truly recessive at the molecular level. VHL acts to repress VEGF transcription by forming a multimeric complex with elongin B/C, CUL-2, and RBX1 (RING-H2 finger protein; a factor that recruits ubiquitin-conjugating enzymes) that is then

degraded in the MCP, thus shutting down VEGF transcription.[298-311,356] It is in this manner that VEGF is normally regulated and when dysregulated, contributes to the formation of microvascularly dense (and thus, oxygen-rich) tumors. The finding of a high microvasular density in tumors formed by the UoC-M1 myeloid leukemic cell line led to an investigation of components of the aforementioned VEGF-regulatory machinery in these cells.

VEGF in UoC-M1 cells

The observations outlined in this chapter regarding the UoC-M1 cell line allude to a proliferative phenotype mediated by angiogenic cytokine (VEGF)[270] secretion both *in vivo* and *in vitro*. After sub-cutaneously injecting immunodeficient mice with the UoC-M1 cell line, solid tumors rapidly developed at the injection site. The observation during necropsy of what appeared to be vascularized tumor masses was confirmed by noting a high microvascular density in H/E stained tumor sections. ELISA of *in vitro* cell culture supernatants demonstrated a profound abundance of VEGF relative to normal, CD34+ controls. The additional observation that UoC-M1 displays two high-affinity VEGF receptor tyrosine kinases on the cell surface suggests that UoC-M1 may be using VEGF as an autocrine promoter of cell growth. RNA message was found to be present in UoC-M1 for three effectors of VEGF transcription: SP1, HIF1-α, and HIF-1β, though mRNA for VEGF repressors was also indicated including key members of the VHL protein complex. Immunoblot analysis of VHL

revealed a reduction in the amount of protein in UoC-M1 relative to controls. While a 50% reduction in VHL protein does not appear to contribute to tumorigenesis (cells with one normal VHL allele do not participate in tumor growth), the effect of protein abundance less than this (but more than zero) is difficult to predict. Perhaps a threshold exists, below which VHL is incapable of sufficiently impairing VEGF transcription, a situation that appears to be the case in UoC-M1. The mechanism by which VHL protein is reduced in UoC-M1 was not determined. Additional experiments designed to determine the effect of VHL overexpression in UoC-M1 have the potential to not only more directly correlate VHL/VEGF levels in the system, but also to contribute basic information to the fundamental nature of VEGF control in hematopoietic cells. Such studies may prove very interesting indeed as the vast majority of literature describing the role of VHL in VEGF regulation has been obtained in the study of endothelial cells alone.

The Role of VEGF in Hematopoietic Neoplasms

Though VEGF-induced proliferation has been described mainly in solid tumors of non-hematopoietic origin, other reports indicate that VEGF may play an important role in MDS and AML,[241-250] a role that is not surprising given the observations that VEGF signaling is key to the development of embryonic hematopoietic cells[344,345] and more mature myeloid progenitors.[357] Dias *et al* have demonstrated autocrine stimulation of leukemic cells via KDR.[250] Work

published by List's group has recently shown that an autocrine VEGF loop may be important to cellular proliferation and secretion of other cytokines in dysplastic myeloid progenitors at the center of the intramedullary space in MDS and AML, a condition known as abnormally localized immature myeloid precursors or ALIP.[249] VEGF and VEGF receptor expression (FLT-1, KDR, or both) was limited to primitive myeloid cells and not seen in lymphoid cells from late-stage MDS/AML patients. Furthermore, colony formation could be enhanced by addition of VEGF to bone marrow mononuclear cells from MDS/AML patients. The effect of VEGF on the abnormal myeloid cells in the studies reported by List's group (and others) is congruent with the observations reported here in the UoC-M1 cell line and support the notion that autocrine stimulation by VEGF may contribute to the proliferative nature of leukemic blasts. Such an effect may proceed along intracellular signaling pathways also shown to be key to leukemic cell proliferation, especially in those cells demonstrating factor-independent *in vitro* growth (such as UoC-M1). One such pathway involves the STAT molecules.[328-334]

VEGF signals intracellularly via multiple pathways including STAT-1, STAT-3, and STAT-5.[325-327] VEGF has been shown to induce activation of the non-receptor tyrosine kinase SRC (the protein produced by the human homolog of the chicken Rous sarcoma virus gene *v-src*)[358] and in turn SRC phosphorylates STAT3.[359] Furthermore, v-src overexpression results in constitutively active STAT-1, 3, and 5.[360] STAT phosphorylation is important to

normal hematopoietic cell homesostasis[361] and when constitutively activated, is associated with factor-independent growth in myeloid leukemic cells.[328-334] While attempts to tie activation of c-KIT to UoC-M1 proliferation were ineffective, anti-phospotyrosine immunoblots constructed in those experiments did reveal the presence of prominently phosphorylated species in the molecular weight range of STAT molecules (ca. 95 kDa).[323,324] This notion was confirmed via immunoblot analysis using phospho-specific antisera for STAT's 1, 3, and 5, all three of which were found to be constitutively activated. Attempts to inhibit STAT phosphorylation with VEGF blockers were partially successful in that more general inhibitors such as herbimycin A and genistein were able to completely and durably inhibit phosphorylation while specific VEGF inhibitors were either only marginally able or incapable of impairing both cellular growth and STAT phosphorylation. These results might reflect either high, uninhibitable levels of VEGF activity in UoC-M1 cells or simply that VEGF signaling does not account for STAT phosphorylation or cellular proliferation. Additional experiments making use of dominant-negative STAT molecules,[325] alternative inhibitory compounds, or reintroduction of genetic material known to be missing in UoC-M1 cells all have the potential to shed further light on this issue. Regarding the latter, reasoning that a complex cytogenetic profile is perhaps contributory to the angiogenic nature of the UoC-M1 leukemic phenotype, reintroduction of chromosomal material via somatic cell hybrid fusion was undertaken in an attempt to suppress either the angiogenic nature, constitutive STAT phosphorylation, or both.

As certain karyotypic anomalies are known to be non-random in MDS/AML including monosomic loss of chromosome 7 (see chapter 1), reintroduction of chromosome 7 specifically was attempted via the rodent A9-7 hybrid. Unfortunately, experiments using both whole cell fusions as well as microcell-mediated chromosome transfer between A9-7 and UoC-M1 were all unsuccessful. Clearly, I am not certain of the reasons for the failure to achieve fusions in 19 separate experiments. One possibility is that UoC-M1 cells may be poor fusion partners. Previous experiments performed with UoC-M1 (and other myeloid cells) have shown these cells to be particularly difficult to fractionate into nuclear and cytoplasmic components (see chapter 3, figure 4). This suggests that the UoC-M1 cells may have an especially robust cell membrane, perhaps making them unsuitable for interacting adequately with fusion partners. Alternatively, successful fusions might well have occurred. However, the result may have been complementation and phenotypic reversion to a hematopoietic cell phenotype closer to the normal state, that is, a cell with a very short half-life in culture because it underwent terminal differentiation or apoptosis. In fact, the normal developmental profile of a primitive hematopoietic cell is to differentiate and then to die.[10-12] While attempts were made to supplement post-fusion media with hematopoietic growth factors (such as GM-CSF and FLT-3) as well as UoC-M1 conditioned media, it is possible that the factor-independent growth of the UoC-M1 cells[189] was abolished (or significantly reduced) by genomic complementation with human chromosome 7. When we began these studies we

hoped that the complex cytogenetic profile of the UoC-M1 cells would allow appreciation of a more subtle phenotypic alteration after reintroduction of chromosome 7 (such as loss of a primitive surface antigen, reduction of VEGF secretion, or abolition of constitutive phosphorylation of one or more STAT molecules). However, this was clearly a high-risk hope, recognized as such before even our first experiment was performed.

In conclusion, the finding that UoC-M1 cells spontaneously secrete an inordinately large amount of a known mitogenic factor (VEGF) in conjunction with expression of activators of VEGF transcription, reduced levels of the major VEGF inhibitor (VHL), the presence of two high-affinity VEGF receptor tyrosine kinases (FLT-1 and KDR), and constitutive phosphorylation of known intracellular transducers of VEGF signaling (STAT 1, 3, and 5) all point to a mechanism by which these leukemic blast cells have become disconnected from normal cues for regulation of growth. That VEGF-specific inhibitors were unable to significantly impair either UoC-M1 growth or STAT phosphorylation at pharmacologically relevant concentrations may speak either to the degree to which the system is inordinately upregulated or to the fact that another, as yet unidentified soluble factor (or intracellular, ligand-independent event) is at the root of the sustained proliferative capacity of the cells. Additional studies making use of ELISA, dominant-negative STAT molecules, alternative VEGF-specific inhibitors (including transgenes), and non-chromosome 7 somatic cell hybrids have the potential to bring clarity to the pathogenesis of neoplasia in this clone.

Figure 4-1

Figure 4-1 Legend: UoC-M1 forms sub-cutaneous tumors in NOD/SCID mice. To determine the growth characteristics of cells *in vivo*, $5x10^6$ UoC-M1 cells were injected sub-cutaneously into a cohort of three NOD/SCID mice. All mice developed masses within 20 days. Arrow indicates epi-scapular nodule at the injection site.

Figure 4-2

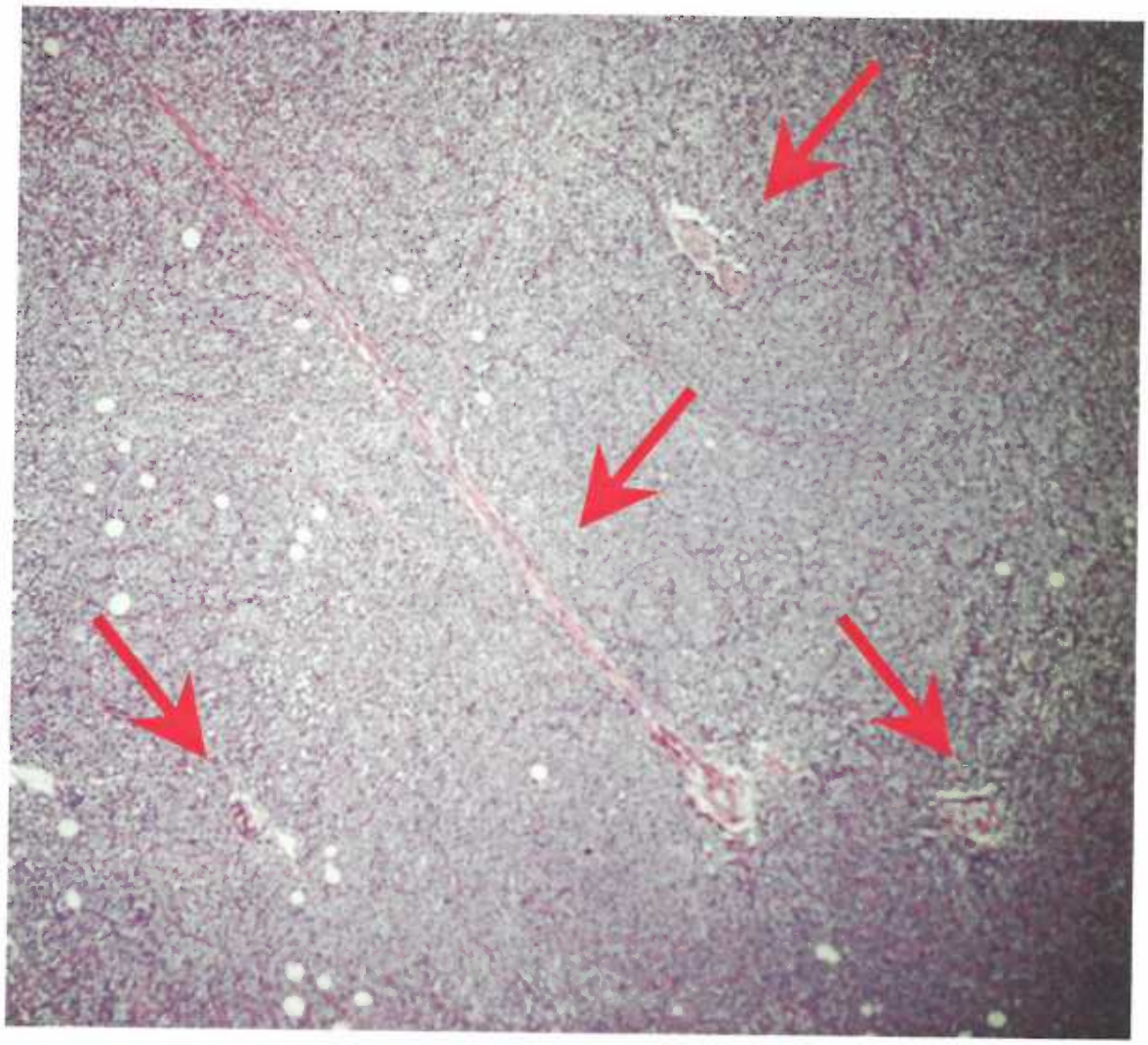


Figure 4-2 Legend: H/E stained tumor sections demonstrate high microvascular density. A similar degree of vascular formation was seen in all three primary animals as well as in secondary and tertiary serial recipients. Tumor sections demonstrated minimal necrosis. Arrows indicate blood vessels in background of tumor cells. Magnification = 40X paraffin sections were produced by the Portland VAMC Pathology Core (Chris Corless, M.D., Ph.D.).

Figure 4-3A

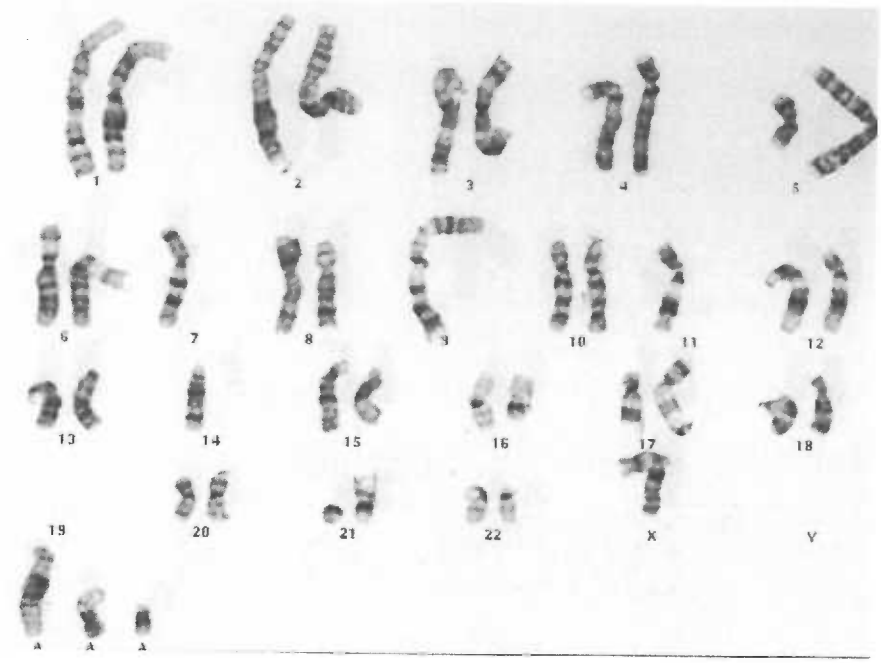
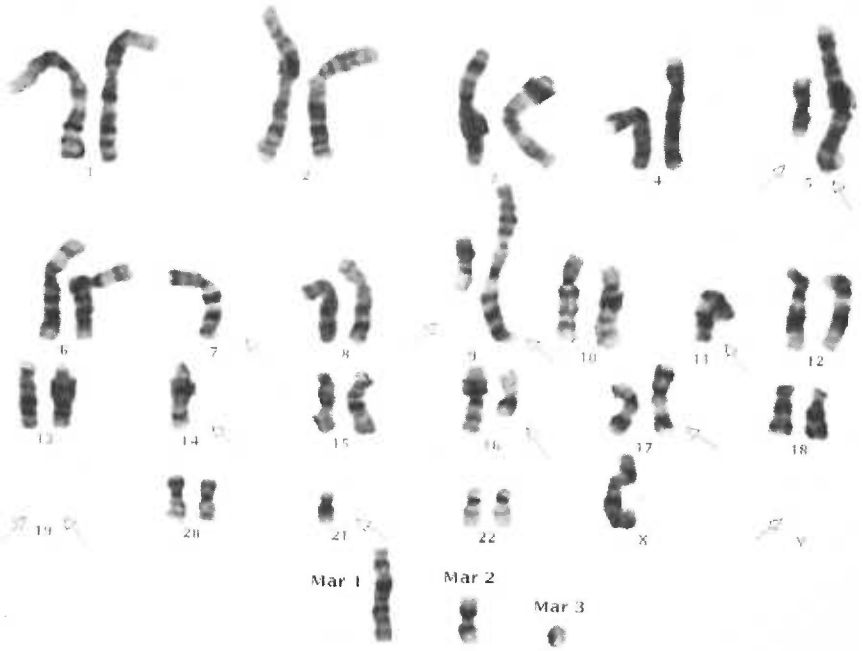


Figure 4-3B



Figures 4-3A and 4-3B Legend: NOD/SCID tumor cell karyotypes are identical to UoC-M1. Compared to the input UoC-M1 cells[189] (4-3A), G-banded tumor cell karyotypes (this from animal MWL002, 4-3B) demonstrate the same complex chromosomal content including 5q-, deletions of 7q, monosomy 11, nullisomy 19, monosomy 21, and loss of Y indicating that the tumor masses were composed of UoC-M1 (and not mouse) cells. Furthermore, no new genetic drift appears to have occurred during *in vivo* proliferation of the cell line. An equivalent karyotype was obtained in mouse MWL003 while mouse MWL001 was not cytogenetically analyzed. G-banded karyotypes were prepared by Carol Reifsteck, OHSU.

Table 4-1: ELISA determinations of cytokine secretion

cytokine	[pg/ml]
VEGF	2700.0
TPO	0.0
TNF-α	0.0
SCF	0.0
IL-6	3.0
IL-4	19.0
IL-1β	6.2
GM-CSF	0.0
FLT-3	9.0

Figure 4-4

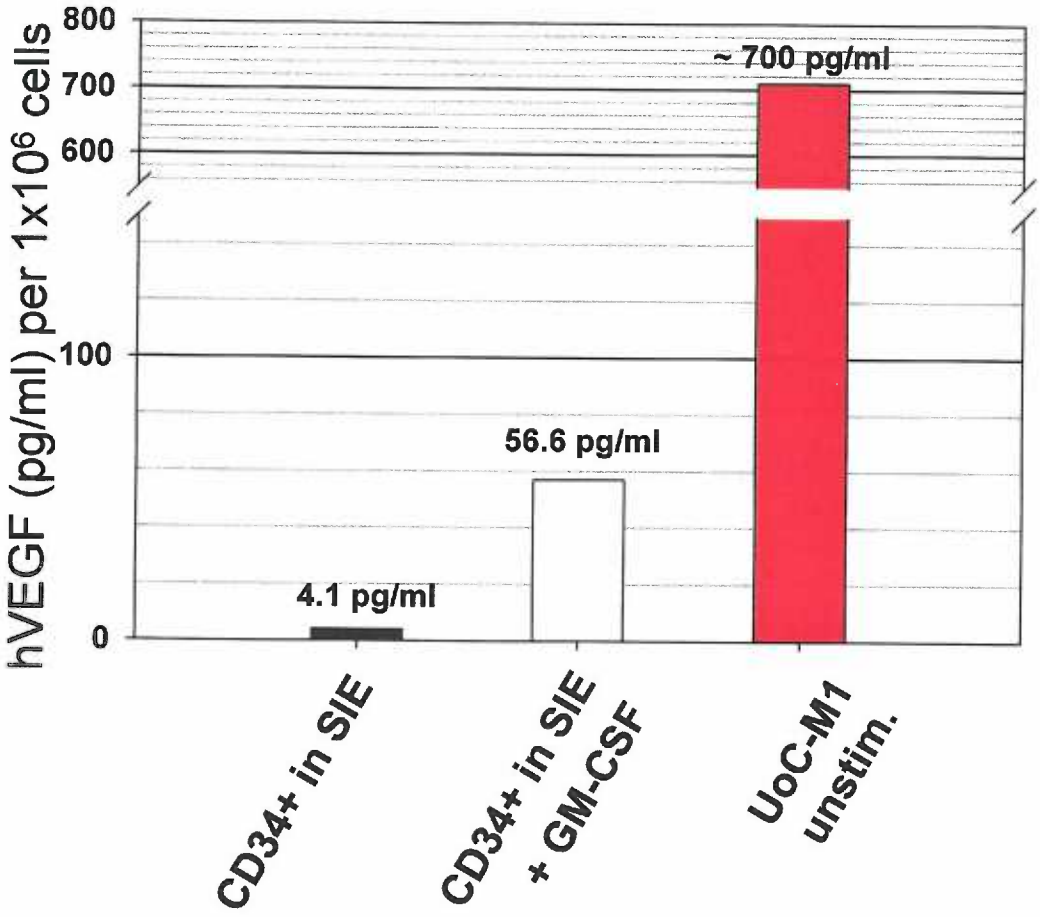

hVEGF (pg/ml) per 1x10^6 cells
800
700
600
100
0
~ 700 pg/ml
56.6 pg/ml
4.1 pg/ml
CD34+ in SIE
CD34+ in SIE + GM-CSF
UoC-M1 unstim.

Figure 4-4 Legend: High level VEGF secretion by blast cells is abnormal. To evaluate VEGF production in UoC-M1 relative to a normal control in a standardized, 72-hour secretion assay, CD34+ cells were obtained and VEGF production quantitated in SIE (hSCF, IL-3, and EPO) and SIE + 100 U/ml of GM-CSF (a factor known to stimulate VEGF production in myeloid cells).[243] Unstimulated CD34+ cells secrete a small amount of VEGF (~ 4 pg/ml per million cells), an amount that could be increased roughly ten-fold with GM-CSF stimulation (~ 56 pg/ml per million cells). However, unstimulated UoC-M1 cells produce over 700 pg/ml per million cells in the same time interval.

Figure 4-5

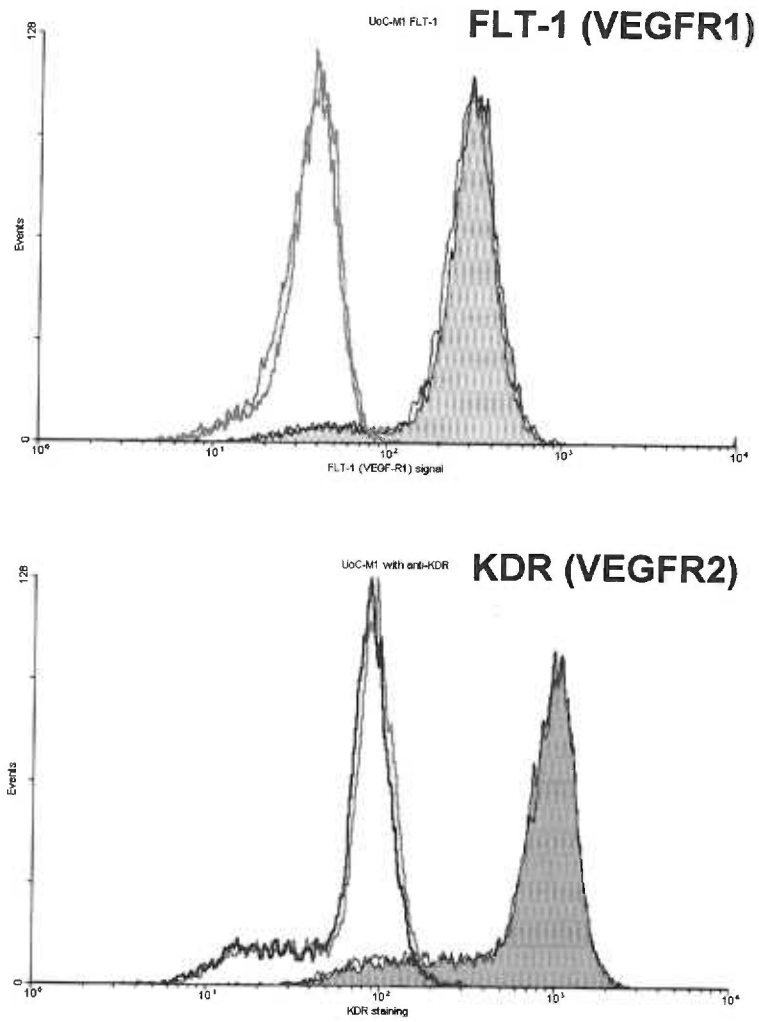

UoC-M1 FLT-1
FLT-1 (VEGFR1)
Events
128
0
10⁰
10¹
10²
10³
10⁴
FLT-1 (VEGF-R1) signal
UoC-M1 with anti-KDR
KDR (VEGFR2)
Events
128
0
10⁰
10¹
10²
10³
10⁴
KDR staining

Figure 4-5 Legend: UoC-M1 has two high-affinity, VEGF-binding, receptor tyrosine kinases. High levels of VEGF were present in UoC-M1 cell culture supernatants. As VEGF is known to be a potent endothelial cell mitogen[270] it is possible that such an activity contributes to the proliferation of UoC-M1 cells. This is especially true given the finding that UoC-M1 displays two high-affinity, receptor tyrosine kinases (right-shifted [FITC-positive] closed areas in each plot), FLT-1 (VEGFR1) and KDR (VEGFR2) for VEGF. Experiments were performed in duplicate and are plotted relative to isotype controls (open areas) for each receptor.

Figure 4-6A

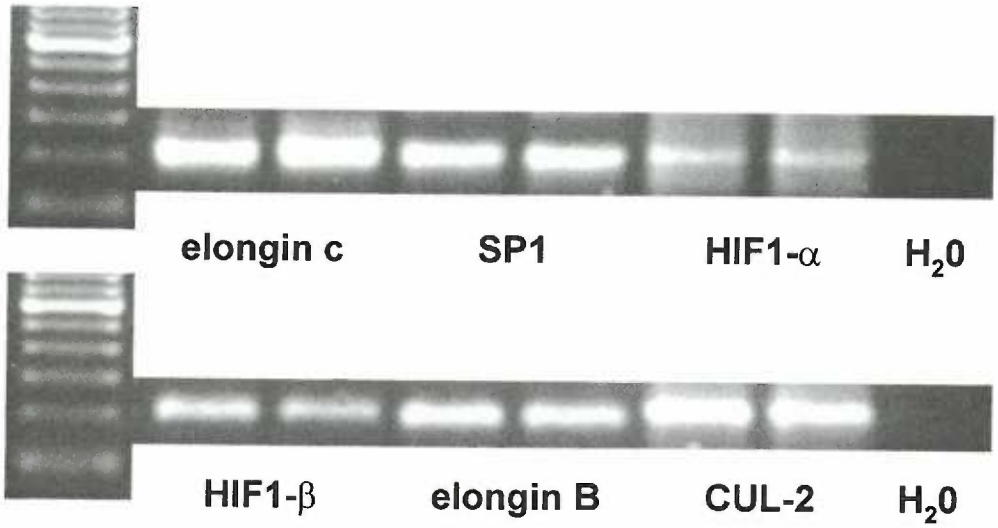


Figure 4-6B

Figure 4-6A and 4-6B Legend: VEGF co-factors and modulators are co-expressed. As shown in figure 4-6A, production of high levels of VEGF in UoC-M1 correlates with expression (RT-PCR) of VEGF transcriptional effectors (*SP1* and *HIF1-α/β*) [288-290,297-301] However, the VEGF repressors *elongin B/C* [299-305] and *CUL-2*[304-308] are also expressed as is the major VEGF repressor, von Hippel-Lindau protein (*VHL*)(not shown).[298,299,301-304,306-311] Samples were amplified and run in duplicate. Far left lanes are 100 bp DNA size standards and far right lanes (H_20), no template control reactions. Figure 4-6B demonstrates that while present, VHL protein (~ 19 kDa) is reduced relative to the CD34+ myeloid leukemic cell line Mo7e and the human embryonic kidney cell line 293 (neither Mo7e nor 293 have been reported to spontaneously secrete VEGF). Each lane was loaded with 80 μg of whole cell extract. Loading continuity was verified by Ponceau-S staining of protein fractions immobilized to nitrocellulose filters prior to probing with VHL antiserum. Such a reduction may account for constitutive production of VEGF by UoC-M1.

Figure 4-7A

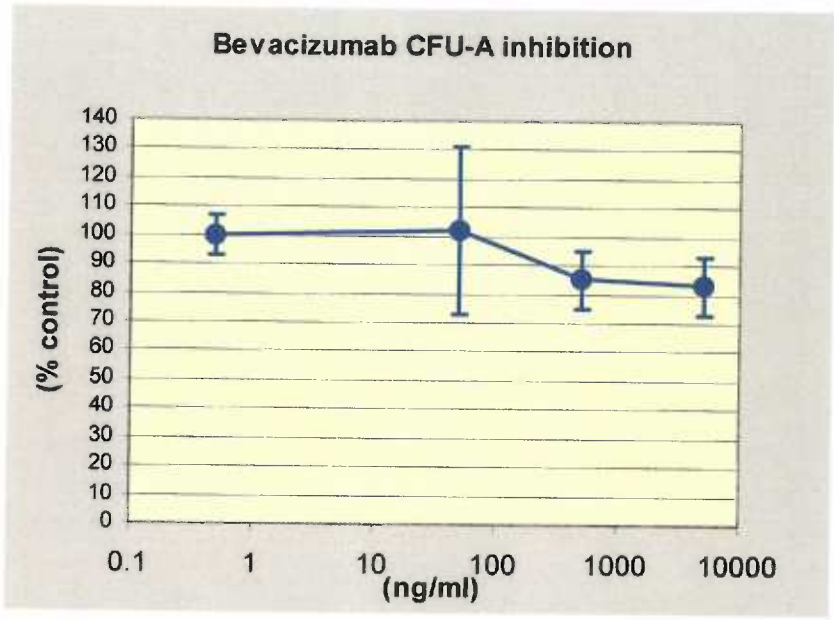


Figure 4-7B

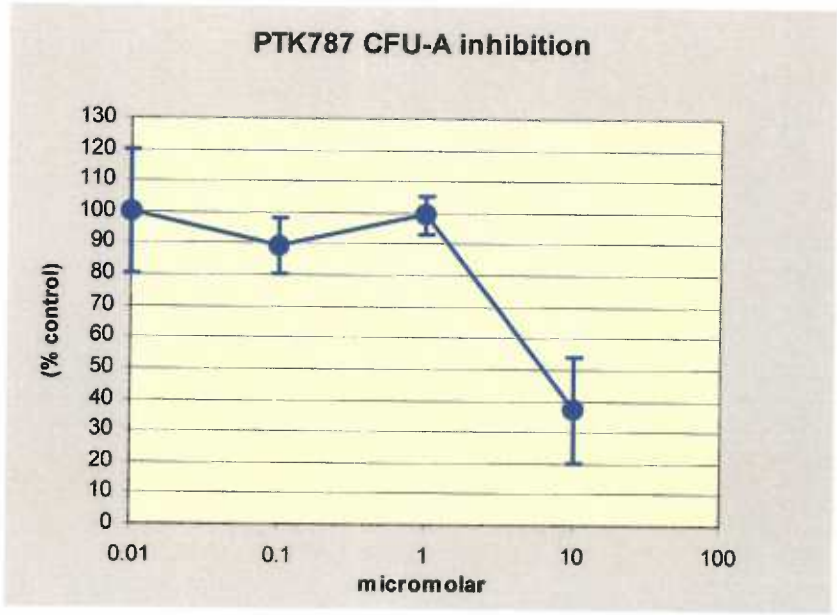


Figure 4-7A and 4-7B Legend: Growth inhibition by VEGF-specific agents. Use of a humanized, VEGF-binding, monoclonal antibody (Bevacizumab) did not result in significant inhibition of UoC-M1 in methylcellulose cultures (figure 4-7A). The highest concentration of antibody used (5,000 ng/ml or 30 nM) was ten-fold greater than the reported IC_{90} for bovine capillary endothelial cells stimulated with 3,000 pg/ml of VEGF.[315] As VEGF is known to bind cell surface heparan proteoglycans,[270] there may be a local concentration of VEGF at the receptor greater than determined via ELISA analysis of cell culture supernatants. Direct inhibition was also attempted at the receptor. Figure 4-7B demonstrates that the VEGF-receptor tyrosine kinase inhibitor PTK787,[320] has an IC_{60} of 10 μM in UoC-M1. This result suggests that VEGF-dependent proliferation may occur in UoC-M1, though concentrations of PTK787 in the micromolar range have also been associated with non-specific interactions and toxicity.[320]

Figure 4-8

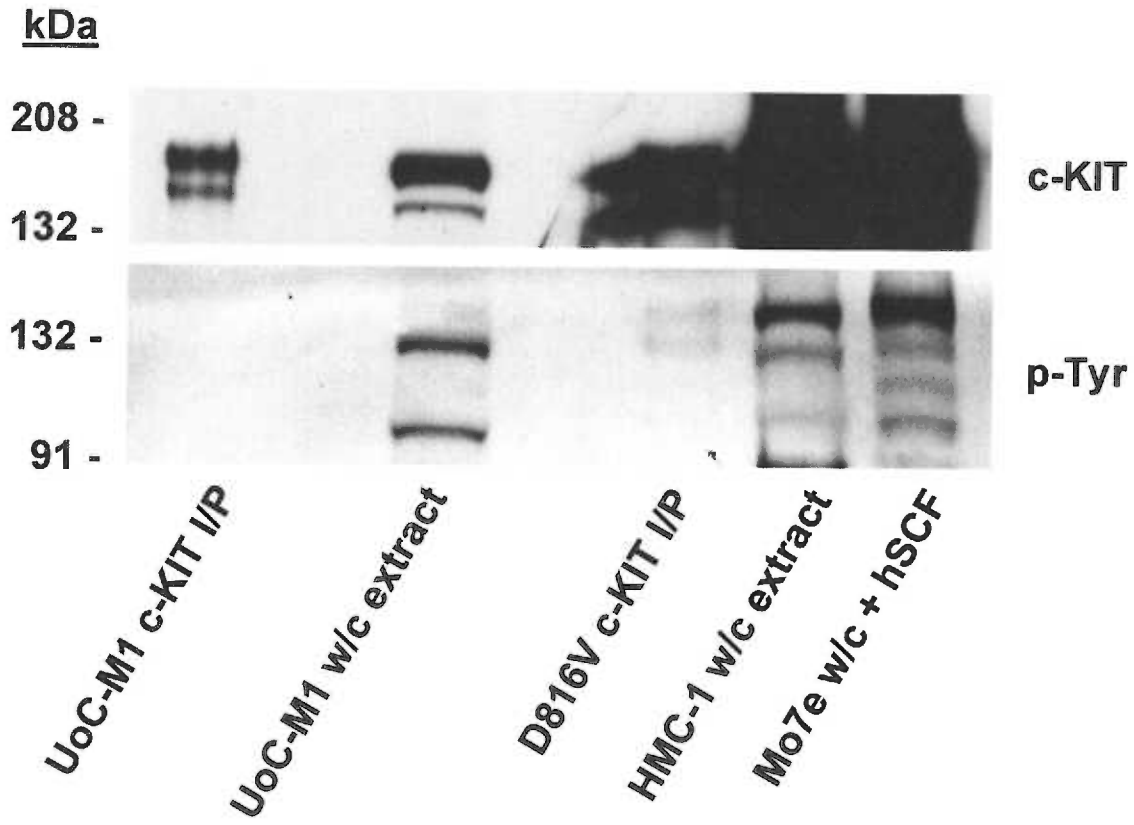

kDa
208 -
132 -
132 -
91 -
c-KIT
p-Tyr
UoC-M1 c-KIT I/P
UoC-M1 w/c extract
D816V c-KIT I/P
HMC-1 w/c extract
Mo7e w/c + hSCF

Figure 4-8 Legend: Immunoprecipitation with anti-c-KIT. Alternative pathways for factor-independent growth in UoC-M1 were sought. UoC-M1 is CD117 (c-KIT) positive though the ligand for c-KIT (stem cell factor [SCF]) is not produced by UoC-M1 (see table 4-1). However, it remained possible that an activating c-KIT mutation could be present in UoC-M1. To determine the activation state of c-KIT, immunoprecipitation was performed with an antibody raised against c-KIT and anti-phosphotyrosine immunoblotting was performed. As shown here, compared to SCF-stimulated Mo7e, HMC-1 (a constitutively c-KIT active mast cell leukemia line), and CHO cells transiently transfected with *c-KIT* bearing an activating mutation (D816V), UoC-M1 does not have activated c-KIT. However, two prominent tyrosine-phosphorylated species (approximately 95 and 130 kDa) were observed in UoC-M1 whole cell extracts. These molecular weights are in the range of phosphorylated Janus kinases (JAK's) and signal transducers and activators of transcription (STAT) molecules.

Figure 4-9

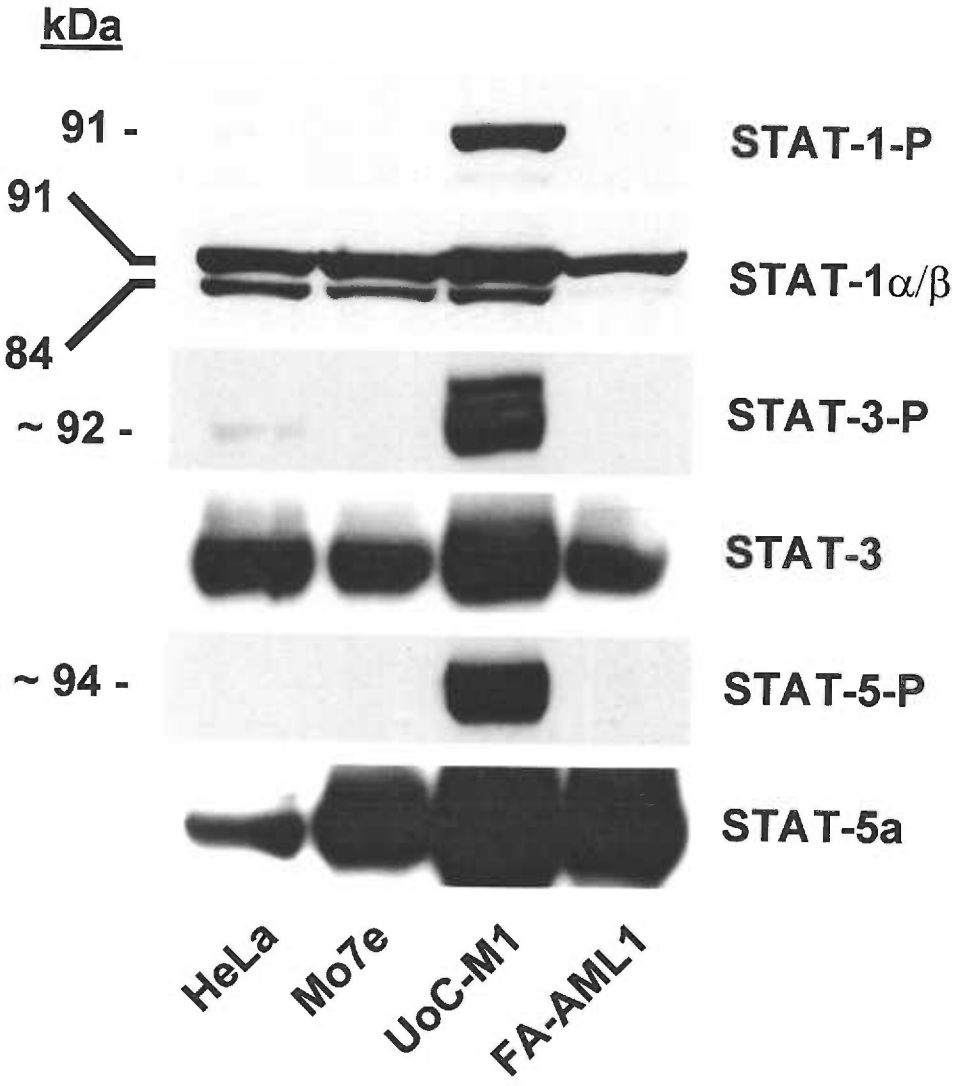

kDa
91 -
91
84
~ 92 -
~ 94 -
STAT-1-P
STAT-1α/β
STAT-3-P
STAT-3
STAT-5-P
STAT-5a
HeLa
Mo7e
UoC-M1
FA-AML1

Figure 4-9 Legend: Ground-state phosphorylation of STAT-1, 3, and 5 in UoC-M1 cells. Anti-phosphotyrosine immunoblots suggested that phosphorylated STAT's may be present in UoC-M1 and as STAT activation (via phosphorylation)[324] has been associated with both VEGF production[325-327] and factor-independent growth of myeloid leukemic cells,[328,329] STAT activation status (phosphorylation) was determined via immunoblot analysis. As shown above, UoC-M1 has constitutive activation of STAT-1, STAT-3, and STAT-5. These STAT's are known to transduce signals through receptors for a variety of cytokines[361] including VEGF.[325-327]

Figure 4-10A

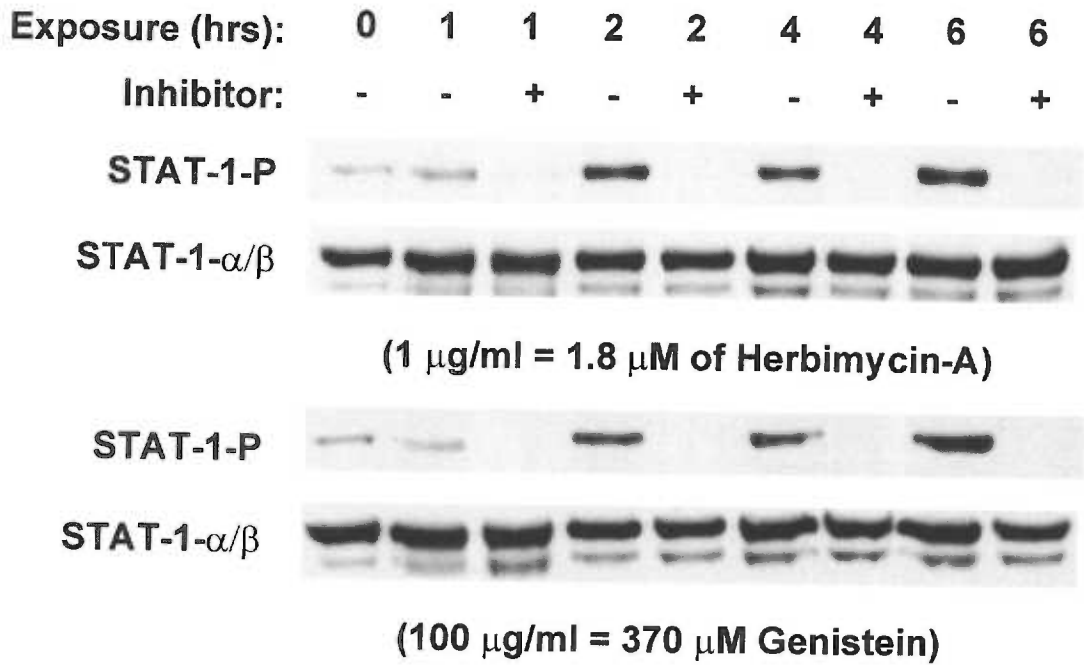


Figure 4-10B

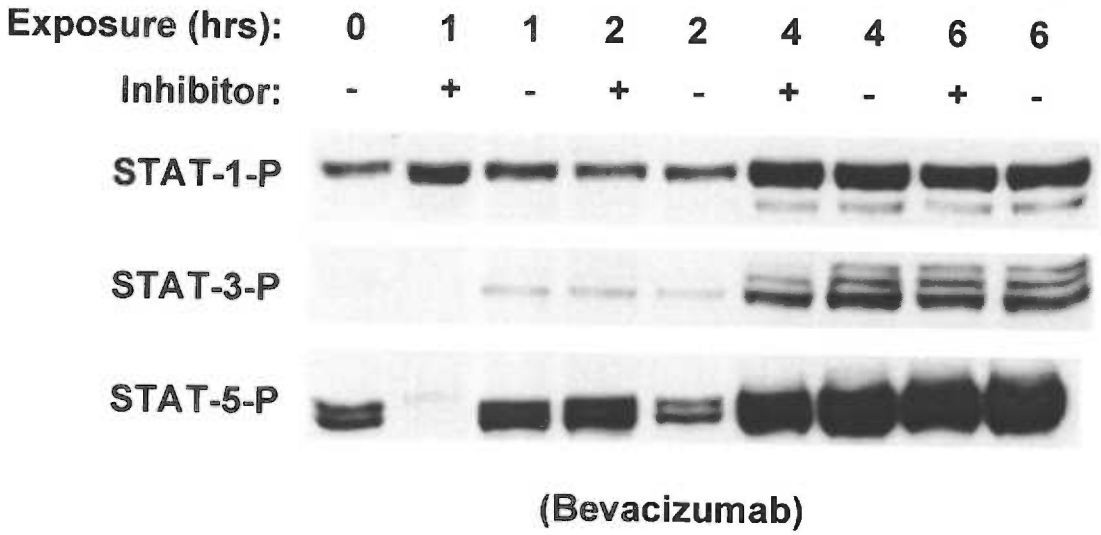


Figure 4-10A and 4-10B Legend: STAT phosphorylation is inhibitable. UoC-M1 cells were subjected to time-course treatment with the tyrosine kinase inhibitors herbimycin A and genistein to determine whether or not STAT signaling could be impaired. As shown in figure 4-10A, both compounds resulted in durable STAT-1 inhibition up to the final time point (6 hours). Similar data was obtained for STAT-3 and STAT-5 (not shown). In an attempt to correlate STAT phosphorylation to VEGF binding events, the monoclonal VEGF inhibitor Bevacizumab was used in an identical time course. As shown in figure 4-10B, STAT-1 phosphorylation could not be impaired with Bevacizumab treatment while STAT-3 and STAT-5 were transiently inhibitable up to one hour following exposure. The transient nature of STAT inhibition likely correlates with the inability of Bevacizumab to inhibit UoC-M1 growth (see figure 4-7A). Of note, the phosphorylation kinetics of STAT-3 and 5 appear slightly different. STAT-3 demonstrates a signal that was lost with cell rinsing (cold PBS) prior to lysis (time point zero, figure 4-10B) and maximal reactivation delayed to between two and four hours compared to STAT-5 that maintained phosphorylation despite rinsing prior to treatment. This observation may suggest that a soluble factor (with low relative affinity) or one with quick on/off kinetics intracellularly is responsible for the STAT-3 phosphorylation while STAT-1 and STAT-5 activation is of a more durable nature or perhaps results from a ligand/receptor interaction with higher affinity.

Figure 4-11A

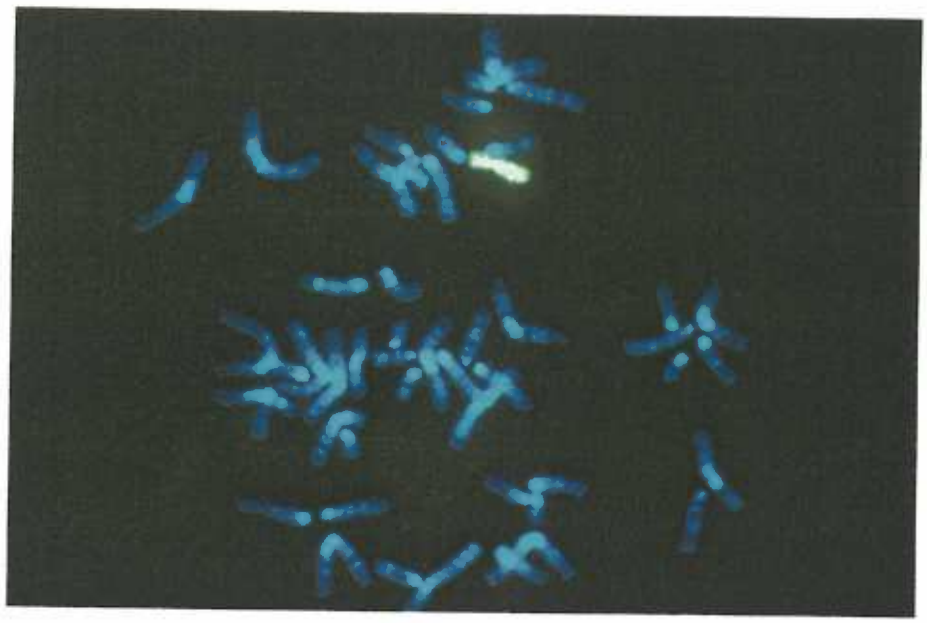


Figure 4-11B

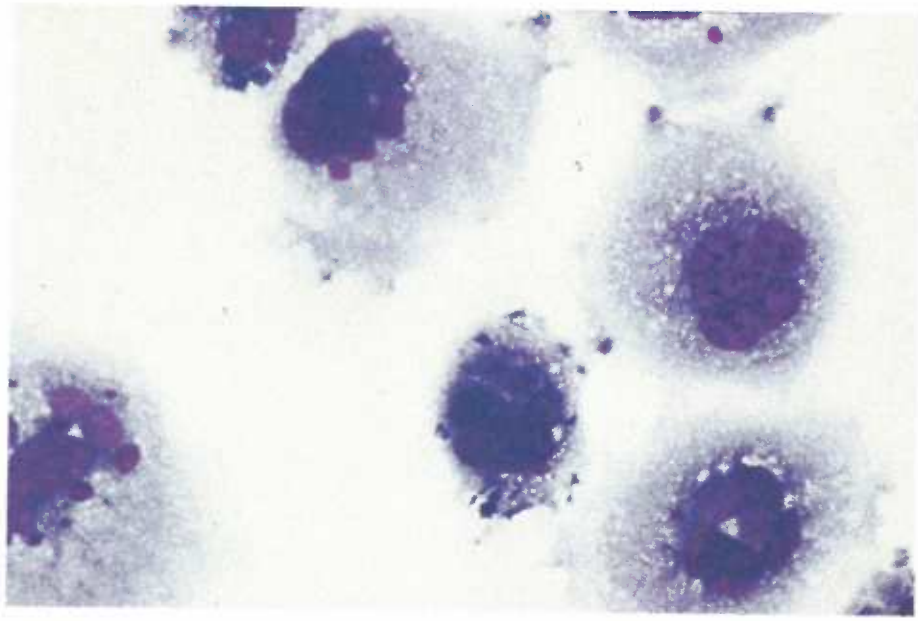


Figure 4-11A and 4-11B Legend: Reagents for chromosomal complementation. To establish a link between the transformed phenotype, STAT phosphorylation, and the aneuploidy present in UoC-M1, complementation with large-scale chromosomal material (whole-fusion and microcell mediated chromosome transfer) was attempted. FISH analysis with a whole-chromosome 7 paint (Vysis, United Kingdom) against DAPI counterstained mouse chromosomes indicates that the rodent somatic cell hybrid A9-7 contains a single human chromosome 7 (green signal) on a mouse background (blue signal) (figure 4-11A). The human chromosome 7 is marked with a dual selection cassette: positive with hygroB (*Hy*) and negative with ganciclovir (*HSV-tk*). Multiple attempts at whole cell fusion were unsuccessful. Reasoning that dominantly acting mouse material may have impaired the ability to obtain viable fused products, microcell-mediated chromosome transfer was also employed. As shown in figure 4-11B, A9 cells can be induced to micronucleate when cultured with colcemid.[257,258] These images are of W/G stained A9-7 cells after culture for 48 hours in 0.08 μg/ml of colcemid. Despite the performance of multiple microcell fusion experiments, no viable fused products were obtained. This suggests that either UoC-M1 cells demonstrate poor fusion kinetics or that reintroduction of chromosome 7 results in apoptosis. FISH analysis was performed by Carol Reifsteck, OHSU.

Figure 4-12

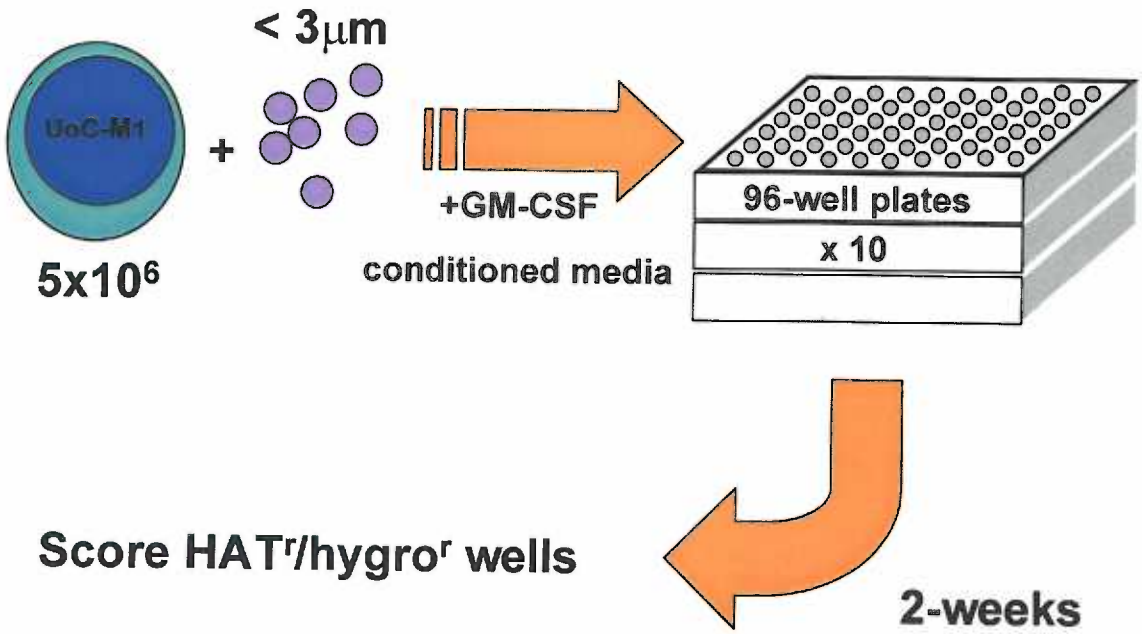

< 3μm
UoC-M1
+
5x10⁶
+GM-CSF
conditioned media
96-well plates
x 10
Score HATʳ/hygroʳ wells
2-weeks

Figure 4-12 Legend: Schema for microcell-mediated chromosome transfer. Microcell-mediated chromosome transfer protocols were optimized to foster maintenance of fused products that may have lost factor-independence by the addition of GM-CSF and 50% UoC-M1 conditioned media. Furthermore, schemes were used to selectively enforce the growth of fused material by use of HAT medium to select against mouse cell contamination (mouse A9 cells are *Hprt*$^{-/-}$) and for the human chromosome 7 via hygromycin-B (the human chromosome 7 in A9-7 is hygro^{r} marked).

Chapter five: Summary and conclusions

The objective of the work presented herein was to define the processes by which leukemic clonal evolution occurs in patients with Fanconi anemia and to determine whether Fanconi protein dysfunction occurs in patients with sporadic acute leukemia. The work presented in each of the four chapters above has; (A) provided a demonstration of a viable model of adaptive evolution in Fanconi anemia that involves the development of apoptotic resistance, (B) identified candidate genes that may be responsible for the adaptive responses, (C) demonstrated that acquired defects of FA protein function may contribute to leukemic progression in cases of sporadic leukemia in non-FA individuals, and (D) suggested molecular mechanisms by which factor-independent proliferation evolves in a particular leukemic clone.

Regarding the first point, the hematopoietic stem cell (HSC) pool in Fanconi anemia exhibits exaggerated apoptotic responses to extracellular cues. This phenotype has been confirmed in our lab and in others as well and is likely the cause of bone marrow failure that occurs in virtually all patients with FA. Those individuals that do not die of severe BMF are at extraordinary risk of MDS and AML. The variety of MDS/AML that develops in these patients is virtually identical to the MDS/AML that occurs in adults who have been exposed to alkylating agents for other disorders (known as secondary MDS/AML). Shared hallmarks include the occurrence of precisely the same type and mix of non-

random cytogenetic defects. We propose that in each case, progenitor cell hypersensitivity to apoptotic cues (as part of the aberrant signaling function observed with FA gene mutations and somehow resulting from prior therapy in non-FA adults) contributes a profound selective pressure for the evolution of apoptosis-resistant HSC's. In FA, an additional element, cytogenetic instability, is present at all times and sets the stage for adaptive mutations. The proof-of-principle for this notion was developed using comparative growth inhibition (IFNγ) profiles for bone marrow progenitors in normal controls, non-FA adults with secondary MDS/AML, FA patients without evidence of malignancy, and finally FA patients that have progressed to MDS or AML. Compared to normal controls, 6 of 6 FA patients without malignancy demonstrated significant IFNγ-hypersensitivity while FA patients progressing to malignancy were IFNγ-resistant. These observations led to a transcriptomal assessment of a unique multiplex pedigree of FA/MDS. This pedigree contained two siblings with FA-A at different stages of disease progression (one with FA/MDS [and IFN-resistant] and another with FA alone [and IFN-hypersensitive]).

Gene expression microarray analysis was performed with low-density bone marrow cell RNA from these siblings seeking to define genes important for the initiation of malignancy in FA. The result of this analysis was a list of 327 genes expressed at greater than a 2-fold difference in pair-wise comparisons. Several rather suggestive candidate genes were identified including *BAX*, *c-ETS*,

and the L-3-phosphoserine phosphatase homolog that are currently being evaluated in our laboratory.

The suggestion that FA-related MDS and AML have key pathogenetic links with non-FA, adult MDS and AML was strengthened by the demonstration that acquired, lineage-restricted defects of FA protein function may serve as progression factors in individuals without evidence of hereditary FA. The case in point was of a nearly 70-year old male that presented with secondary AML with characteristic complex cytogenetic defects in his leukemic clone. As is almost universal in such cases, his disease was intractable and described a short and quickly fatal course. From this patient two cell lines were derived at the University of Chicago; a spontaneously immortalized myeloid cell line (UoC-M1) with cytogenetics comparable to the patient's leukemia and an EBV immortalized lymphoblastoid cell line (4081). Each line was subjected to exhaustive analyses of FA protein function.

The results of these studies demonstrated that mitomycin C sensitivity existed in the UoC-M1 myeloid cell line but not 4081 cells. A defect of FANCA protein function was identified in UoC-M1 cells but not 4081 cells. Specifically, we discovered a loss of nuclear FANCA and FANCG proteins (these two proteins are known to stabilize one another) as well as inability to form FANCD2-L (a post-translational modification of the FANCD2 gene) in UoC-M1 cells. None of these defects were present in 4081. Retroviral transduction with *FANCA*

restored MMC resistance, increased cellular protein levels of both FANCA and FANCG, and resulted in FANCA nuclear accumulation. However, genomic DNA sequencing was unable to demonstrate mutations of *FANCA*, QRT-PCR indicated that the *FANCA* mRNA was abundant, and Western blot analysis showed that FANCA was of the proper molecular weight. A secondary defect of FANCA involving protein complex stabilization or nuclear transport is most likely. The discovery of the particular defect involved is beyond the scope of the work described in this thesis. However, it is clear that in this case, FA protein dysfunction occurred as a somatic event during the evolution of this leukemic clone and based upon the normal gene sequences and the finding of normal FA protein function in the B-cells of the same patient (not part of the leukemic clone), it is not likely that this patient had inherited FA. We have argued above that somatic losses of FA function would not serve as an efficient leukemia-initiating event given that the phenotype of FA mutations in hematopoietic cells is to promote apoptosis. We do propose that such dysfunction could serve as a progression factor for already initiated leukemic cells and could profoundly contribute to cytogenetic instability.

Finally, *in vivo* studies with the UoC-M1 cell line conducted in immunodeficient mice revealed a highly-angiogenic phenotype that derived from constitutive secretion of vascular endothelial cell growth factor (VEGF). This notion stemmed from the discovery of high level VEGF production by this cell line, expression in this line of two-high affinity receptor tyrosine kinases for VEGF

(FLT-1 and KDR) coupled with a reduction in protein levels for the major VEGF-inhibitory factor von Hippel-Lindau protein (VHL), and demonstration of constitutive activation (phosphorylation) of key transcription factors (STAT's 1, 3, and 5) known to be activated by VEGF. However, two VEGF-specific inhibitors, one a humanized monoclonal blocking antibody to VEGF and the other a VEGF receptor-specific tyrosine kinase inhibitor, had only a minor impact on cellular proliferation and STAT activation. These observations suggest that an as yet unidentified factor may be responsible for both the constitutive VEGF production and factor-independent cell growth of the UoC-M1 cell line.

Taken together, the work presented in this thesis focuses specifically on the FA pathway and clarifies biological issues of leukemogenesis not only in patients with FA mutations but in adults with secondary leukemia as well. These issues include, in FA patients, the occurrence of somatic adaptations to an inherently apoptotic phenotype and in non FA patients, the occurrence of FA protein dysfunction as a somatic event in patients with sporadic secondary AML and the discovery of signaling mechanisms that disconnect cell proliferation from modulatory controls. Because the model of AML in Fanconi anemia and of FA protein dysfunction in sporadic AML has revealed both early and late events in leukemogenesis, we argue that further study of secondary leukemia is in order using FA genes and proteins as tools for scientific discovery.

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