DEVELOPMENT AND APPLICATION OF AN *IN VITRO* CULTURE MODEL FOR

PRIMARY HUMAN T-ALL CELLS

by

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Abstract

T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive hematologic malignancy that affects both children and adults. Optimization of chemotherapy regimens has led to steady improvements in outcome for pediatric patients over the last 5 decades, with a long-term survival rate of 80%. However, the five-year survival rate for adults is still only 35-40% and there is a poor prognosis for relapse patients (Goldstone et al., 2008). Further improvements in outcome will undoubtedly require introduction of novel approaches and more specific targeted therapies. Research efforts in this area have been hampered by the lack of a reproducible model for *in vitro* growth of human T-ALL blasts. Most efforts to date have relied heavily upon established cell lines, which can be a useful tool to study malignancies but do not necessarily always represent bona fide disease biology, and in vivo studies involving patient samples expanded as xenografts in immunocompromised mice, which are costly, time-consuming and complicated by non-cell autonomous effects. Current methods for *in vitro* culture of patient T-ALL samples yield variable performance with high rates of apoptosis and less than robust proliferation. Development of an efficient and reproducible *in vitro* culture method for growth of primary human T-ALL blasts would greatly enhance the ability to test and validate novel therapies by allowing for direct assay for sensitivity/resistance of patient cells which have not been subject to extensive manipulation or selection.

In this work we report an *in vitro* co-culture system using defined, serum-free media and a stromal feeder cell layer which supports robust growth and minimal apoptosis of patient T-ALL blasts. We have shown that the stromal feeder cell layer and supplemental IL-

7 cytokine is critical for sustained patient T-ALL blast growth in this model. Finally, we have demonstrated the utility of this culture system as a platform that will facilitate ongoing efforts to identify growth factors/cytokines required for maintenance of leukemia cell self-renewal activity, aid in the study of signaling pathways important in T-ALL pathogenesis and maintenance, and allow for prospective testing of novel compounds for therapeutic efficacy on patients' own tumor cells, thus enabling implementation of personalized medicine initiatives.

Preface

All experiments were conducted by A. Yost except for the collaborations listed below. Dr. V. Giambra provided assistance with the human T-ALL xenografts in immunocompromised mice.

The western blot shRNA validation, shRNA knockdown of T-ALL cell lines and the siRNA knockdown of TYK2 in patient T-ALL blasts reported in Chapter 3 were done by Dr. Jeffery Tyner at the Oregon Health and Science University and Dr. Takaomi Sanda at the Dana-Farber Cancer Institute.

All work on the patient T-ALL blast sensitivity to the novel therapeutic, borrelidin, as reported in Chapter 3, was done in collaboration with Darya Habibi, a graduate student in the laboratory of Dr. Chris Ong at the University of British Columbia.

The results presented in Chapter 3 on Borrelidin have been published. D. Habibi, N. Ogloff, R.B. Jalili, A. Yost, A.P. Weng, A. Ghahary, C.J. Ong. Borrelidin, a small molecule nitrile-containing macrolide inhibitor of threonyl-tRNA synthetase, is a potent inducer of apoptosis in acute lymphoblastic leukemia. *Invest New Drugs*. 2011.

Primary samples were obtained at initial diagnosis with informed consent from patients or their legal guardians under approved institutional review board protocols (Human ethics protocol #H06-00028) and following guidelines established by the Declaration of Helsinki.

Mice were housed in the BC Cancer Research Centre Animal Resource Centre. All animal use was approved by the animal care committee of the University of British Columbia (Animal Research Protocol #A09-0771), and animals were maintained and euthanized under humane conditions in accordance with the guidelines of the Canadian Council on Animal Care.

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Abbreviations

7-AAD: 7-Aminoactinomycin D

Ab: Antibody

ADAM: proteins containing a disintegrin and a metalloprotease domain

AHR: Aryl hydrocarbon receptor

ALL: Acute lymphoblastic leukemia

AML: Acute myeloid leukemia

ANK: Ankyrin

ARNT: Aryl hydrocarbon receptor nuclear translocator

B-CLL: B-cell chronic lymphocytic leukemia

BrdU: Bromodeoxyuridine (5-bromo-2'-deoxyuridine)

CAM-DR: Cell adhesion mediated drug resistance

CD: Cluster of differentiation

CDK8: Cyclin-dependent kinase 8

CFSE: Carboxyfluorescein diacetate succinimidyl ester

CI: Combination index

CLL: Chronic lymphocytic leukemia

CMML: Chronic myelomonocytic leukemia

CNS: Central nervous system

CSC: Cancer stem cell

CSL: CBF-1, Suppressor of Hairless, Lag-1

CTB: Cell Titer Blue

DL1: Delta-like-1

DNA: Deoxyribonucleic acid

DMSO: Dimethyl sulfoxide

DSL: Delta, Serrate, Lag2

ECN: Extracellular notch

EGF: Epidermal growth factor

FBS: Fetal bovine serum

FBW7: F-box and WD repeat domain-containing 7

Flt3L: Fms-like tyrosine kinase 3 ligand

FTOC: Fetal thymic organ culture

Gy: Gray

HBSS: Hank's Balanced Salt Solution

HD: heterodimerization domain

HES: Hairy and enhancer of split

HIF-1: Hypoxia inducible factor 1

HOXA: Homeobox protein A

HSC: hematopoietic stem cell

IC_{50}: Half maximal inhibitory concentration

ICN: Intracellular notch

IGF: Insulin-like growth factor

IGF1R: Insulin-like growth factor 1 receptor

IL-2: Interleukin 2

IL-3 : Interleukin 3

IL-4 : Interleukin 4

IL-7: Interleukin 7

IL-9: Interleukin 9

IL-15: Interleukin 15

IL-21: Interleukin 21

InsR: Insulin receptor

IRS : Insulin receptor substrate

LSC: Leukemia stem cell

LYL1: Lymphoblastic leukemia derived sequence 1

mAb: Monoclonal antibody

MAM: Mastermind

MAML1: Mastermind-like 1

MCL: Mantle cell lymphoma

MEM: Minimum essential medium

mTOR: Mammalian target of rapamycin

MYC: Myelocytomatosis oncogene

NOD/SCID: Non-obese diabetic/severe combined immunodeficiency

NSG: Non-obese diabetic-severe combined immunodeficiency/IL-2 receptor gamma null

PBS: Phosphate-buffered saline

PCR: Polymerase chain reaction

PEST: proline (P), glutamic acid (E), serine (S) and threonine (T) rich

PI: Propidium Iodide

PI3K: Phosphoinositide 3-kinase

PTEN: Phosphatase and tensin homolog

RAM: RBP-jk-associated molecules

SCF: Stem cell factor

SR1: StemRegenin 1

SSC: Squamous cell carcinoma

TAD: Transcriptional activation domain

TAL1: T-cell acute lymphocytic leukemia 1

T-ALL: T-cell acute lymphoblastic leukemia

TCR: T-cell receptor

TLX1/HOX11: T-cell leukemia homeobox 1

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A special thanks to my supervisor, Dr. Andy Weng for all of his mentorship and training. I would also like to thank my supervisory committee, Dr. Kevin Bennewith and Dr. Aly Karsan, for their helpful comments, advice and encouragements. Also thank you to my external examiner Dr. Fumio Takei and my defense committee chair Dr. Sharon Gorski for taking time out of their busy schedules to help guide me in the completion of this thesis. Big thanks also goes to all the members of the Weng lab and my friends in the TFL for helping answer all my questions and keeping life fun and interesting while I completed this degree. Thank you to my wonderful family and friends for all your love and support, I could not have done this without you!

Chapter 1

Introduction

1.1 T-cell Acute Lymphoblastic Leukemia

1.1.1 Clinical characterization and diagnosis

Acute lymphoblastic leukemia (ALL) is an aggressive hematologic malignancy that affects both children and adults. It makes up about 12% of all leukemias and is the most common malignancy diagnosed in children and adolescents. Its peak incidence is at 2-5 years of age, with another peak later in life, sometime after 50 years of age (Jemal et al., 2006). It is a clonal disease of the bone marrow in which early lymphoid precursors proliferate and replace the normal hematopoietic cells of the marrow. Clinically it is characterized by hyperleukocytosis (high white blood cells counts), increased number of blast cells and enlarged mediastinal lymph nodes (Grabher et al., 2006). The tumor cells correspond to immature lymphoid precursors; around 80% are B lineage, and 20% are T lineage, called B-ALL and T-ALL respectively. T-ALL makes up around 15% of pediatric and 25% of adult ALL cases (Koch and Radtke, 2011). It is currently clinically treated with combination cytotoxic chemotherapy in which multiple antileukemic drugs are administered in various combinations (Silverman et al., 2001). The chemotherapy regimen generally consists of three phases; induction, intensification and maintenance.

Current chemotherapy regimens cure 80-90% of children and 30-40% of adults (Hernandez et al., 2010). However relapse patients and those refractory to initial treatment

have a very poor prognosis, and this combined with the low cure rate for adult patients, highlights the need for new treatment strategies. The rates of cure, or complete remission, for children have been steadily improving over the last 50 years mainly through the optimization of chemotherapy regimens (Pui and Evans, 2006) but further improvements will undoubtedly require introduction of novel approaches and more specific, targeted therapies. In recent years we have learned through technological advances such as high-resolution genome-wide screening, that ALL is a heterogeneous disease with distinct manifestations (Faderl et al., 2010). Analysis has shown that this heterogeneity can be partially related to arrest at distinct stages of development (De Keersmaecker et al., 2005). In T-ALL this refers to arrest at different stages in normal thymocyte development, such as the earliest stage, immature double negative (CD4-CD8-) or the later, mature single positive (CD4+CD8- or CD4-CD8+) T-cell (Figure 1.1). This heterogeneity suggests that further improvements to cure rate may require implementation of a more personalized medicine approach, with different treatment strategies for different patients. Therapeutic targeting strategies are further complicated by the spread of the disease throughout the body and involvement of the central nervous system (CNS), which is often seen in relapse cases (Pui, 2006). In order to improve patient prognosis and develop new, targeted therapies we must further enhance our knowledge of the molecular mechanisms that cause and drive the disease.

T Cell Development
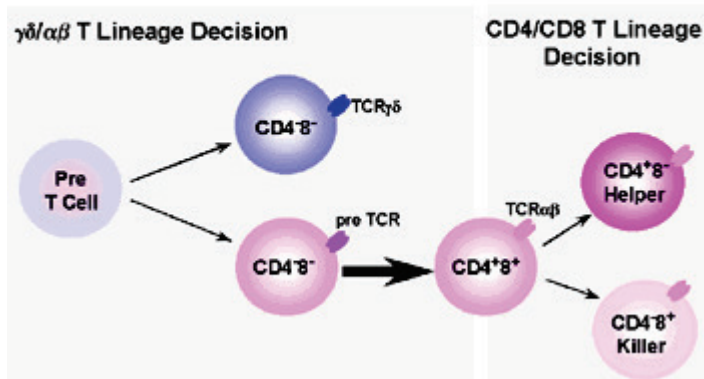
Figure 1.1 T-cell development

Normal T-cell development occurs in the thymus where the cells undergo selection and differentiation from Pre-T cells to mature, single positive T-cells. Figure from http://www.niaid.nih.gov/LabsAndResources/labs/aboutlabs/lcmi/tcelldevelopmentsection/pages/fowlkes.aspx, ©B.J. Fowlkes, 2006, accessed February 2012.

1.1.2 Genetic abnormalities found in T-ALL

In the last several decades sequencing and cytogenetic analysis have increased our knowledge of T-ALL and revealed that aberrant expression of many different transcriptional regulators play a large role in this disease. These aberrancies often result in the incorrect rearrangement of the T cell receptor (TCR) genes (Raimondi, 1993). This may lead to the juxtaposition of strong TCR gene enhancers and promoters with transcriptional regulators, which results in their abnormally high expression (Van Vlierberghe et al., 2008). There are many different transcription factors which have been found to have aberrant expression in T-ALL, such as TAL1, LYL1, TLX1/HOX11, members of the HOXA gene family, and MYC to name a few (Aifantis et al., 2008). Chromosomal translocations that do not involve TCR loci have also been reported in T-ALL cases. These translocations generally lead to fusion

genes and chimeric proteins, and gene inactivation or duplication (Koch and Radtke, 2011). Interestingly more than 50% of T-ALL patients, including all major molecular oncogenic subtypes, have mutations leading to the hyper activation of the Notch1 pathway, implicating this signaling pathway to be very important in the pathogenesis of this disease (Weng et al., 2004). The importance of Notch signaling in T-ALL is also supported by the fact that mutations in other Notch pathway genes, such as F-box and WD repeat domain-containing 7 (FBW7), have been described which lead to increased Notch signaling in the malignant cells (O'Neil et al., 2007).

1.2 Notch Signaling

1.2.1 Notch gene and receptors

The Notch gene was first described in 1919 while studying *Drosophila melanogaster* and was named for the partial-loss-of function phenotype of an indentation, or notch, caused in the fly blade wings when mutated (Mohr, 1919). It wasn't until 60 years later that molecular cloning studies showed that the Notch gene encodes a transmembrane protein which functions as a receptor that signals through ligand binding with neighboring cells (Wharton et al., 1985; Fehon et al., 1990). In mammals, there are four members of the Notch family (Notch 1-4) that are expressed differentially in a variety of tissues. While Notch 1 and 3 are expressed in T-cells, Notch 2 is predominantly expressed in B-cell progenitors (Saito et al., 2003), and Notch 4 expression is found mostly in the endothelial vasculature (Hainaud et al., 2006). The Notch receptors all have similar structures with repeated extracellular EGF

domains, nuclear localization signals and C-terminal PEST domains (Figure 1.2, panel A). They contain RAM domains which associate with the CSL protein and ANK domains which associate with other proteins to form the transcription complex (Chiba, 2006). The largest difference between the Notch receptors is found in variations in the PEST domain, which negatively regulates the half-life of Notch (Aster et al., 2000). The Notch receptor forms a heterodimer and is composed of two subunits, the N-terminal extracellular domain (ECN) and a transmembrane C- terminal intracellular domain (ICN). It is activated through binding of the extracellular portion with a Notch ligand. These ligands contain EGF repeats that are capable of binding the EGF repeats found on the Notch receptors.

In mammals there are five different ligands that bind the Notch1 receptor, Delta 1, 3 and 4 and Jagged 1 and 2 (Figure 1.2, panel B). These ligands have been reported to be expressed in the bone marrow, fetal liver and the thymus (Felli et al., 1999; Schmitt et al., 2004). What drives Notch-ligand biding specificity is still unknown in the majority cases although a few methods of modification that alter binding specificity have been reported. For example, Notch signaling is modulated by the Fringe family of proteins, which add N-acetylglucosamine to certain EGF-like repeats on the Notch receptor which promotes Notch signaling in response to Delta ligands and inhibits Jagged ligand mediated signaling (Haines and Irvine, 2003). However, the physiologic consequences of this modification have not yet been characterized.

During T lymphopoiesis in the thymus *in vivo*, Notch signaling via interaction with DL4 ligand has been shown to be essential even though both DL4 and DL1 ligands are expressed (Mohtashami and Zuniga-Pflucker, 2006; Hozumi et al., 2008; Koch et al., 2008).

However, in order address to which of these ligands is more effective in inducing T-cell lineage differentiation from immature progenitors *in vitro*, stromal bone marrow derived cell lines were engineered to express varying levels of either DL1 or DL4 and co-cultured with primary T-ALL cells. Stromal cells expressing high levels of either ligand were able to give rise to T lineage cells with a similar efficiency, but at lower levels DL4 maintained the ability to support T lineage commitment whereas DL1 was not (Mohtashami et al., 2010). These findings show that while DL4 is the relevant ligand for T lymphopoiesis *in vivo* and is more potent than DL1 in supporting T lineage commitment *in vitro*, both DL1 and DL4 can support *in vitro* development when expressed at high levels.

1.2.2 Notch signaling pathway

The Notch signaling pathway has been found to be an evolutionarily conserved pathway in both vertebrates and invertebrates and is used as a mechanism to control cell fate through local cell interactions (Artavanis-Tsakonas et al., 1999). It is a core signaling system that is involved in embryonic development and processes such as cell-fate decisions, apoptosis and proliferation (Bray, 2006). Notch signaling has been shown to induce the expression of early differentiation markers and cell cycle arrest (Rangarajan et al., 2001), play an important role in the regulation of tissue homeostasis, be involved at many levels in hematopoiesis (Radtke et al., 2004), and is necessary for the support and maintenance of hematopoietic stem cells (Varnum-Finney et al., 2000; Kumano et al., 2003).

Notch signaling is activated by direct cell-to-cell interaction between a cell with a Notch receptor and one carrying a ligand (Figure 1.3). Notch ligands are transmembrane cell surface proteins that are characterized by the presence of a DSL (Delta, Serrate and Lag2)

domain (Figure 1.2, Panel B). The binding of the Notch ligand to the extracellular subunit of

the receptor leads to activation of the pathway and also causes the ligand to be endocytosed

into the cell with the receptor (Parks et al., 2000). The ligand is then either recycled at the

cell surface or degraded within the cell (Le Borgne et al., 2005). This transmembrane, direct

cell contact model allows cells to communicate directly with their neighbors. Once a Notch

receptor and ligand have bound, a series of proteolytic cleavage events is initiated and ends

in the intracellular portion of the Notch receptor (ICN) relocating to the nucleus and acting as

a direct transcriptional activator. During maturation and trafficking to the cell surface the

Notch receptor, which is originally translated as a large 300kD peptide, moves to the Golgi

apparatus and is glycosylated by Fringe and processed by a furin-like protease to produce a

heterodimeric receptor (Logeat et al., 1998; D'Souza et al., 2010). From there the receptor

moves to the cell surface where it is situated as a transmembrane protein with both

extracellular and intracellular domains. Ligand binding to the extracellular portion causes a

mechanical pull that exposes the part of the receptor at the cell membrane to cleavage by

ADAM-like metalloproteases (Brou et al., 2000). This is followed by an intramembrane

cleavage event by γ-secretase that releases ICN so it is free to translocate to the nucleus

(Fortini, 2002). In the nucleus it binds directly to DNA in a complex with the CSL DNA

binding protein (CBF-1, Suppressor of hairless, Lag-1) and transcriptional co-activators like

Mastermind (MAM) and leads to increased expression of its target genes. These target genes

include MYC, Deltex, pre-Tα and the hairy and enhancer of split (HES) family to name a

few (Reizis and Leder, 2002; Chadwick et al., 2009). Notch signaling is terminated by

polyubiquination by FBW7 ubiquitin ligase, also known as Sel-10, and its subsequent

proteasomal degradation (Oberg et al., 2001). This ubiquitination is strongly enhanced by the

expression of CycC: CDK8, which is localized to ICN through interaction with the Notch binding partner MAM (Fryer et al., 2002). Purified, recombinant CycC: CDK8 can also ubiquitinate the TAD and PEST domains of the Notch receptor thereby tagging it for degradation (Fryer et al., 2004). This dual role for MAM in Notch signaling, in which it is an integral part of the complex to initiate transcription of Notch target genes and also aids in Notch receptor degradation, suggests that it is an important regulator of Notch signaling.

A)
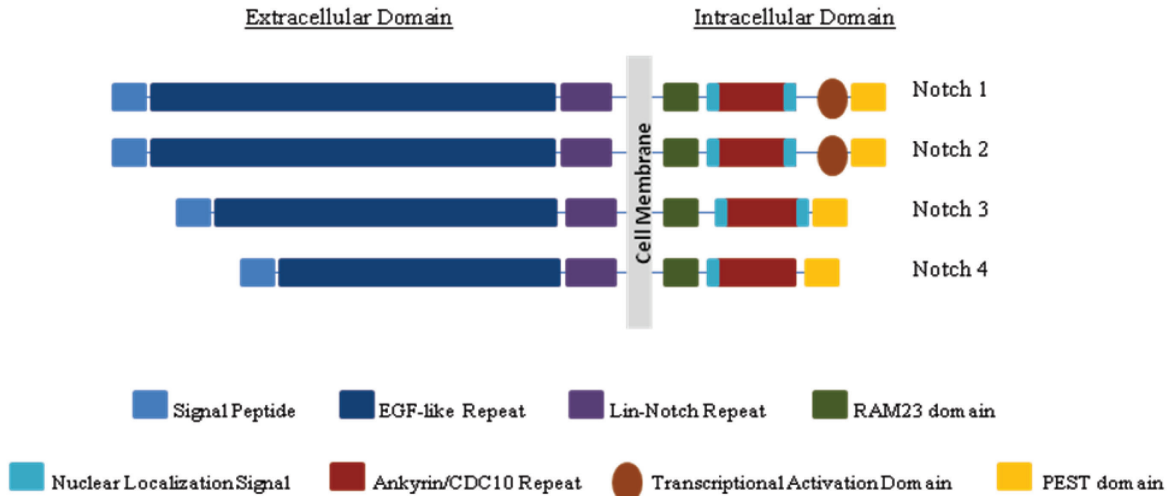
Notch Receptors
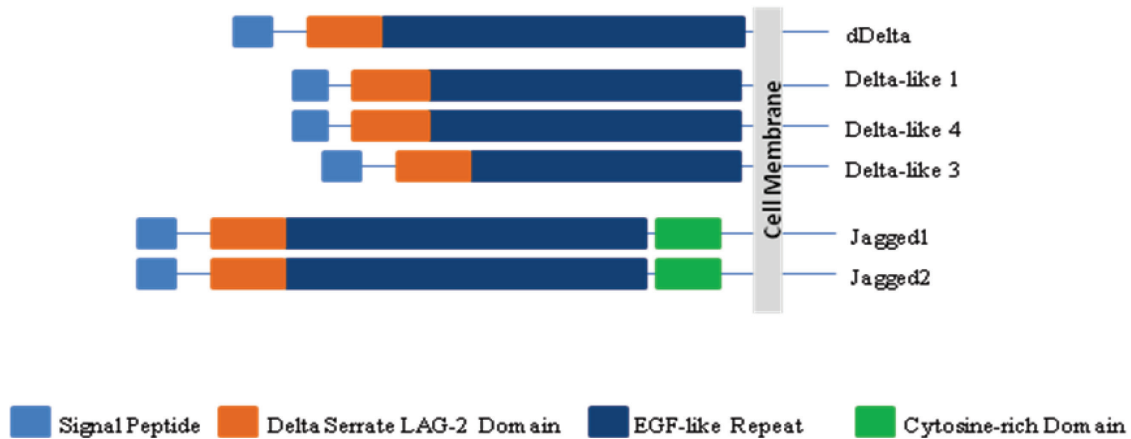
Figure 1.2 Notch receptor and ligand structures

Domains present in human Notch receptor and ligand structures. Figures adapted from http://www.trojantec.com/site.84.articles.en.html. ©Trojantec, 2007, accessed January 2012.

Notch Signalling Pathway
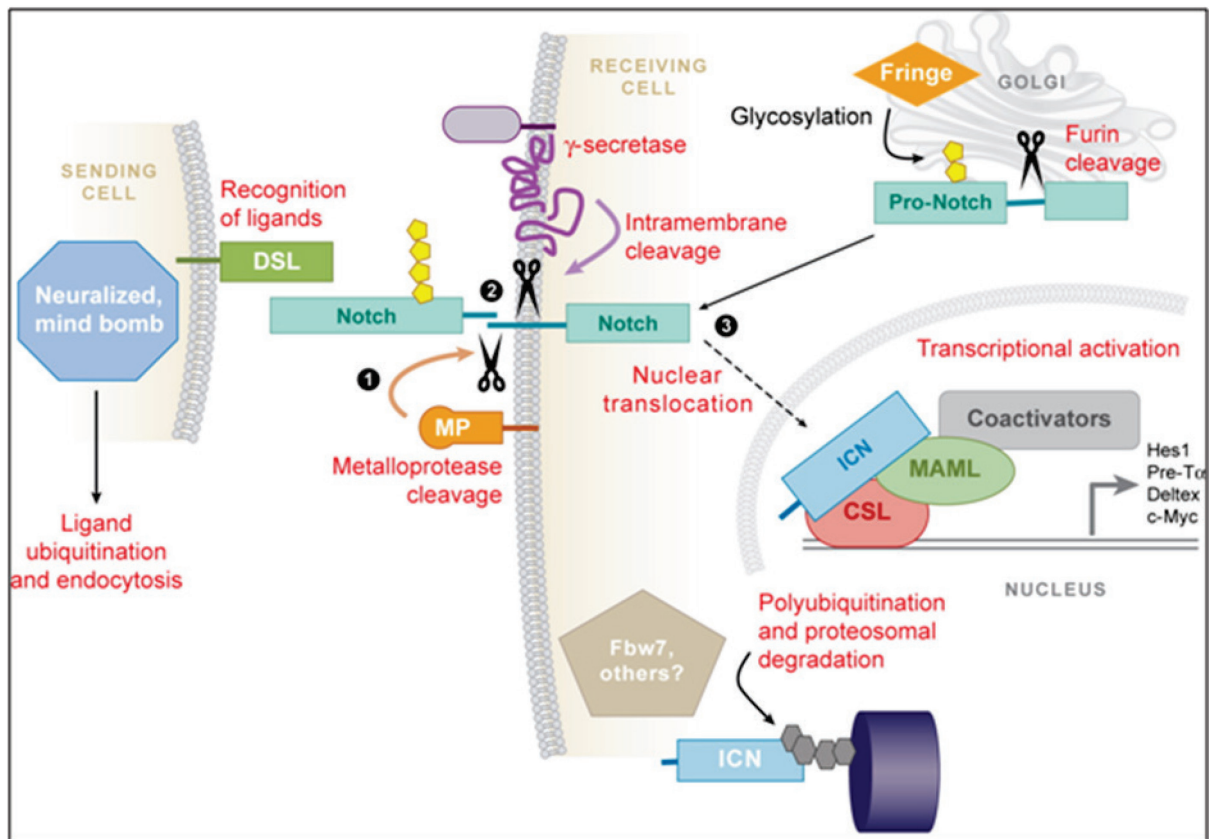
Figure 1.3 Notch signaling pathway

      Diagram of the Notch signaling pathway described in section 1.2.2. After receptor/ligand binding there is a metalloprotease cleavage at the heterodimerization domain (1) and then a subsequent cleavage event by γ-secretase (2). This frees ICN to translocate to the nucleus (3) to initiate transcription of its target genes. Figure adapted from Notch Signaling in Leukemia (Aster et al., 2008). © S.C. Blacklow, 2008, accessed January 2012.

1.2.3 Notch signaling in T-ALL

Interestingly and fittingly, the human Notch1 gene was originally discovered from a chromosomal translocation (t (7;9)(q34;q34.3)) in a case of T-ALL (Ellisen et al., 1991). The translocation results in aberrant Notch signaling. However this translocation turned out to be a very rare event and it was more than 10 years later before a role for Notch1 in T-ALL pathogenesis and maintenance was conclusively described. It is now known that activating Notch1 point mutations, insertions and deletions are frequently present in both adult and childhood T-ALL (Pear and Aster, 2004; Weng et al., 2004). These mutations are most commonly found in the heterodimerization (HD) and PEST domains of the receptor. The mutations in the HD domains result in hypersensitivity to ligand or ligand independent signaling (Malecki et al., 2006). On the other hand PEST domain mutations result in increased Notch signaling due to impaired receptor degradation (Weng et al., 2004). As mentioned earlier, some human T-ALLs are also found to have FBW7 mutations, which also function to increase Notch signaling through impaired receptor degradation prolonging Notch1 half-life (Thompson et al., 2007). This work was primarily done using T-ALL cell lines and testing the effects on growth and proliferation of γ-secretase inhibitors and MAML1, a dominant negative protein that forms a complex with Notch1 and CSL in the nucleus to block transcription (Weng et al., 2003). They found that proliferation and growth were severely inhibited by the blockage of the Notch signaling pathway in several lines. Several downstream targets have been directly implicated in T-ALL pathogenesis, notably c-MYC, a well characterized oncogene which has been shown to be a direct target of Notch1 and plays a role in the growth of T-ALL cells (Weng et al., 2006). The mTOR pathway,

which is known to play a role in cell growth and proliferation (Wullschleger et al., 2006), has also been shown to be positively regulated by Notch1 signaling, most likely through MYC as an intermediate (Chan et al., 2007). Recent work looking into further roles of Notch in T-ALL has shown that Notch1 acts together with chemokine receptor pathways and can provide the T-ALL cells with chemotactic abilities and influence tumor cell progression and localization (Mirandola et al., 2011). Taken together these findings all support the role of and implicate Notch1 as major player in the pathogenesis and maintenance of the disease (Ferrando, 2009; Medyouf et al., 2011; Mirandola et al., 2011; Xiang et al., 2011).

1.2.4 Notch signaling in other hematologic malignancies

Given the extensive usage and the necessity of this signaling pathway in normal biology, it is not surprising that aberrations in the Notch receptor and its ligand have been implicated in several diseases and malignancies. It is well established in hematologic malignancies and although it is particularly associated with T-ALL, a role has been described for aberrant Notch signaling in many other types of leukemia and lymphoma as well. It has recently been reported that 8-12% of chronic lymphocytic leukemia (CLL) patients contain activating Notch1 mutations. Furthermore Notch1 mutational status in CLL is an independent predictor of poor survival and is correlated with adverse biological features, Richter transformation, and chemorefractory disease (Fabbri et al., 2011; Puente et al., 2011). Expression of Notch1 and its ligands Delta1 and Jagged1 have also been reported to be elevated in acute myeloid leukemia (AML) and this higher expression and subsequent activation of the pathway is correlated with poor prognosis (Xu et al., 2011). Recurrent activating mutations in Notch1 have also been described in mantle cell lymphoma (MCL)

and are associated with overall poor survival in this disease as well (Kridel et al., 2012). It has also been shown that Notch1 and its ligands are constitutively expressed in B-CLL cells and this increased Notch signaling plays a role in B-CLL cell survival and protection from apoptosis (Rosati et al., 2009). Interestingly, Notch has also been shown to have tumor suppressor function in several B-cell malignancies by functioning exactly opposite to this and inhibiting growth and inducing apoptosis when constitutively activated (Zweidler-McKay et al., 2005). This dual role for Notch as an oncogene and a tumor suppressor is seen in many malignancies and the effect of aberrant Notch signaling is thought to be highly context and tissue specific. Although in the studies referred to above activation of Notch signaling was reported to have tumor suppressor function, it has been recently reported that inactivation of the Notch pathway is seen in some chronic myelomonocytic leukemia (CMML) patients and this inactivation can lead to CMML development (Klinakis et al., 2011).

1.2.5 Notch signaling in solid tumors

Aberrancies in the Notch pathway have also been described in many types of solid tumors. It has also been shown in this system to have both oncogenic and tumor suppressor roles depending on the tissue and context. Notch1 over expression has been observed in human breast cancer and is associated with poor prognosis (Reedijk et al., 2005). In lung cancer, a chromosomal translocation resulting in over expression of Notch3 has been found, and over expression is frequently seen in human non-small-cell lung cancer (Dang et al., 2000). It has been shown that this tumorigenic role may be in part due to over expression of Notch3 leading to the inhibition of terminal differentiation (Dang et al., 2003). Other examples of solid tumors in which Notch signaling plays an oncogenic role are prostate

(Santagata et al., 2004), pancreatic (Miyamoto et al., 2003) glioblastoma (Brennan et al., 2009), and ovarian (Park et al., 2006). Roles for Notch as a tumor suppressor have also been described in several different contexts as well. In the skin it has been suggested that Notch expression induces differentiation and cell cycle arrest (Nguyen et al., 2006) and inhibition of Notch signaling has been shown to promote squamous cell carcinoma (SSC) (Proweller et al., 2006). It was recently reported that inactivating somatic mutations of Notch1 and Notch2 were found in around 75% of cutaneous SSCs and a smaller fraction of lung SSCs (Wang et al., 2011). These findings indicate that Notch receptor loss of function plays a large role in SSCs and possibly other epithelial malignancies. Notch has also been described as having both oncogenic (Ferrari-Toninelli et al., 2010) and tumor suppressive (Zage et al., 2011) roles in neuroblastoma. These findings serve to support the complex and varying, but important role Notch signaling plays in the development and maintenance of human malignancies.

1.3 T-ALL Models of Human Disease

In order to develop therapies for human diseases and malignancies we must first understand how they arise and how they function. This requires representative disease modeling outside of the human body, which can pose a challenge to researchers. There are several strategies for this, which all have their pros and cons, as no modeling system can exactly recreate human biology.

1.3.1 Tumor cell line models

Much has been learned from *in vitro* studies using long-term maintained patient-derived cell lines. Immortalized, human cancer cell lines have been a crucial tool in building our understanding of the molecular mechanisms involved in cancer formation and maintenance and are used to investigate the potential efficacy of new drugs (Greshock et al., 2007). They are cost effective, easily accessible and manipulatable, and experimentally easy to use. However, these studies also have their limitations as to correlation with actual human disease. This is because these cell lines have become adapted to growth *in vitro* over a long period of culture and may have gained or lost functions that are not representative of the biology seen in human disease. For example, a study done in human T-ALL cell lines reported that PTEN loss correlated with resistance to Notch inhibition (Palomero et al., 2007). However when studies looked at this same pathway in an in vivo mouse model and in primary human T-ALL samples they found no correlation between PTEN status and resistance to Notch inhibition (Medyouf et al., 2010). As this exemplifies, cell lines are a useful tool, but may not be a suitable tool for gaining a deeper understanding of the overall growth pattern of T-ALL blasts in the body.

1.3.2 Mouse models

Mouse models of T-ALL have also greatly contributed to the knowledge of T-ALL development and molecular biology. These models can be broken down into two categories, mouse leukemia models and mice used as xenograft hosts for human T-ALL cells. Transgenic mouse models are commonly developed through several methods; oncogenes can be integrated into random sites in the genome, or they can be conditionally mutated so that

the mutation is induced at a specific time or lineage, or lastly retroviral transduction can be used to rapidly generate a series of genetically related leukemias (Frese and Tuveson, 2007). These models are used to perform controlled experiments not possible in humans and have the added benefit that mice have a defined genotype and syngenic siblings that can be used as experimental controls. Mouse models allow for the direct regulation of genetic diversity, easy access to and genetic manipulation of primary tumor cells, the use of well defined and accepted assays and the generation of large, well-defined cohorts to facilitate statistical analysis. However, these assays are quite expensive and time consuming and have a number of caveats as well. The first concern is the tumor modeling itself, because genetic engineered malignancies can mimic those found in patient samples, but is not necessarily directly representative of patient biology and may not represent bona fide disease initiating events as seen in human cancers. Second are the potential differences in mouse pharmacology and toxicology that make it difficult to directly relate the results of therapeutic trials run in mice to their potential efficacy in humans.

In order to more closely mimic real human disease several groups have created mouse models by transplanting human T-ALL cells as a xenograft into immunodeficient mice and then studying the disease as it progresses within the mouse (Cox et al., 2007; Armstrong et al., 2009; Chiu et al., 2010; Medyouf et al., 2011). While these have the advantage of using human tumor cells, they still have the same potential caveats arising from the differences in mouse and human host biology, making it difficult to interpret the efficacy of potential therapeutics. However, these models do allow for the massive expansion of human T-ALL cells that is not currently possible otherwise, and these cells can then be studied not only in the mouse but also taken out and cultured *in vitro*.

1.4 Culture Systems for Human T-ALL Blasts

Many different groups have cultured patient T-ALL cells *in vitro,* using several different techniques, but as of yet no one model has stood out. While most of these systems have achieved some degree of success in the culture of patient T-ALL blasts none has been used as a 'gold standard' in the field. Some of the past methods used for *in vitro* growth of primary human T-ALL blasts include: suspension cultures using standard basal media without addition of supplemental cytokines or growth factors (Styczynski et al., 2000), suspension cultures with the addition of supplemental cytokines and growth factors (Makrynikola et al., 1991; O'Connor et al., 1991; Karawajew et al., 2000), co-cultures with a stromal cell feeder layer (Armstrong et al., 2009; Chiu et al., 2010), culture in different oxygen environments- hypoxia versus normoxia (Cox et al., 2007), and even using a novel fetal thymic organ culture (FTOC) assay (Ma et al., 2002).

<u>1.4.1 Fetal thymic organ culture</u>

The FTOC culture is designed using thymus lobes taken from NOD/SCID mouse fetuses. The lobes are depleted of endogenous hematopoietic cells and then plated with leukemic cells taken from patient bone marrow in a formulated FTOC media. After two days of incubation the lobes are transferred to a filter membrane on a sponge soaked in media and cultured for 2-10 weeks (Ma et al., 2002). This system showed continuous proliferation of T-ALL cells from both fresh and cryopreserved samples and did not require the addition of any exogenous factors. Furthermore the FTOC-cultured samples were unable to grow in suspension cultures with or without the additions of cytokines showing that the culture system did not create a cancer cell line and is indeed an assay that can be used to study

patient T-ALL cell biology directly. However this procedure is costly, time consuming and technically difficult to perform, making it less than ideal for common widespread use for the study of patient T-ALL cells.

1.4.2 Suspension culture

Dr. Blair's lab reported an *in vitro* suspension culture system for growth and proliferation of patient T-ALL blasts (Cox et al., 2007). This culture model was adapted from suspension cultures previously reported in their lab to sustain the growth of AML and B-ALL patient blasts (Sutherland et al., 1996; Cox et al., 2004). Their system was tested on 13 primary patient samples which were cultured *in vitro* for 4-6 weeks in IMDM basal media supplemented with insulin, transferrin, human albumin serum and cytokines/growth factors IL3, IL-7 and SCF. The cells were kept at 37°C in 5% CO_2 and 5% O_2. In this study eight out of thirteen cases expanded in a range of 5-25 fold growth. This model is different than many others in that they found hypoxic conditions (3-5% O_2) to be better for T-ALL blast growth than the standard 20% O_2 used for the culture of most cells in the laboratory. However, others have reported on difficulties in reliably and reproducibly maintaining or expanding T-ALL samples under similar *in vitro* conditions and the degree of proliferation and cell growth achieved with this system is not optimal.

1.4.3 Co-culture models

Both Dr. Dick (Chiu et al., 2010) and Dr. Pflumio's (Armstrong et al., 2009) labs reported *in vitro* culture of patient T-ALL in a co-culture system where the T-ALL cells were cultured on top of a feeder layer of mouse stromal cells. They used stromal cells engineered

to expressed the Notch ligand Delta-like 1 (DL1) and compared the growth of patient cells on top of this feeder layer directly against growth on a feeder layer not expressing the ligand. They used different mouse stromal feeder cell lines (OP9 and MS5 respectively); both of their results showed that sustained levels of Notch activation lead to higher levels of *in vitro* proliferation for the patient T-ALL blasts. This held true for the majority of patient cases tested which further emphasizes the importance of the Notch signaling pathway in T-ALL. Dr. Dick's lab used a standard media (alpha MEM) supplemented with 20% fetal bovine serum (FBS) and cytokines IL-7 and Flt3L. However, they did not achieve much growth *in vitro* in this system and saw only 1.5 fold growth over a culture period of four weeks. Dr. Pflumio's group used the same basal media supplemented with 10% FBS, 10% human AB serum, insulin and cytokines SCF, Flt3 and IL-7. They observed up to 12 fold growth of samples after one month in culture, but many of their samples went through an acute loss of viability in the beginning days of *in vitro* culture before proliferating indicating the outgrowth of minor or sub-clones. This clonal outgrowth makes the *in vitro* culture less representative of normal T-ALL cell biology within the body, and highlights the need for a culture system that supports the growth of primary patients samples and gives rise to a cell population that is representative of what is seen in human disease.

1.4.4 Cytokines/growth factors

Interleukin 2 (IL-2) is a cytokine signaling molecule in the immune system that was originally described as a T cell growth factor that was essential for the growth and development of T cells in the thymus (Morgan et al., 1976). It was the first type I cytokine to be cloned and the first type I cytokine for which its receptor was cloned (Taniguchi et al.,

1983; Leonard et al., 1984). It signals through a receptor complex consisting of IL-2 specific receptor (IL-2R) α, IL-2Rβ and a common gamma chain (γc) (Kim et al., 2006). The gamma chain is shared by all members of this family of cytokines, which include IL-4, IL-7, IL-9, IL-15 and IL-21. Binding of IL-2 to IL-2R activates Ras/MAPK, JAK/Stat and PI 3-kinase/Akt signaling modules leading to a number of downstream effects (Lin and Leonard, 2000). Previous studies have shown that IL-2R activation to be important for T-ALL growth *in vitro,* as well as for growth stimulation of normal T cells (Lowenberg and Touw, 1986). Previous work in our lab (unpublished) has also shown the addition of supplemental IL-2 to be advantageous for *in vitro* mouse and human T-ALL cell growth in many instances.

Interleukin 7 (IL-7) is a cytokine signaling molecule in the same family as IL-2. It was first described in 1988 as a factor that promoted the growth of murine B-cell precursors in a bone marrow stromal co-culture system (Namen et al., 1988). It is now know to be essential for lymphocyte development and survival. It is produced in the bone marrow and thymic stroma, as well as the non-lymphoid cells in various lymphoid organs (Barata et al., 2005; Alves et al., 2009). Its presence in these microenvironments where T-cells develop allow it to directly modulate growth. IL-7 signals through interaction with its receptor (IL-7R) which consists of two components, the IL-7R α chain (also called CD127) and the common gamma chain that is shared between all receptors in the family. Studies have shown that *in vitro* thymic epithelium and bone marrow stromal cells are able to support T-ALL cell growth through the production of IL-7 (Scupoli et al., 2003; Scupoli et al., 2007) and that IL-7 induces proliferation in most primary T-ALL samples (Barata et al., 2004a). Furthermore it has been reported that IL-7-dependent growth is modulated through the activation of the PI3K/Akt signaling pathway (Barata et al., 2004b).

Stem cell factor (SCF) is a stromal derived cytokine that is synthesized by mesenchymal cells within the bone marrow such as stromal fibroblasts and endothelial cells (Zhu and Emerson, 2002). It is also known as kit ligand, mast cell growth factor or steel factor. SCF has been reported to be constitutively expressed in the bone marrow stromal cells and is essential for the survival, proliferation, adhesion/migration and differentiation of hematopoietic stem cells (HSCs) (Broudy, 1997). It signals through binding its receptor, c-kit. C-Kit is expressed in the human hematopoietic system by the majority of CD34+ cells in the bone marrow and megakaryocytes (Papayannopoulou et al., 1991; Avraham et al., 1992). It was added as a supplement to our basal medium based on previously reported *in vitro* models for T-ALL growth and because it is known to support the survival and proliferation of HSCs.

Insulin-like growth factor 1 (IGF-1) is the ligand for insulin-like growth factor 1 receptor (IGF1R), which is a member of the tyrosine kinase class of receptors (Ullrich and Schlessinger, 1990). IGF1R shares a high homology with the insulin receptor (InsR) and both are widely expressed in normal tissues (Pollak, 2008). Both IGF1R and InsR are composed of an extracellular α-chain that is involved in ligand binding and an intracellular β-chain that has tyrosine kinase activity (De Meyts, 2004). Both IGF1R and InsR exist as homodimers, but can also form heterodimers with one another, or "hybrid" receptors (Benyoucef et al., 2007). Signaling downstream of these receptors is similar but not identical. The activated receptor leads to the phosporylation of the members of the insulin receptor substrate (IRS) family of proteins due to the kinase activity. This then leads to the activation of PI3K, AKT and other downstream pathways leading to cell growth and survival (LeRoith, 2000). In contrast to insulin, which is produced almost exclusively by pancreatic β-cells, IGF-1 is

expressed by many cell types although the main site of production is in the liver. IGF-1 is also frequently expressed in neoplastic tissue and it is thought that it can influence malignant cells through autocrine, paracrine or endocrine mechanisms (Pollak, 2008). In a T-ALL context IGF-1 has been reported to support the proliferation and growth of T-ALL cells (Medyouf et al., 2011).

1.5 Thesis Objectives

Optimization of *in vitro* culture conditions that support growth and survival of patient T-ALL blasts will enable us to address several questions surrounding T-ALL biology/pathophysiology including patterns of proliferation with respect to lineage subsets/stages of differentiation, dependence of stromal cell-derived signals, and responsiveness to various cytokines and growth factors. It will also be a valuable tool in our search for the factors that maintain leukemia stem cells and allow us to gain more insight into their biology. An *in vitro* model would also greatly enhance efficient testing and validation of novel therapies by allowing direct assay of primary human cells. This will allow us to develop and optimize more targeted therapies and better learn how to target leukemia stem cells in order to help reduce instances of relapse. Ultimately, we hope to be able to use *in vitro* culture systems in the clinic to test the sensitivity of the patients' own tumor cells to potential therapies and thereby enable a personalized medicine approach to cancer treatment.

Aim 1: To improve upon existing *in vitro* culture models for patient T-ALL blasts and create a robust, reproducible system for blast survival/expansion

Aim 2: To apply this culture model to directly answer biological and therapeutic questions using patient T-ALL blasts.

Chapter 2

Materials and Methods

2.1 Cell Culture and Reagents

2.1.1 Primary human T-ALL samples

      Cryopreserved lymphoblast samples were provided by collaborating institutions in Canada (BC Children's Hospital), the United States (Karmanos Cancer Center and MD Anderson Cancer Center), and France (Hopital Andre Trousseau). Primary diagnostic bone marrow aspirate samples were obtained at initial diagnosis or relapse with informed consent from patients or their legal guardians under approved institutional review board protocols and following guidelines established by the Declaration of Helsinki.

2.1.2 Culture and irradiation of MS5-DL1 stromal feeder cells

      The MS5-DL1 stromal feeder cells were grown in MEM alpha medium (Invitrogen) supplemented with 5% fetal bovine serum (Invitrogen, lot #306399), 2 mM Glutamax (Invitrogen) and 1X penicillin-streptomycin (StemCell Technologies). For experiments using irradiated feeder cells they were irradiated at a dose of 50 Gy. This dose was determined after testing a dose range from 20-100 Gy. It damages the DNA of the feeder cells enough so that they cannot divide, but are still healthy enough to adhere to the plate and stay alive for around 7 days. Cells were plated into tissue culture treated plastic12-well plates at a density of 8.5 x 10^4/well in 2 ml of media. When 6-well plates were used the cells were plated at 2.2 x 10^5/well in 3 ml of media. Feeder cells were allowed to settle and adhere for a minimum of

4 hours before T-ALL cells were placed on top of them. When co-cultured with primary human T-ALL cells the entire culture was grown in one of the two mediums listed below for growth of primary T-ALL cells. The T-ALL cells were passaged onto freshly irradiated feeders every 4-6 days.

2.1.3 Culture of primary human T-ALL cells

Primary human T-ALL cells were cultured in either MEM alpha medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen, lot#306399), 10% human AB serum (Mediatech), 50 ng/ml mouse SCF, 20 ng/ml mouse Flt3, 10 ng/ml mouse IL-7 (all from Peprotech) and 20 nM human Insulin (BCCA) or WIT-L medium (a kind gift of Dr Tan Ince, Interdisciplinary Stem Cell Institute, University of Miami) supplemented with 50 ng/ml mouse SCF, 10 ng/ml human IGF-1, 10ng/ml human IL-2 and 10 ng/ml mouse IL-7 (all from Peprotech). When co-cultured with the MS5-DL1 stromal cells one of the two above mediums was used, as stated in each experiment. Cells were seeded into tissue culture treated plastic 12-well plates (Becton Dickinson) at 1 x 10^6/ml in 2 ml of media. All experiments were done in triplicate. Cytokine/growth factor cross reactivity as determined from Peprotech specifications is summarized in Table 2.1 below.

Cytokine/growth factor	Mouse and human cross reactivity
Recombinant murine SCF	-Mouse and human The ED50 as determined by the dose-dependent stimulation of the proliferation of human TF-1 cells is < 10 ng/ml, corresponding to a specific activity of > 1 x 10^5 units/mg.
Recombinant human SCF	-Human The ED50 as determined by the dose-dependent stimulation of the proliferation of human TF-1 cells is ≤ 2.0 ng/ml, corresponding to a specific activity of ≥ 5 x 10^5 units/mg.
Recombinant murine IGF1	-Mouse The ED_{50} as determined by a cell proliferation assay using murine FDC-P1 cells is < 2.0 ng/ml, corresponding to a specific activity of > 5 x 10^5 units/mg
Recombinant human IGF1	-Mouse and Human The ED_{50} as determined by a cell proliferation assay using murine FDC-P1 cells is ≤ 2.0 ng/ml, corresponding to a specific activity of ≥ 5 x 10^5 units/mg.
Recombinant murine IL-2	-Mouse and Human The ED_{50} as determined by the dose dependent stimulation of murine CTLL-2 cells is < 0.2 ng/ml, corresponding to a specific activity of > 5 x 10^6 units/mg.
Recombinant human IL-2	-Mouse and Human The ED_{50} as determined by the dose-dependent stimulation of murine CTLL-2 cells is ≤ 0.1 ng/ml, corresponding to a specific activity of ≥ 1 x 10^7 units/mg.
Recombinant murine IL-7	-Mouse and Human The ED_{50} as determined by the dose-dependent stimulation of the proliferation of murine 2E8 cells is < 0.2 ng/ml, corresponding to a specific activity of > 5 x 10^6 units/mg.
Recombinant human IL-7	-Mouse and Human The ED_{50} as determined by the dose-dependent stimulation of the proliferation of murine 2E8 cells is ≤ 0.5 ng/ml, corresponding to a specific activity of ≥ 2 x 10^6 units/mg.
Recombinant murine Flt3L	-Mouse and Human Determined by the dose-dependent stimulation of the proliferation of human AML5 cells. The expected ED_{50} for this effect is 5.0 – 8.0 ng/ml.
Recombinant human Flt3L	-Mouse and Human The ED_{50} as determined by the dose-dependent stimulation of the proliferation of human AML5 cells is ≤ 1.0 ng/ml, corresponding to a specific activity of ≥ 1 x 10^6 units/mg.

Table 2.1 Cytokine cross reactivity

<u>2.1.4 IgDL1 coated tissue culture plates</u>

12-well plates of tissue culture plastic were coated with immobilized DL1 ligand (Delta1$^{\text{ext-IgG}}$, kind gift of Dr. Irwin Bernstein, Fred Hutchinson Cancer Research Center) (Varnum-Finney et al., 2003). The IgDL1 was serially diluted in PBS and then 500 μl was plated at concentrations of 0.2 μg/ml, 1 μg/ml, and 2 μg/ml respectively. After 3-6 hours the plates were washed with PBS to ensure that no un-adhered IgDL1 remained and the primary human T-ALL cells were then plated on top in one of the culture mediums previously described. All experiments were done in triplicate.

<u>2.1.5 MS5-DL1 conditioned media</u>

MS5-DL1 cells were irradiated at 50 Gy and plated in MEM alpha medium (Invitrogen) supplemented by 5% FBS (Invitrogen, lot #306399). After the cells had adhered the medium was removed and replaced with WIT-L basal medium supplemented with 50 ng/ml mouse SCF, 10 ng/ml human IGF-1, 10 ng/ml human IL-2 and 10 ng/ml mouse IL-7 (all from Peprotech). After four days the medium was removed and filtered through a 0.45 μm sterile filter (Millipore, cat# SLHV033RB). This conditioned media was used in a 2:1 ratio with freshly made WIT-L medium supplemented with cytokines for all conditioned media experiments.

2.2 Expansion of Primary Human T-ALL Samples in Immunodeficient Mice

NOD-*scid*/IL2rg$^{-/-}$ (NSG) mice were used as recipients for human T-ALL xenografts. Mice were housed in specific pathogen-free animal facilities at the BC Cancer Agency

Animal Resource Center and all protocols were approved by institutional guidelines. The mice were sub lethally irradiated at 200 rads and then injected intravenously via lateral tail vein with 5-10 million primary human T-ALL cells. Mice were monitored daily and those developing clinical signs of disease were sacrificed immediately and tissues harvested. Bone marrow from leukemic animals was harvested by flushing intramedullary cavities of femurs and tibias with 3% fetal bovine serum in PBS. Cell suspensions were filtered through 70 μm nylon mesh cell strainers (BD Falcon, cat# 352350) and red blood cells lysed with ammonium chloride solution as per the manufacturer's protocol (Stem CellTechnologies). Cells were then resuspended in 3% fetal bovine serum/PBS for subsequent processing. Splenocytes were harvested by mechanical disruption of explanted spleens in 3% fetal bovine serum/PBS, followed by filtration through 70μm nylon mesh and red blood cell lysis as above. These cells were then resuspended in fetal bovine serum (Invitrogen) supplemented with 10% DMSO and stored at -135°C until used.

2.3 Flow Cytometry

Acquisition was performed on a FACSCalibur or FACSCanto II (BD Biosciences) and data were analyzed using FlowJo software (TreeStar).

2.3.1 Surface markers and cell viability

Patient T-ALL cells were stained with fluorochrome conjugated antibodies against CD3, clone UCHT1 (cat# 561416); CD4, clone RPA-T4 (cat# 560345); CD5, clone L17F12 (cat# 341089); CD8, clone SK1 (cat# 348793); CD19, clone HIB19 (cat# 560353); CD25, clone M-A251 (cat# 557753); CD45, clone HI30 (cat# 560777) (BD Biosciences); CD34,

clone 4H11 (cat# 12-0349-41); CD44, clone IM7 (cat# 35-0441-81); CD150, clone

A12(7D4) (cat# 11-1509-73) (eBiosciences); CD1a, clone HI149 (cat# 300121) and CD7,

clone CD7-6B7 (cat# 343107) (BioLegend). BD Biosciences antibodies were used at 2:50ul

and all others were used at 1:50ul. Not all samples were stained with every marker. Cell

viability was assessed in parallel flow cytometry for propidium iodide (PI) dye exclusion.

2.3.2 Activated caspase 3

Patient T-ALL cells were harvested from co-culture, filtered through a 48µm sterile

square mesh filter (Sefar Nitex, cat# 03-48/31) and re-suspended in WIT-L. Activated

caspase 3 staining was preformed according to the manufacturer's instructions (Caspase-3-

Detection kit, RED-DEVD-FMK; Calbiochem).

2.3.3 BrdU

Cultured cells filtered through a 48 µm sterile square mesh (Sefar Nitex, cat# 03-

48/31) and then pulsed with 10 µM bromodeoxyuridine (BrdU) for 6 hours *in vitro* prior to

harvest. Cells were fixed and stained according to the manufacturer's instructions (BrdU flow

kit; BD Biosciences) using an anti-BrdU antibody(Alexa Fluor® 647; Invitrogen) and 7-

AAD (BD).

2.4 Resazurin Reduction Assay

Cell Titer Blue (Promega) was added to co-cultures, 5 ul/well for a 12-well plate and

1ul/well for a 96-well plate, and allowed to incubate for 2-6 hours depending on the number

and type of cells present in the well. The cultures were then analyzed using a Tecan GENios

Flourometric plate reader. The plate reader was set to read from the bottom at 535 nm excitation and 590 nm emission wavelengths, at optimal gain with an integration time of 80 µs and a lag time of 0 µs. The plate was orbitally shaken in the machine for 5 sec prior to the read.

2.5 Statistics

Quantitative data were analyzed using GraphPad Prism 5 software. Significance was determined using the student *t*-test.

2.6 Drugs

Small molecule dual IGF1R/Insulin receptor inhibitor, BMS-754807, was obtained under Material Transfer Agreement from the manufacturer. It was re-suspended in DMSO (Sigma) at 10 mM, and then serially diluted in Hank's Balanced Salt Solution (Invitrogen) prior to addition to culture media.

Dexamethasone (Sigma, cat# D4902) was reconstituted in 100% ethanol for a stock solution of 63.7 mM. It was used at 10 nM, 100 nM, 1 uM, 10 uM, 100 uM with serial dilutions carried out in WIT-L medium. Doxorubicin (Sigma, cat# D1515) was reconstituted in water to a stock solution concentration of 5 mg/ml. For experiments it was diluted in WIT-L and used at 0.5 µg/ml and 1 µg/ml.

Borrelidin (Alexis Biochemicals, ALX-380-102) was supplied by our collaborator Chris Ong. Patients' T-ALL cells were plated on top of an irradiated MS5-DL1 stromal feeder layer in a 24-well plate, treated with a titration of Borrelidin (0-150 ng/ml) and

incubated for 48 hours at 37°C in a 5% CO_2, 20% O_2 atmosphere. Cells were then strained through a 48μm sterile square mesh (Sefar Nitex, cat# 03-48/31) into FACS tubes, pelleted and re-suspended in Staining Buffer (1X PBS + 0.5% BSA + 0.1% NaAzide) and 2 μg/ml PI (Sigma). The viability of the cells was then analyzed on a FACS calibur flow cytometer with gating based on size to exclude the MS5-DL1 feeder cells.

The degree of synergism between BMS-754807 and Doxorubicin was determined using CalcuSyn Version 2.0 (Biosoft, Cambridge, United Kingdom).

2.7 Genomic DNA Extraction, Amplification and Sequencing

Patient T-ALL cells were harvested from co-culture and filtered through a 48 μm filter (Sefar Nitex, cat# 03-48/31). Genomic DNA was extracted according to the manufacturer's instructions (PureLink Genomic DNA kit; Invitrogen).

PCR was performed using Platinum Taq High Fidelity (Invitrogen) on a Dyad Disciple thermal cycler (Bio-Rad). Gene-specific primer sequences were as follows: human NOTCH1 exon26 (FW5'-TTT GAA TTC GAA GGC CAG TGC AAG TAA GG-3', RV5'-TTT GGA TCC GAG AGT TGC GGG GAT TGA C-3'); human NOTCH1 exon27 (FW5'-TTT GAA TTC CAG CCC CTC TCT GAT TGT-3', RV5'-TTT GGA TCC TGC AGG CAG AGC CTG TT-3'); human NOTCH1 exon34pest (FW5'-TTT GAA TTC GCT GCA CAG TAG CCT TGC T-3', RV5'-TTT GGA TCC TCT CCT GGG GCA GAA TAG TG-3'); human NOTCH1 exon34pestB418 (FW5'-TTT GAA TTC GAG CTT CCT GAG TGG AGA GC-3', RV5'- TTT GGA TCC AAG GCT TGG GAA AGG AAG C-3'); human FBW7 exon9 (FW5'-TGA TGG GAT CAT TTT ATA CGG ATG-3', RV5'-GAC AAA ACG CTA TGG CTT TCC-3'); human FBW7 exon10 (FW5'- CCC AAC TTC CCA TTC

CCT TA-3', RV5'- TTT CTT CAT GCC AAT TTT AAC G-3'); human PTEN exon7

(FW5'-TGA CAG TTT GAC AGT TAA AGG-3', RV5'-GGA TAT TTC TCC CAA TGA

AAG-3'); human IL7R exon6 (FW5'-CAA CTT TCA GGA AAT AAT AAG TGG-3',

RV5'-TAA ATT CGT GAA ATG CCT TAA TCC-3'). Human primers were confirmed not

to amplify mouse sequences. Human PTEN primers were designed to prevent amplification

of the PTEN pseudogene on chromosome 9p21.

FBW7 exons 9 and 10 were amplified using the following PCR program: 94°C 3

minutes, [94°C 30 sec, 53°C 30 sec, 68°C 45 sec] repeat for 35 cycles, then 68°C 7 minutes.

PCR was performed using Platinum Taq High Fidelity (Invitrogen) as specified by the

manufacturer.

Notch exon 26 was amplified using the following PCR program: 94°C 2 minutes,

[94°C 30 sec, 56°C 30 sec, 68°C 45 sec] repeat for 15 cycles, [94°C 15 sec, 56°C 15 sec,

68°C 45 sec] repeat for 25 cycles, then 68°C 7 minutes. Notch exons 27, 34A and 34B were

amplified using the following PCR program: 94°C 2 minutes, [94°C 30 sec, 56°C 30 sec,

68°C 30 sec] repeat for 15 cycles, [94°C 15 sec, 56°C 15 sec, 68°C 30 sec] repeat for 25

cycles, then 68°C 7 minutes. PCR was performed using Platinum Taq High Fidelity

(Invitrogen) as specified by the manufacturer with the addition of 3.75ul DMSO/50ul

reaction for all Notch exons.

PTEN exon 7 and IL7R exon 6 were amplified using the following PCR program:

94°C 3 minutes, [94°C 30 sec, 51°C 30 sec, 68°C 40 sec] repeat for 35 cycles, then 68°C 7

minutes. PCR was performed using Platinum Taq High Fidelity (Invitrogen) as specified by

the manufacturer with the addition of 3.75ul DMSO/50ul reaction for the IL7R protocol.

Sequencing was done by McGill University and Genome Quebec using the forward primers listed above except for human FBW7 exon 9 and 10. The sequences used for these were as follows: human FBW7 exon9 (FW5'- TTT AAA TCA CTT TTC CTT TCT ACC C-3'); human FBW7 exon10 (FW5'- TGA CTA AAT CTA CCA TGT TTT CTC A-3'). Analysis was done using Vector NTI software (Life Technologies).

Chapter 3

Results

3.1 Establishing a Culture System for the Growth of Primary Human T-ALL Blasts

3.1.1 Defined, serum-free basal media

The *in vitro* culture models for T-ALL mentioned previously had the caveats of either being technically challenging, such as the FTOC system, or difficultly in reliable growth and reproducibility. In order to improve upon these existing models we began by choosing a defined, serum-free medium to replace the fetal calf and human serum-containing basal media. The use of this media in our culture system will improve overall reproducibility of experimental results by eliminating variation in media composition due to differences between serum lots. The difference between lots of serum can vary greatly making it hard to compare results and causing confusion as some serum-supplemented mediums will support growth and others will not, depending on the lot of serum used. The medium we chose has been optimized for the *in vitro* growth of malignant human cells and is similar to the basic formulation as described by Weinberg's lab (Ince et al., 2007) called WIT. Although there are many serum-free mediums readily available on the market, we chose this particular one due to preliminary studies in Dr. Aster's lab that suggested it worked for the culture of patient T-ALL blasts (personal communication with Dr. Aster). The formulation we used is called WIT-L and it has been slightly modified to support growth of patient leukemic blasts specifically. These modifications include the removal of all growth factors besides insulin

from the prior formulations. We also chose to use our own cytokine/growth factor cocktail designed to enhance T-ALL blast growth. The supplemental cytokine/growth factor cocktail we used for these experiments was SCF, IL-2, IL-7 and IGF. This was designed based on previous experience in the lab and literature review (Armstrong et al., 2009; Chiu et al., 2010; Medyouf et al., 2011).

3.1.2 Co-culture with a stromal feeder layer

We chose to use the WIT-L media in a co-culture system with a murine stromal cell line that has been engineered to express DL1 (MS5-DL1) as a feeder layer for the primary human T-ALL blasts. These cells express DL1 at a very high level, making up for any possible qualitative differences between DL1 and DL4 Notch stimulation. This decision was based on previous reports, which showed greatly increased proliferation of human T-ALL blasts in the presence of exogenous Notch stimulation and showed that some T-ALL samples will expand only in the presence of DL1 and will simply die when cultured on feeder cells which do not express it (Armstrong et al., 2009; Chiu et al., 2010). For these experiments we used sub-lethally irradiated feeder cells so that their growth would not be confused with that of the T-ALL blasts in our growth and proliferation assays. In all experiments the T-ALL blasts were passaged onto a freshly irradiated feeder layer every 4-6 days to insure that they were always in a co-culture with viable feeder cells. The WIT-L co-culture model for the growth of primary patient T-ALL blasts was set up as in Figure 3.1.
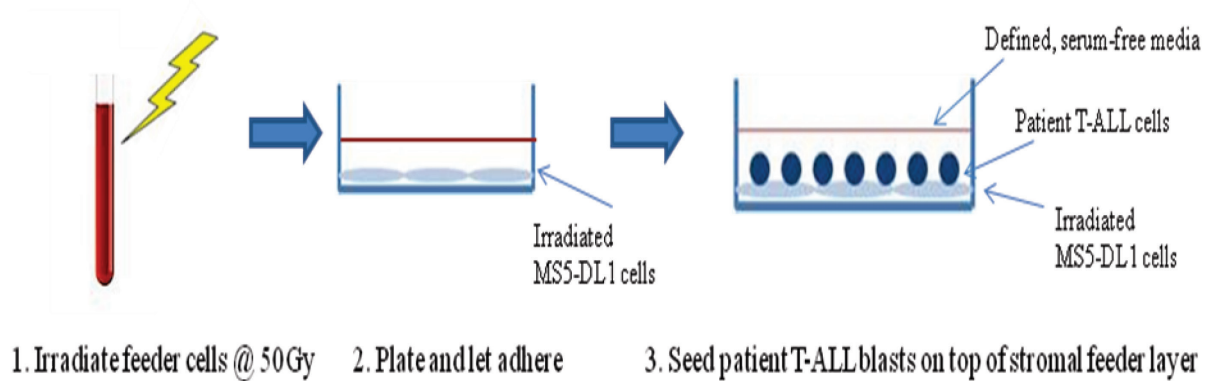
Defined, serum-free media
Patient T-ALL cells
Irradiated MS5-DL1 cells
Irradiated MS5-DL1 cells

1. Irradiate feeder cells @ 50Gy   2. Plate and let adhere   3. Seed patient T-ALL blasts on top of stromal feeder layer

Figure 3.1 WIT-L culture system schema

Stromal feeder layer preparation and set up of the co-culture model.

3.1.3 Patient T-ALL blast sources

As mentioned earlier, T-ALL is a relatively rare malignancy and as such it can be difficult to obtain patient T-ALL samples for research use. When setting up a new culture system it is necessary to have enough starting material to appropriately test and optimize the system. In order to do this we chose to use two different sources of patient T-ALL cells. We used samples that had been cryopreserved directly after being taken from the patient and samples that had been expanded as a xenograft in immunodeficient mice and then cryopreserved. This second method allows for the expansion of the sample while in the mouse, giving us much more material to work with. This method has been used to study human T-ALL by several other groups (Cox et al., 2007; Armstrong et al., 2009; Chiu et al., 2010). Recent work (Clappier et al., 2011) reported that expansion in immunodeficient mice selects for and expands a more aggressive malignancy that is similar to the disease seen in relapse patients. They did this work by comparing genome-wide profiling of patient T-ALL samples at time of diagnosis, after xenograft into immunodeficient mice, and after relapse in

the patient (Figure 3.2). Comparison of these paired samples showed that expansion in immunodeficient mice can recapitulate the process of clonal selection seen in human relapse disease and that establishment of human leukemia in immunodeficient mice expands a more aggressive leukemia that is similar to the process of relapse in patients. Although it is possible the mouse model may be exerting different selective pressure on the human xenografts, resulting in a disease which is not the same as what would be seen in human disease progression, this work gave us confidence that the use of xenograft expanded T-ALL samples as a material with which to test and optimize our culture system was still relevant to human biology.
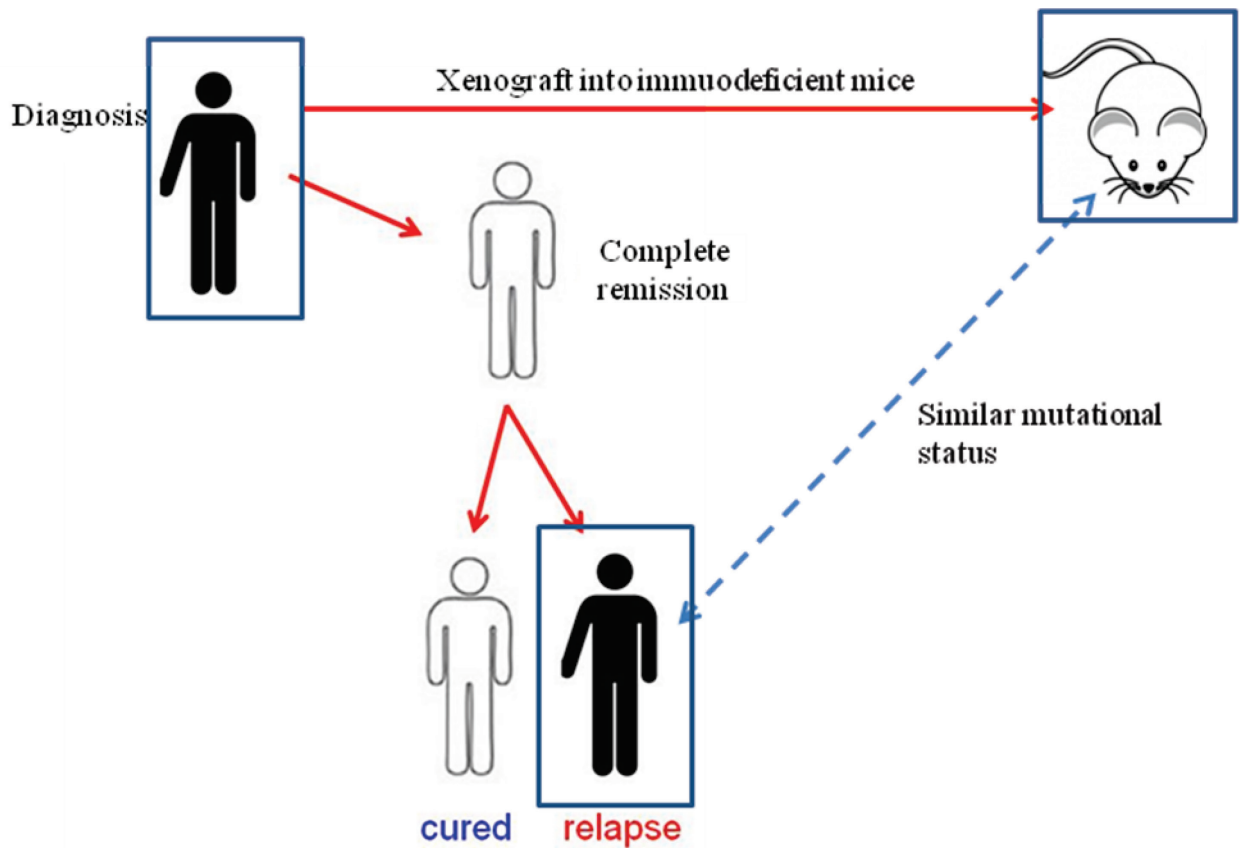
Figure 3.2 Comparison of primary patient T-ALL samples with samples xenograft expanded in immunodeficient mice

Samples were taken from patients at the time of diagnosis and sequenced for mutational status as well as xenografted into immunodeficient mice. After the primary leukemia had expanded in the mouse it was sequenced again for mutational status. In the case of disease relapse samples were also taken from the relapsed patient and sequenced for mutational status. The mutations present at diagnosis, after expansion in a mouse, and at relapse were then compared. It was shown that the mutations gained during human relapse closely resembled those gained during expansion in a mouse, suggesting that expansion in a mouse can recapitulate the process of relapse seen in humans.

3.1.4 Initial testing of the culture model

In order to test the efficacy of our system we performed a head-to-head growth comparison of primary human T-lymphoblasts cultured on an irradiated MS5-DL1 feeder layer in the WIT-L media against the previously described serum containing media (Armstrong et al., 2009), which we will call Pflumio conditions. The basal media were supplemented with the addition of cytokines/growth factors as shown in Table 3.1. To ensure that the cells were always sitting atop a viable feeder layer the T-ALL cells were passaged every 4-6 days onto freshly irradiated MS5-DL1 cells. For this comparison we used lymphoblasts obtained directly from patient bone marrow and peripheral blood samples at diagnosis (Figure 3.3, panel A) as well as following expansion in immunodeficient mice (Figure 3.3, panel B). We looked at the characterization of all primary samples used in this study and have reported it in Table 3.2. After 10-12 days of culture in the above conditions the cells were analyzed for growth using a resazurin reduction assay. This is an assay in which the metabolic capacity of the cell is used to measure growth and viability. A redox dye, resazurin, is introduced into the cultures and live cells, or those with a functioning metabolism, will convert the dye into a fluorescent end product, resorufin, which can be detected by a fluorometer. As nonviable cells rapidly lose metabolic capacity, they will not generate a signal in this assay. In all cases the cells grown in the serum-free WIT-L medium showed significantly better growth and viability than those cultured in the serum-containing Pflumio medium conditions.

A)

D115-1-2

M18-1-5

M22-7-1

M71-10-3
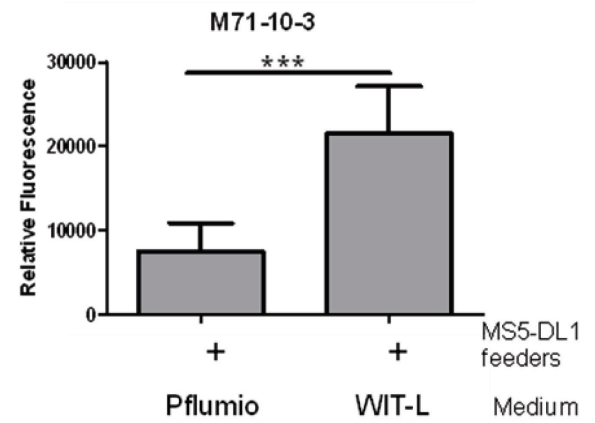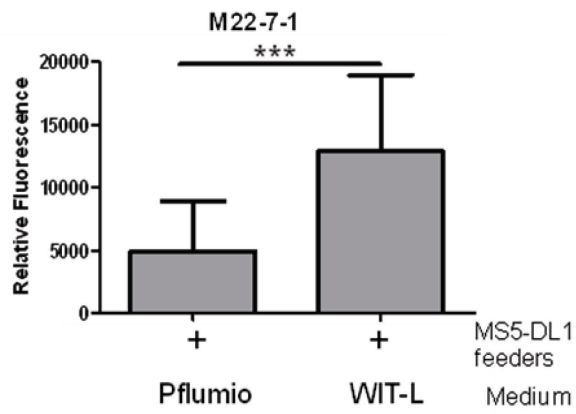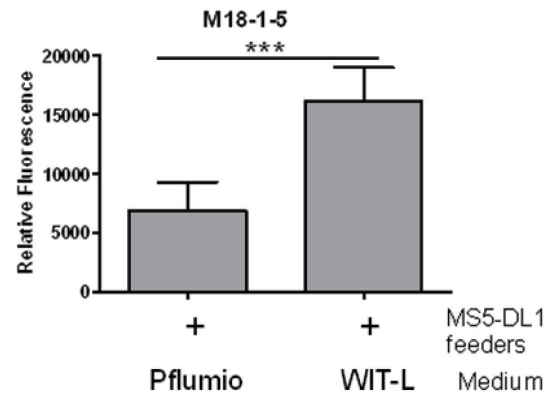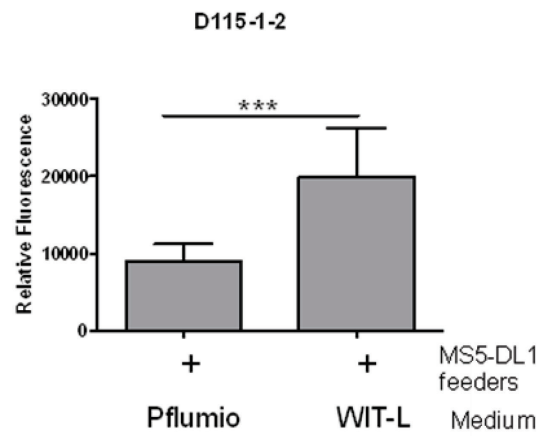
B)
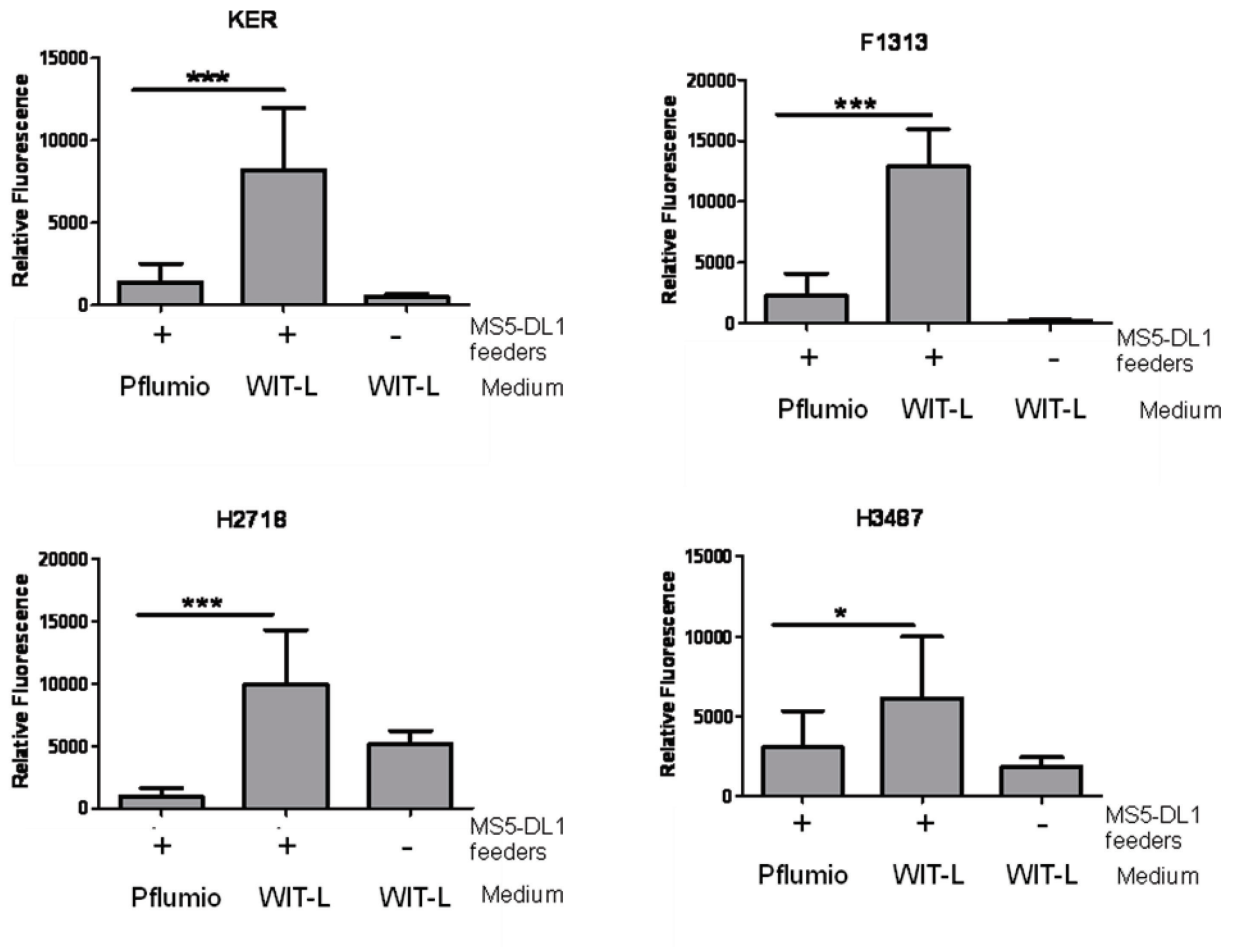
Figure 3.3 WIT-L serum-free medium supports improved growth of patient T-ALL blasts

Cell growth assay performed on primary (A) and xenograft-expanded (B) patient T-ALL cells. Cryoperserved cells were pre-cultured on irradiated MS5-DL1 feeders in Pflumio medium for 2-3 days, and then passaged onto freshly irradiated feeders in either WITL or Pflumio medium. Cells were assayed for growth using a resazurin reduction assay 4-6 days later. Cells were passaged a second time under identical media conditions and growth measured again. Data shown is a composite of passages 1 and 2. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

	Pflumio	**WIT-L**
Basal media	αMEM	50% F12 + 50% M199
		Organic supplements
Human serum	10% AB serum	-
Calf serum	10% FCS	-
Insulin (human; MW 5808)	20nM *(= 116.16 ng/ml)*	*none added (10 ug/ml already in WIT-L)*
SCF	50ng/ml	50 ng/ml
Flt3L	20 ng/ml	-
IGF-1	-	10 ng/ml
IL-2	-	10 ng/ml
IL-7	10 ng/ml	10 ng/ml

Table 3.1 Summary of media formulations

| Sample ID	Diagnosis v Relapse	Age/Sex of patient	Notch1 HD mutation	Notch1 PEST mutation	PTEN mutation	FBXW7 mutation
KER	Diagnosis	<18	WT	WT	WT	R479Q
M18	Diagnosis	6/M	WT	P2513L	WT	WT
M22	Diagnosis	7/M	WT	WT	229 KGTGRQVHVL* / 233 PGKTSSCTLSSLSR YLCVVISK*	WT
M30	Diagnosis	12/F	WT	Q2520*	WT	WT
M71	Diagnosis	<18	L1586P	WT	WT	WT
D115	Diagnosis	<18	WT	Q2459*	245 YQFMFLVW*	WT
D135	Diagnosis	<18	WT	2506 DLLPP*	233 EEKTSSCTLSSLSR YLCVVISK*	WT
F1313	Diagnosis	<18	ND	ND	ND	ND
H2718	Relapse	19/M	ND	ND	235 GKTSSCTLSSLSRY LCVVISK*	ND
H3487	Relapse	31/F	ND	ND	WT	ND
H2908	Relapse	28/M	ND	ND	WT	ND
H3255	Relapse	36/M	ND	ND	ND	ND
H3379	Relapse	28/M	ND	ND	ND	ND

* Stop codon

Table 3.2 Characterization of primary patient T-ALL samples

3.1.5 Assessment of cell growth/culture expansion

   To quantify fold expansion of the primary cultures in our system we also performed manual counts of live cells with a hemocytometer using trypan blue exclusion dye. Trypan blue is a diazo dye that selectively stains dead tissues or cells, but is not taken up by live cells, making it possible to assess the number of live cells present in a culture. These counts were performed every 4-6 days when the cells were passaged onto freshly irradiated feeders. The cells were first filtered through a 48 micron filter to get rid of any clumped cells. As the MS5-DL1 cells have quite a different morphology than the primary T-lymphoblasts they are easily distinguishable under a microscope making it possible to obtain accurate counts of the number of viable T-lymphoblasts present. Absolute numbers of lymphoblasts increased 3-4 fold with the first two weeks of culture *in vitro* for the primary samples (Fig 3.4, panel A) and from 7-130 fold for the samples that had been expanded in immunodeficient mice prior to *in vitro* culture (Fig 3.4, panel B). The upward trend of these growth curves seen in the first two weeks of culture suggests that the growth represents an expansion of bulk patient cells as opposed to outgrowth of a minor clone following massive cell death in the culture. We saw this upward growth trend in most samples, but not all, as seen in sample M22-7-1 (Figure 3.4). It is possible that in some cases such as this one, in which we see expansion, a decrease in cell numbers and then more expansion, that after an initial phase of bulk expansion the culture will begin to die, and when it begins to expand again that this growth is most likely representative of the outgrowth of a clone.

A)

44
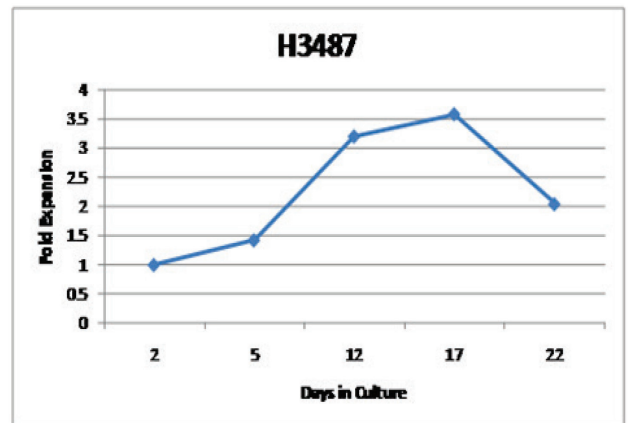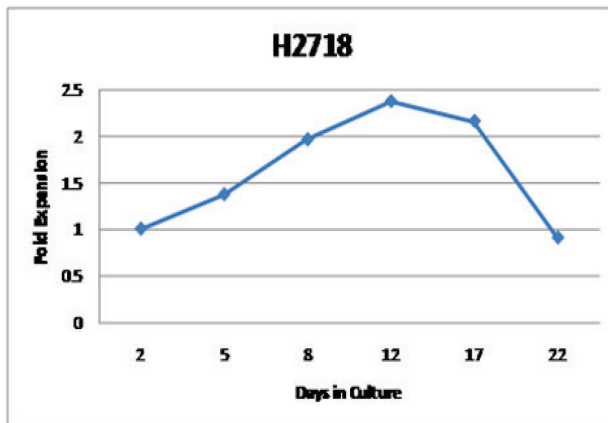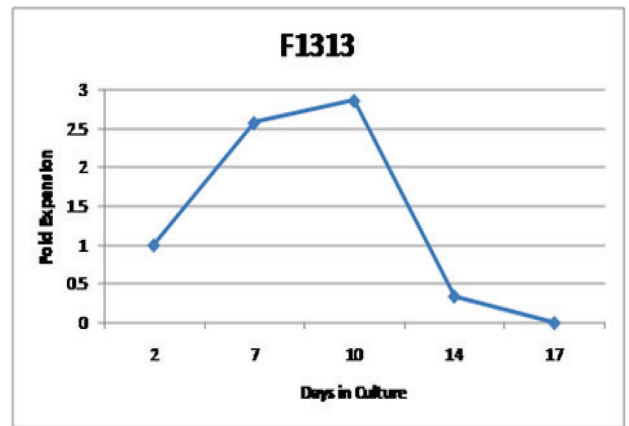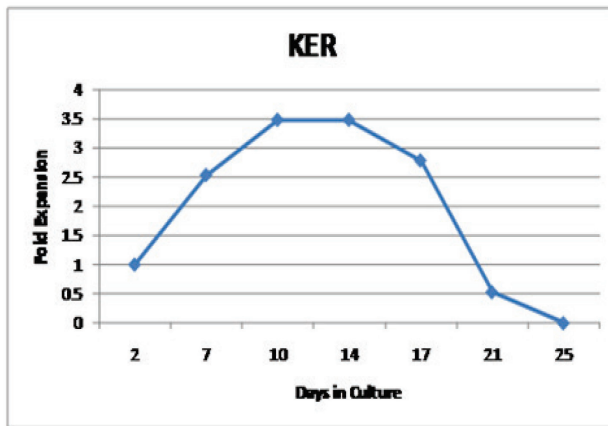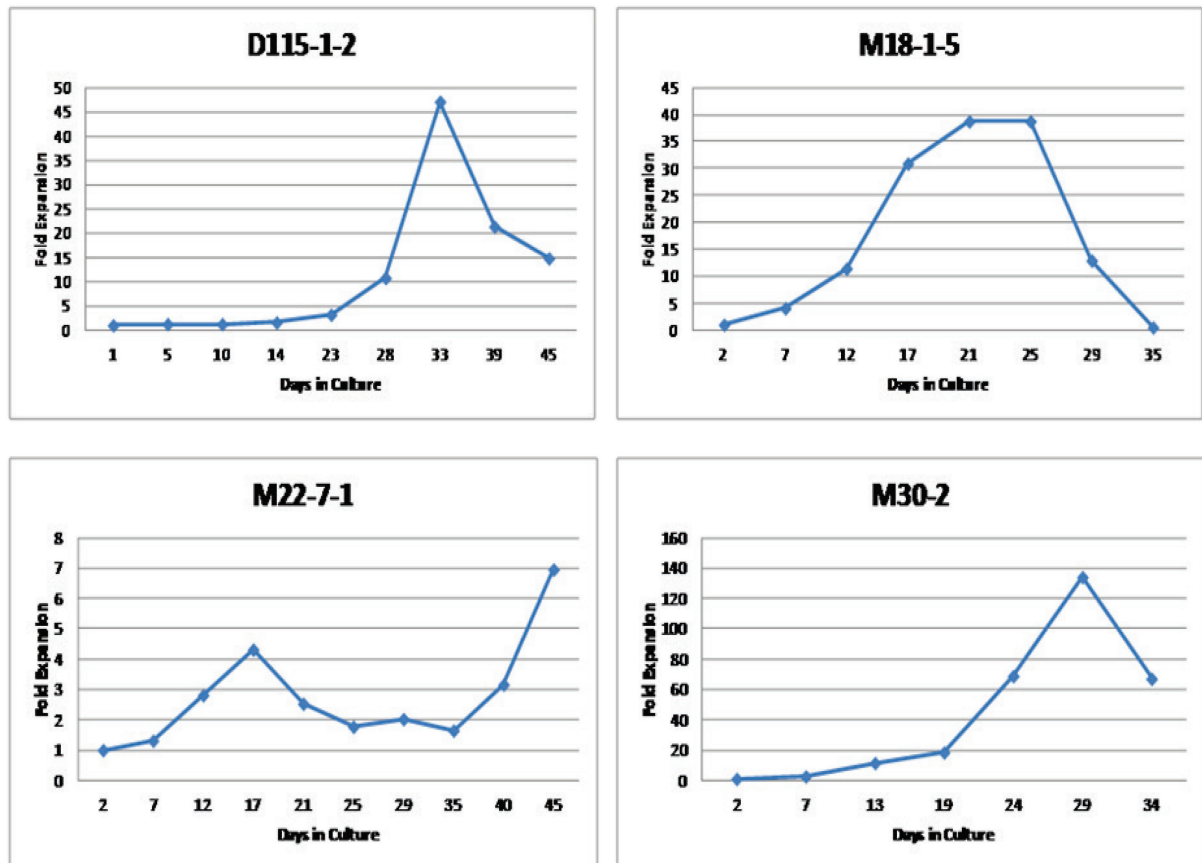
Figure 3.4 WIT-L supports expansion of primary human T-ALL cultures up to one month

Cell growth curves of primary (A) and xenograft-expanded (B) patient T-ALL cells. 1 x 10^6 viable cells were seeded into each well of a 12-well dish and passaged every 4-6 days onto fresh feeders. Viable cells were counted manually by trypan blue exclusion at each passage and reseeded at 1 x 10^6.

3.1.6 Assessment of culture composition

        To evaluate immunophenotype and confirm leukemia cell expansion, we analyzed the

samples by flow cytometry with a panel of extracellular markers at several different time

points throughout the culture period (Figure 3.5). We used CD45 as a leukocyte marker,

CD3, CD7, CD4 and CD8 as T cell markers and CD5 and CD19 as B cell markers with

gating as shown in Figure 3.6. In all cases immunophenotyping confirmed that our culture

system was in fact sustaining the growth of T-ALL blasts..
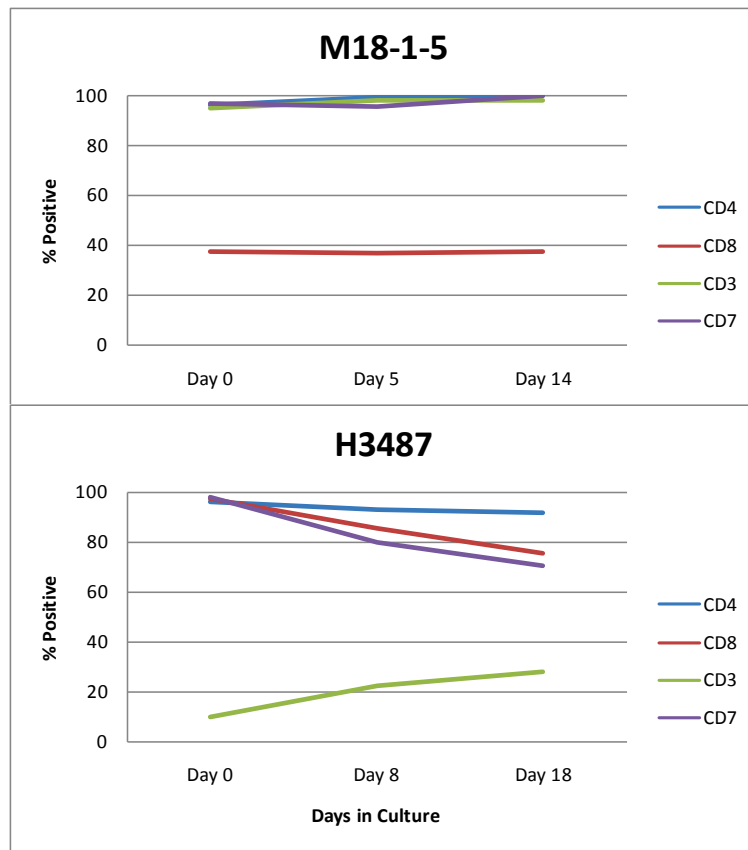
Figure 3.5 Immunophenotype of patient T-ALL samples over time *in vitro*

        Immunophenotype as determined by flow cytometry for T-cell markers on patient T-
ALL samples. Cells were immunophenotyped at thaw and after expansion *in vitro*.
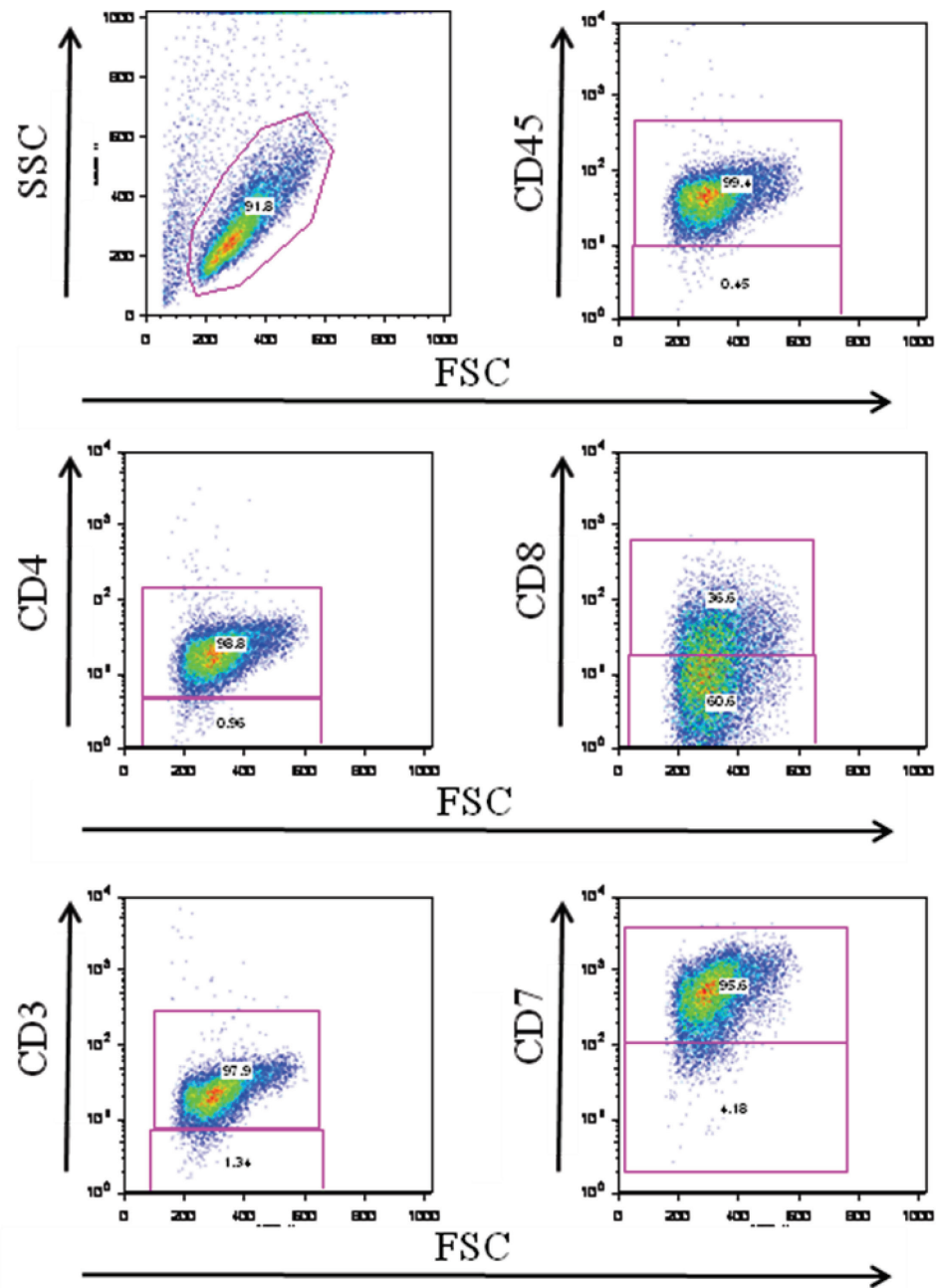
Figure 3.6 Example of gating for flow cytometry immunophenotyping of primary samples

  Example of staining with some of the markers used and the gating that was done to assess the cell population. This same flow panel and analysis was used at multiple time points during the *in vitro* culture period, beginning when the cryopreserved sample was thawed. Shown here is sample M18-1-5 at day 6 *in vitro* stained with markers CD45, CD4, CD8, CD3 and CD7.

3.1.7 Assessment of cell viability

With all of our samples we observed that after around two weeks of culture the T-ALL blasts began to die. In order to further elucidate the reason for this observed death we stained the cells with a marker for apoptosis and then analyzed the cultures by flow cytometry (Fig 3.7). Apoptosis, or programmed cell death, is a highly regulated mechanism of cellular suicide that is activated in response to various distress signals. Apoptosis is mediated through activation of the caspase cascade. There are two types of caspases, the initiator caspases and the effector caspases, which are activated in that order. They are expressed in the cytoplasm in a non-active pro-form which requires a cleavage step for activation; once an initiator caspase is activated it can then activate an effector caspase which eventually results in apoptosis (Nunez et al., 1998). For this assay we used a permeable, non-toxic inhibitor that binds irreversibly to activated caspase-3 in apoptotic cells. As caspase 3 is an effector caspase, and plays a key role in the initiation of the apoptotic process, this assay allows us to determine the amount of apoptosis present in the culture. In the samples tested we were unable to detect any significant amount of apoptosis in the culture and therefore hypothesized that the death observed is most likely occurring through a non-apoptotic mechanism such as autophagy or necrosis.
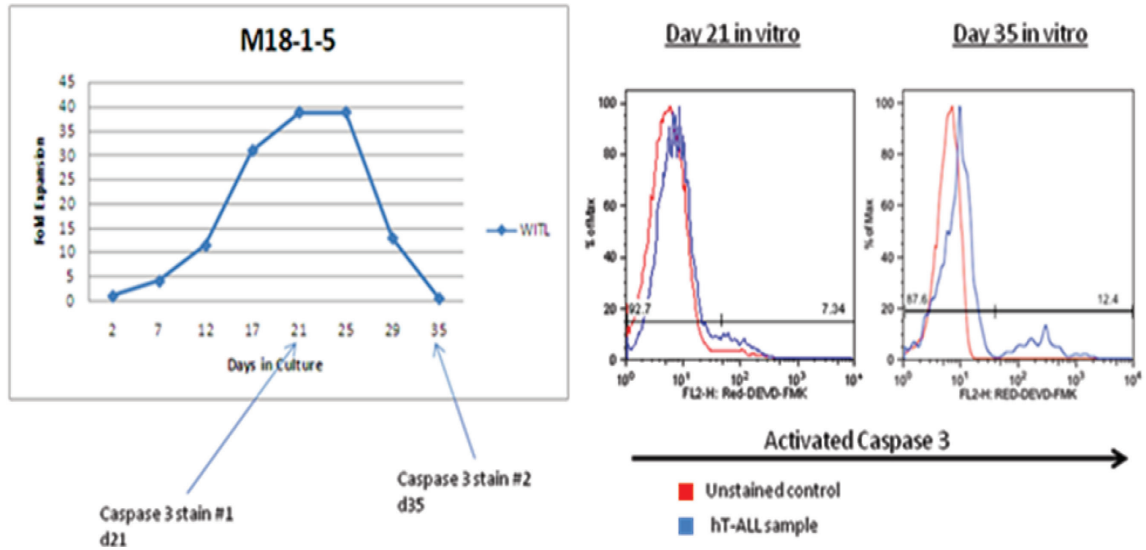
Figure 3.7 Apoptosis is not a major contributor to cell death in WIT-L patient T-ALL cultures

Cells growth curve and activated caspase-3 staining for a xenograft expanded patient T-ALL sample. 1 x 10^6 viable cells were seeded into each well of a 12-well dish and passaged every 4-6 days onto fresh feeders. Viable cells were counted manually by trypan blue exclusion at each passage and reseeded at 1 x 10^6. At days 21 and 35 of *in vitro* culture staining for activated caspase-3 was done to assess the amount of apoptosis in the culture. At both time points negligible amounts of apoptosis were detected.

3.1.8 Assessment of cell proliferation

As a further validation that our system was in fact promoting growth and proliferation

of T-ALL blast cultures, we also looked at the percentage of cycling cells in the culture

with a bromodeoxyuridine (BrdU) assay (Figure 3.8). This is an assay in which BrdU is

pulsed into a culture of dividing cells. It is then incorporated into the newly synthesized

DNA strands in the place of thymidine during DNA replication. The culture is then

stained with an antibody specific for BrdU conjugated to a fluorochrome and analyzed by

flow cytometry. We then know that the cells which are labeled with the BrdU specific

antibody were actively replicating their DNA during the assay. Our results show the cultures do have actively cycling cells and are in fact proliferating. This is shown by the number of cells in S phase during the BrdU pulse. In the sample shown below around 15% of viable cells were actively dividing during the pulse. The Sub G1 fraction is roughly equivalent to the amount of debris and dead cells present in the culture, indicating that the sample below is about 80% viable.
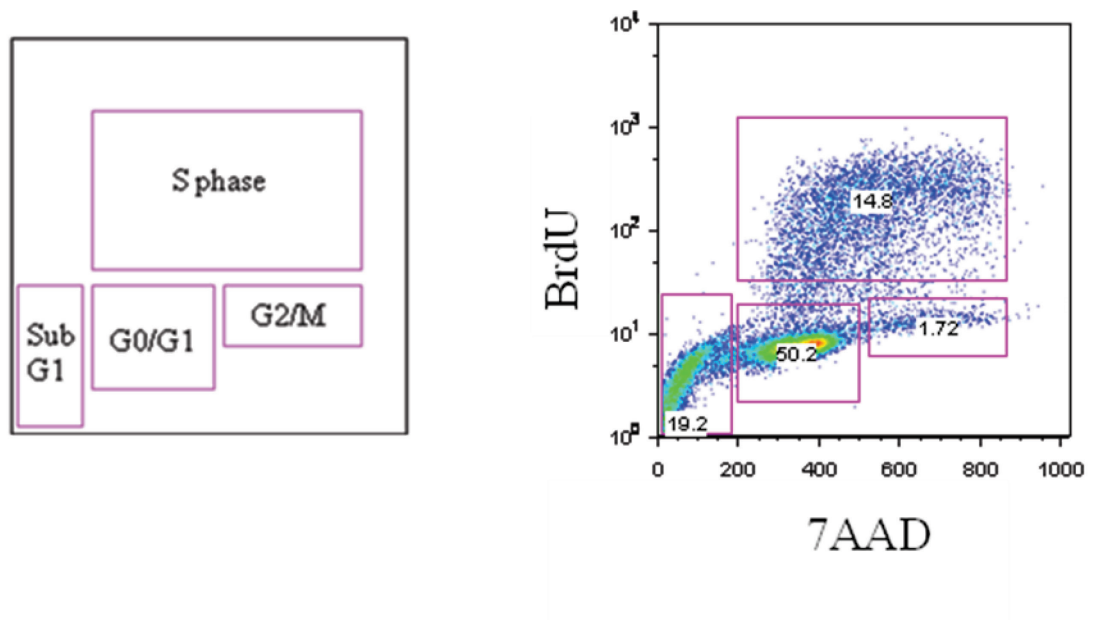
Figure 3.8 Patient T-ALL *in vitro* cultures containing actively cycling cells

BrdU labeling of a xenograft expanded human T-ALL sample. Cells were pulsed with BrdU for 6 hours and then fixed and stained with anti-BrdU Ab and DNA marker 7-AAD.

3.2 Dissecting the Contribution of Each Component of the WIT-L Co-culture System

3.2.1 Cytokine/growth factor dependence

In order to elucidate which components of our model were essential for supporting the growth of primary T-ALL blasts we next looked at the dependence of the T-ALL blasts on the addition of supplemental cytokines to the media. This was done by plating each sample in replicate with either the complete cytokine/growth factor cocktail or a variation of it. We began by simply subtracting one cytokine at a time to see which ones were essential for supporting growth. The complete cytokine cocktail used in these studies includes SCF, IGF-1, IL-2 and IL-7. After 10-12 days of culture the cells were analyzed for viability using the resazurin reduction assay previously mentioned. In all of our trials we found that the primary T-ALL blasts are dependent on IL-7 for growth in this model, and to a lesser extent IL-2, although IL-7 alone appears to be sufficient to sustain growth (Figure 3.9). Given this knowledge, we can now attempt to find out the reason for this dependence on IL-7 for growth and quickly and easily test the effect of other cytokines and growth factors in supporting bulk patient T-ALL cell growth.

A)
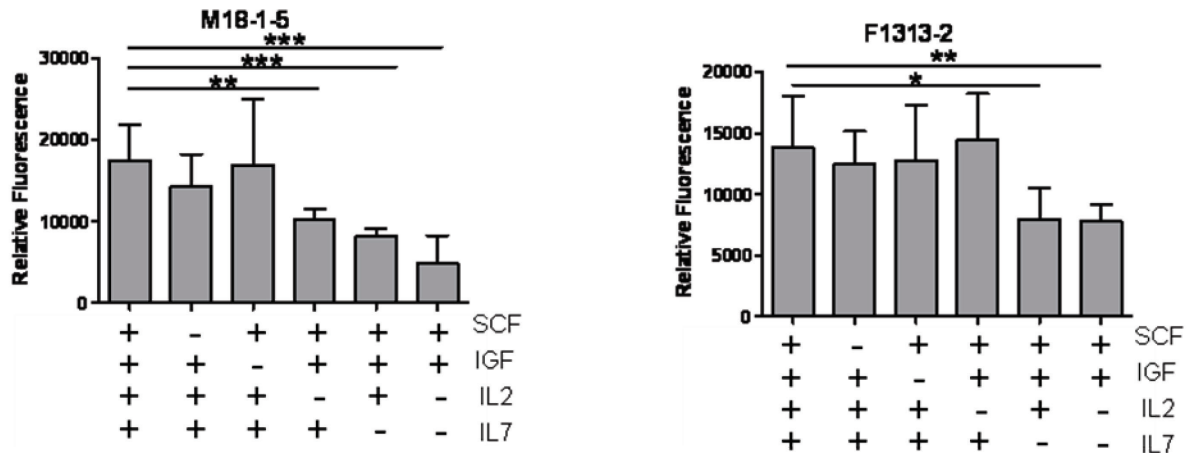
B)
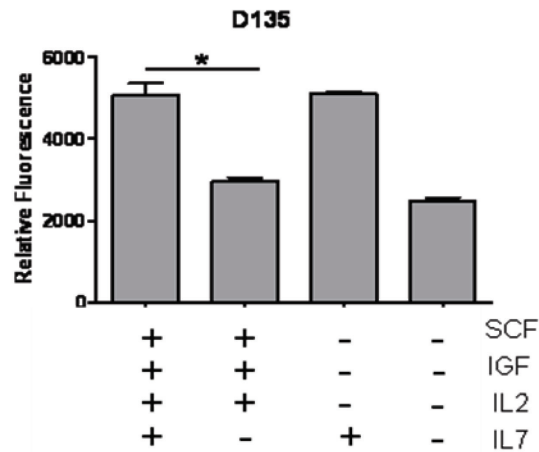
Figure 3.9 Patient T-ALL samples are dependent on IL-7 for growth *in vitro*

Cell growth assay preformed on primary (A) and xenograft-expanded (B) patient T-ALL cells. Cells were cultured and passaged as described in Figure 3.1.3. Cytokines/growth factors were subtracted out of the complete medium and cells were assayed for growth using a resazurin reduction assay. Data shown is a composite of passages 1 and 2. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

<u>3.2.2 Feeder dependence</u>

We next looked at the necessity of the feeders in our model. We started by comparing primary human T-ALL cells in the WITL media with the complete cytokine cocktail growing on top of an MS5-DL1 stromal feeder cell layer as compared to those growing on bare plastic in a tissue culture dish. After 10-12 days the cells were analyzed for viability using the resazurin reduction assay. Our results show that the cultures are completely feeder dependent (Figure 3.10, panel A). As discussed previously, the feeders used in this culture method have been engineered to express DL1 ligand, which stimulates Notch activation. To determine if the DL1 ligand alone is enough to sustain T-ALL blast growth we cultured the cells in the absence of feeders, but on plates coated with DL1 ligand. We measured cell growth and proliferation to determine if the presence of DL1 ligand alone abrogated the previously observed feeder dependence. We used IgDL1, a fusion protein previously described for use *in vitro* (Dallas et al., 2007). When we cultured the cells on bare plastic coated with the DL1 ligand for 10-12 days and then analyzed them for growth using the resazurin reduction assay, we found that the T-ALL cells were not able to grow on the IgDL1 coated plates, indicating that they were still feeder-dependent (Figure 3.10, panel B). Interestingly, the cells died significantly more quickly in the presence of ligand alone as compared to bare plastic. We hypothesized that this may be because the stimulation of the Notch pathway gives a proliferative signal, pushing the cells into cycle even when they are not in an environment that is able to support growth. This would cause the cells to die faster. The cells on bare plastic would not be receiving this signal, and so while not actively proliferating they would stay viable and maintain metabolic activity longer, which would give a stronger signal in the resazurin reduction assay. This signifies that the feeder cell layer contributes something to

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the culture besides just Notch activation through the DL1 ligand. We next hypothesized that the contribution of the feeder layer may be in the form of a secreted soluble factor. In order to test this hypothesis, we made conditioned media from the MS5-DL1 feeders. We then used this conditioned media on top of the IgDL1 coated plates to see if these two components together could be used in the place of a feeder layer. We found that even when cultured in the presence of DL1 ligand and conditioned media the human primary T-ALL cells did not exhibit the growth seen when co-cultured with the stromal feeder layer (Figure 3.10, panel C). The cells did not immediately die in these conditions, and were metabolically active enough to give a signal in the resazurin reduction assay. However these cells were not actively growing, they never expanded in number and died within a week of the data shown, indicating that they were lacking a proliferative signal in the absence of the stromal feeder layer. This leads to the very interesting and complex question of what the feeder layer is providing that is essential for T-ALL blast growth *in vitro*. MS5 cells have been previously reported to secrete a number of cytokines and growth factors as well as extracellular matrix proteins (Suzuki et al., 1992; Arock et al., 1994; Nishino et al., 1995). As the conditioned medium alone did not support proliferation of primary T-ALL cells, we have hypothesized that the growth support signals may be coming from interaction with extracellular matrix proteins or some other non-soluble factor. This is a question we plan to continue looking into in future experiments and hope to use the knowledge gained from those experiments to further optimize our *in vitro* model.

A)

KER

F1313

B)

F1313-2

M18-1-2
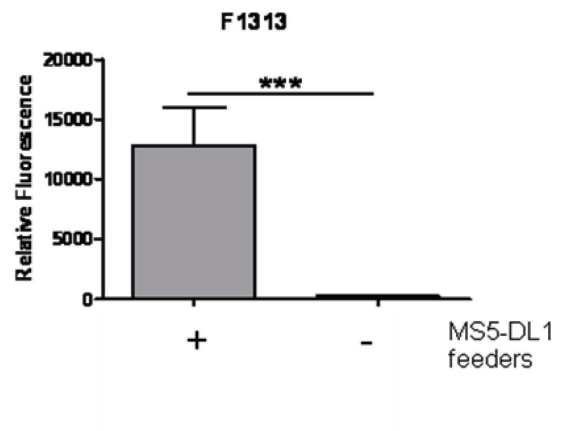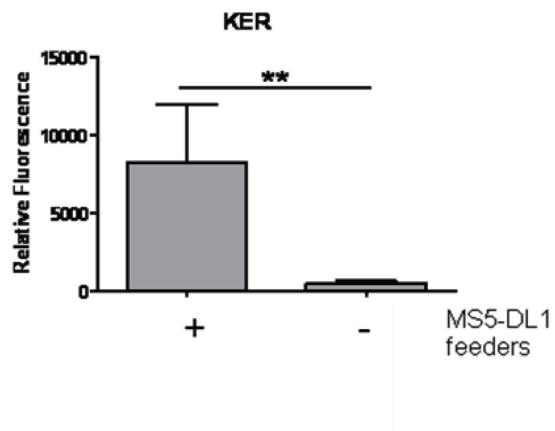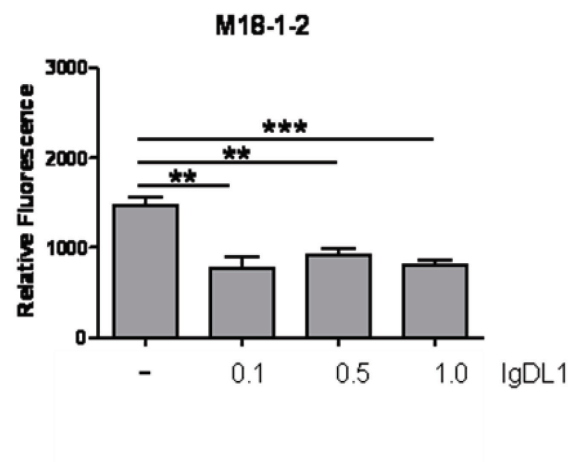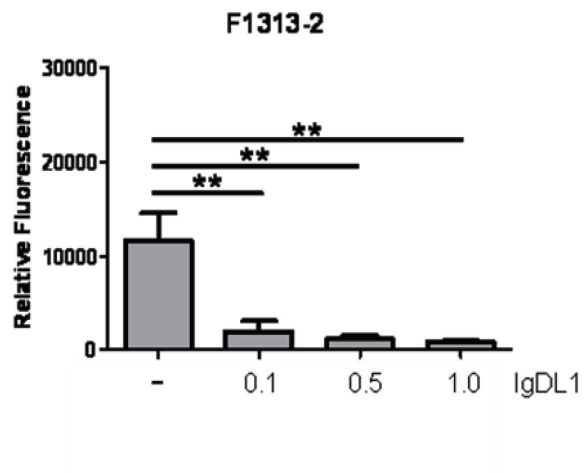
C)
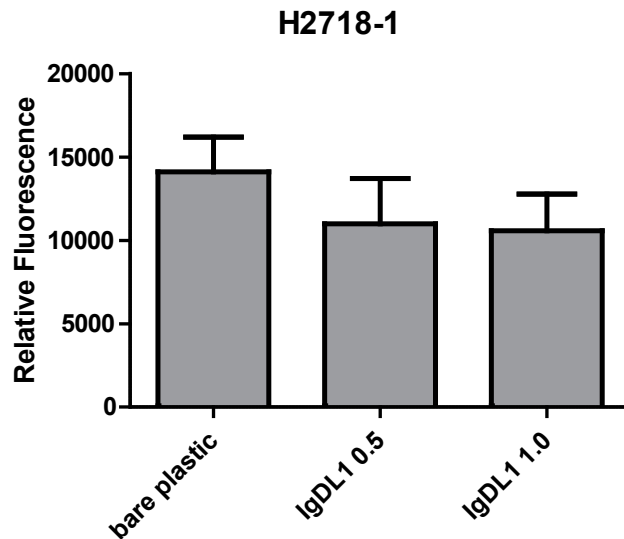
Figure 3.10 Stromal interactions are necessary for growth of patient T-ALL samples *in vitro*

Cell growth assay performed on patient T-ALL samples. Cells were culture on bare plastic plates or on top of an MS5-DL1 feeder layer. The cultures were passaged and assayed for growth as described in Figure 3.1.1. Data shown is a composite of passages 1 and 2 (A). Cell Xenograft-expanded patient T-ALL cells were cultured in WIT-L media (B) or in MS5-DL1 conditioned WIT-L media (C) on either bare plastic or plates coated with varying concentrations of immobilized DL1 ligand. Cells were cultured for 11 days and then assayed for growth with a resazurin reduction assay. *, p<0.05; **, p<0.01; ***, p<0.001.*

3.3 Application of the System to Biological Questions

We began this project with the goal of optimizing an *in vitro* culture system for primary human T-ALL blasts in order to have a robust reproducible model which we could use as a platform to directly ask biologically relevant questions of primary human cells. We have begun some preliminary drugs studies using novel therapeutic agents in combination with

standard chemotherapeutics, collaborated with other labs that wanted to try their assays on primary human cells, and used the system as a platform to begin testing possible candidates for the confirmation of self-renewal properties to these cultures. Experiments addressing this last point will be invaluable in helping to expand our knowledge of the leukemia stem cell field. Excitingly, the drug studies will also allow us to work towards the goal of creating personalized medicine and treatment regimens for cancer patients. In theory, if we could take a patient's cells at diagnosis and using this model, keep them alive and growing in culture while prospectively testing possible treatments directly on the patient's own cells, it would greatly aid in a physician's decision about which course of therapy to proceed with for treatment. Moving from the bench to bedside in this manner is the goal of our research, and with this culture model we have taken some preliminary steps in that direction.

3.3.1 Optimizing the culture system to support long-term growth and self-renewal activity

Although we showed that our system is able to support the short term growth of the bulk cells of a primary patient T-ALL sample, we were next interested in how to extend the length of the culture period and ideally support self-renewal activity. This would provide a very valuable tool for the study of T-ALL cells with self-renewal capability, or leukemia stem cells (LSCs). As of now, the only way to study stem cell activity is in a mouse model, which while it can provide valuable information has its caveats as well, namely being the expense incurred, length of time necessary to perform the experiments and inability to directly view and manipulate the cells. Studying leukemic stem cells *in vitro* would allow for the direct manipulation of these cells and easier testing of possible targeted therapies. We began a preliminary investigation into how to expand our culture model in order to support

long-term growth and self-renewal activity by the addition of a small molecule, called

StemRegenin 1 (SR1). SR1 is an aryl hydrocarbon receptor (AHR) antagonist that has been

previously described to promote expansion of human hematopoietic stem cells *in vitro*

(Boitano et al., 2010). We hypothesized that the addition of this molecule may aid in the

support of the cells with self renewal capacity in our cultures. Interestingly, we found that the

addition of SR1 was not able to confer long-term growth on our culture but it did increase the

magnitude of the expansion of the culture (Figure 3.11).

We hypothesize that this may be due to an ability of SR1 to play a role in the bulk

expansion of hematopoietic progenitor cells as well as the expansion of cells with self-

renewal capacity. The increase in bulk expansion that we see may be occurring through many

different pathways, one of which is hypoxia inducible factor 1 (HIF-1) signaling. HIF-1 is a

DNA-binding complex that regulates gene expression according to oxygen levels and has

been reported to be a heterodimer of two proteins called HIF-1α and HIF-1β (Wang and

Semenza, 1995). Interestingly, HIF-1β is identical to the previously identified aryl

hydrocarbon receptor nuclear translocator (ARNT), which has been reported as an essential

molecule for the xenobiotic response, a transcriptional response to certain environmental

hydrocarbons (Hankinson, 1995). Since SR1 is an AHR antagonist, and an AHR/ARNT

heterodimerization and several subsequent steps are required for AHR gene activation, SR1

may be effecting many downstream events (Swanson, 2002). In support of this theory, it has

previously been reported that the HIF-1 pathway regulates the proliferation of embryonic

mulitlineage hematopoietic progenitors and that this pathway requires the HIF-1α/ARNT

heterodimer (Adelman et al., 1999). While this is only one of many ways in which SR1 could

be increasing the magnitude of expansion in the culture, we would like to study the

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mechanism further to determine why it did not support self-renewal capacity in this model. This will in turn help us to better understand what might support self-renewal capacity and long-term growth in this model. Although this preliminary study did not yield an easy mechanism to support long term growth in this system, the WIT-L culture model provides a great platform for asking more questions of this nature and further exploring the topic.
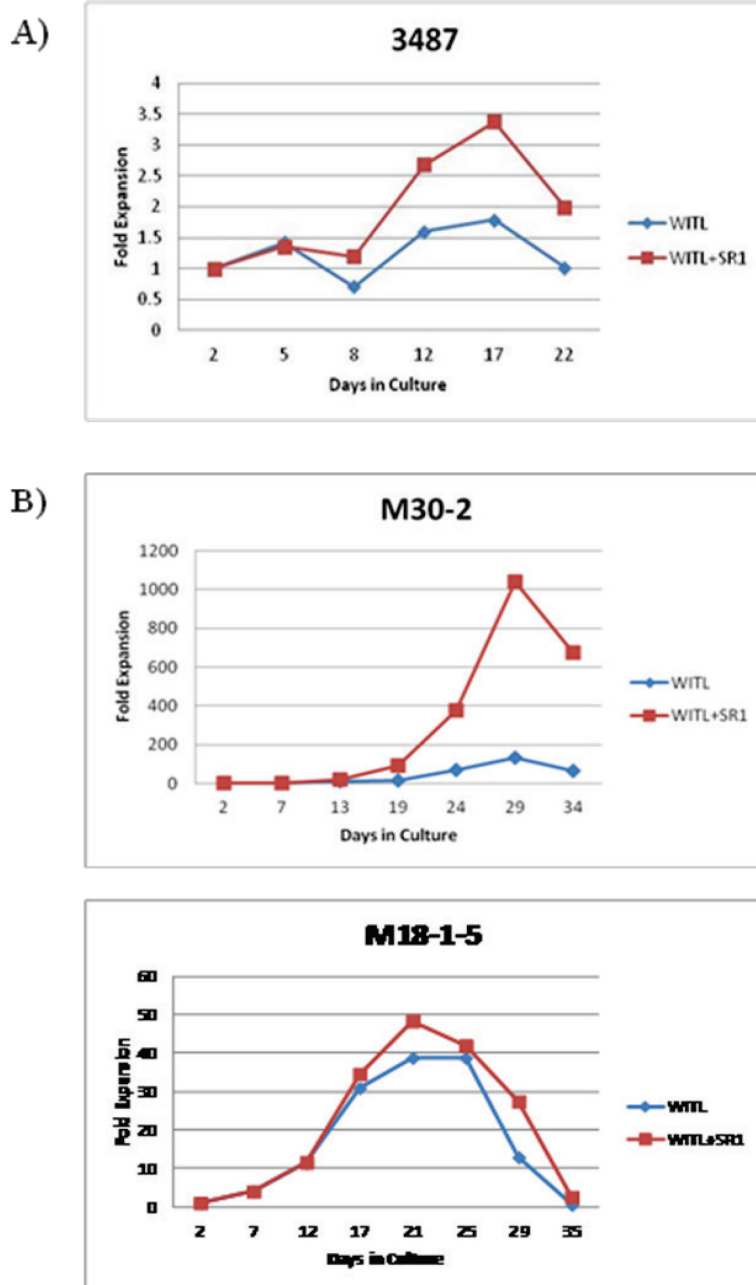
Figure 3.11 Addition of the small molecule SR1 to patient T-ALL cultures *in vitro* enhances the magnitude of expansion

Cell growth curves of primary (A) and xenograft-expanded (B) patient T-ALL cells. 1 x 10^6 viable cells were seeded into each well of a 12-well dish and passaged every 4-6 days onto fresh feeders. Cells were cultured in WIT-L medium with supplemental growth factors +/- the addition of SR1 (with SR1 shown in red). Viable cells were counted manually by trypan blue exclusion at each passage and reseeded at 1 x 10^6.

3.3.2 Sensitivity to conventional chemotherapeutics

We began testing our system as a platform for assessing drug sensitivity of primary patient samples by using two standard chemotherapeutics, dexamethasone and doxorubicin. Dexamethasone is a glucocorticoid that specifically induces apoptosis in malignant lymphoblasts and has constituted a central role in the treatment of lymphoid malignancies, particularly acute lymphoblastic leukemia, for decades (Gu et al., 2010). Unfortunately, clinically glucocorticoid resistance occurs in 10-30% of untreated ALL patients and is seen more frequently in T-ALL than in B-ALL (Kaspers et al., 1994). As resistance to this drug is a frequent and troubling problem in T-ALL, determining the sensitivity of a patient's own leukemic cells to the drug could be an important prognostic indicator and be beneficial in aiding the physician to determine course of therapy. Doxorubicin, another standard chemotherapeutic in the treatment of T-ALL, is an anthracycline antibiotic that functions by intercalating DNA and therefore causing cell death. However it has significant toxicity, it is especially associated with increased risk of cardiomyopathy, and reducing dosage or finding better, less toxic therapies would highly benefit patients (Lipshultz et al., 2010).

A range of clinically relevant doses were chosen for each drug and then the effect of the drug on the MS5-DL1 feeders cells alone was measured to assist in determining appropriate drug dosage for our experiments (Figure 3.12). We chose to carry out our experiments using drug doses that were both clinically relevant and had minimal effect on the stromal feeder cells.
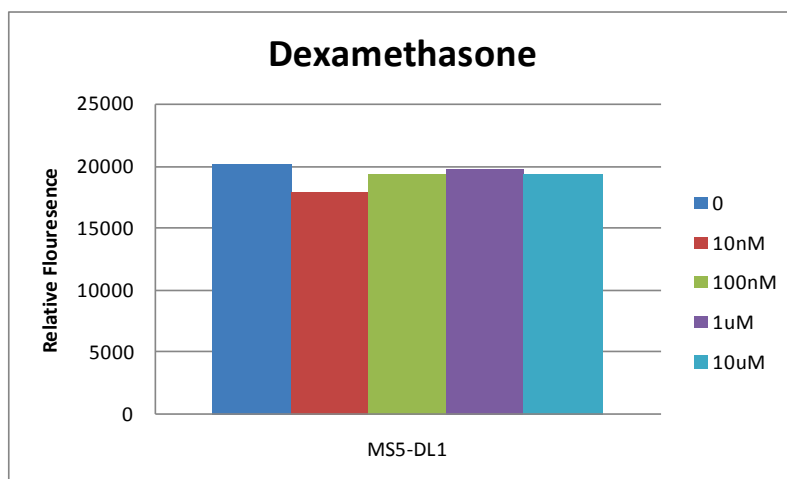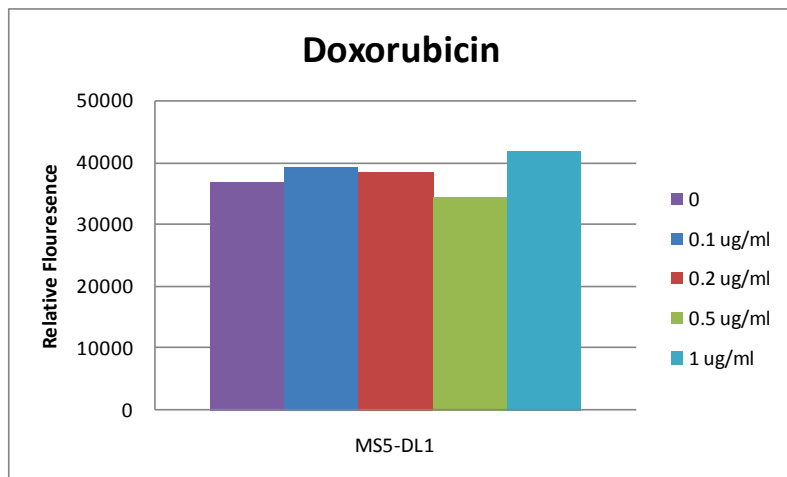
Figure 3.12 MS5-DL1 stromal feeder layer drug sensitivity to common chemotherapeutics

Cell growth assay performed on MS5-DL1 stromal feeder cells. Cells were irradiated at 50 Gy, then plated in MEMα medium and allowed to adhere for 2-6 hours. Drugs were added in several different doses and cells were cultured in their presence for 2-3 days. Cells were then assayed for growth using a resazurin reduction assay.

Once the appropriate doses were chosen for each therapeutic we tested the sensitivity of xenograft-expanded patient T-ALL samples. Drugs were added to a co-culture of T-ALL and MS5-DL1 stromal cells in WIT-L medium and then incubated for 66 hours. Cell viability was then assessed using the resazurin reduction assay. All doses were done in triplicate. All samples tested were extremely Dexamethasone sensitive at all doses, but sensitive to Doxorubicin in a dose dependent manner (Figure 3.13). However, even though the drugs did not have a strong effect on the MS5-DL1 feeder cells alone, the effects of these drugs on the T-ALL blast-stromal feeder layer interaction is not possible to elucidate. Although we believe that we are observing a cytotoxic effect on the patient T-ALL cells as a result of drug treatment, it is possible that the drug damages the feeder layer cells in way that they no longer provide the growth signals needed to support the T-ALL cells. This would cause cell death in the same way that we see when we try and culture the cells in the absence of the feeder layer. As there are these two formal possibilities as to the mechanism of death in the culture it is difficult to come to a strong conclusion regarding the efficacy of the treatment as a cytotoxic agent on patient cells alone. However, as shown in Figure 3.12, the drugs alone, at the doses used in this assay, do not seem to have a strong effect on the feeder layer and we believe that we are seeing at least some cytotoxic effect of the drug on the T-ALL cells alone.

Figure 3.13 Drug sensitivity of patient T-ALL cells to standard chemotherapeutics

Cell growth assay performed on xenograft-expanded patient T-ALL samples. Human T-ALL cells were plated on top of an MS5-DL1 feeder later in WIT-L medium. Cells were treated with a titration of doses for either Dexamethasone or Doxorubicin and then cultured for 66 hours. Cells were then assayed for growth using a resazurin reduction assay to determine sensitivity to the drug. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

3.3.3 Targeted signaling pathways

We also used this culture model to test the efficacy of a novel therapeutic, BMS-754807, which is a dual insulin receptor (IR)/Insulin-like growth factor 1 receptor (IGF1R) inhibitor. We also targeted this same pathway using a monoclonal antibody directed towards IGF1R, CP-751,871. The IR/IGF1R inhibitor, BMS-754807, was chosen because our lab has previously shown the IGF1R pathway to be important in T-ALL and we were interested in seeing if inhibiting this pathway could induce cell death in human T-ALL cells (Medyouf et al., 2011). The antibody also has the added benefits of being more specific than the dual inhibitor and it should have no effect on the feeder cell layer. We began again by testing the effects of the drug on the feeder cell layer alone and found that BMS-754807 kills the feeder

layer cells in a dose dependent manner (Figure 3.14). Because of this we chose very low

doses when treating xenograft expanded T-ALL cells. We found that both the dual IGF1R/IR

receptor drug and the IGF1R targeted antibody significantly decreased the growth of patient

T-ALL cells in a dose dependent manner (Figure 3.15). We also used BMS-754807 in

combination with the chemotherapy drugs tested earlier. Because of the extreme sensitivity

of the samples tested to Dexamethasone we did not choose to use it in combination with any

of the other therapeutics, as the death caused by it alone would mask any other effects.

However, interestingly when we combined Doxorubicin and BMS-754807, both at low

doses, we found some weak synergy between the two drugs (Figure 3.16). However, this test

for synergy was only done on one sample in triplicate and synergism was only seen at one of

the three doses of drug used. In order to make a strong conclusion in regards to the effect of

these two drugs in combination several more experiments would need to be performed.
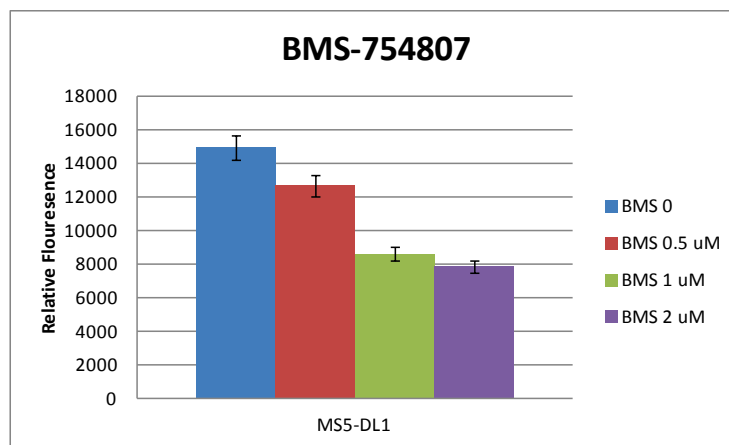
Figure 3.14 MS5-DL1 feeder layer sensitivity to an IGF1R/IR inhibitor

   Cell growth assay performed on MS5-DL1 stromal feeder cells. Cells were irradiated at 50 Gy, then plated in MEMα medium and allowed to adhere for 2-6 hours. BMS-754807 was added in several doses and cells were cultured in its presence for 2-3 days. Cells were then assayed for growth using a resazurin reduction assay.
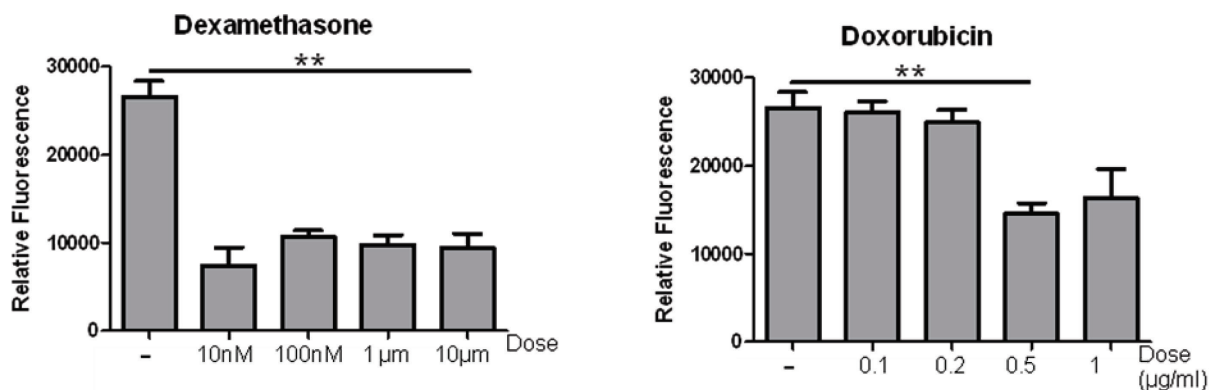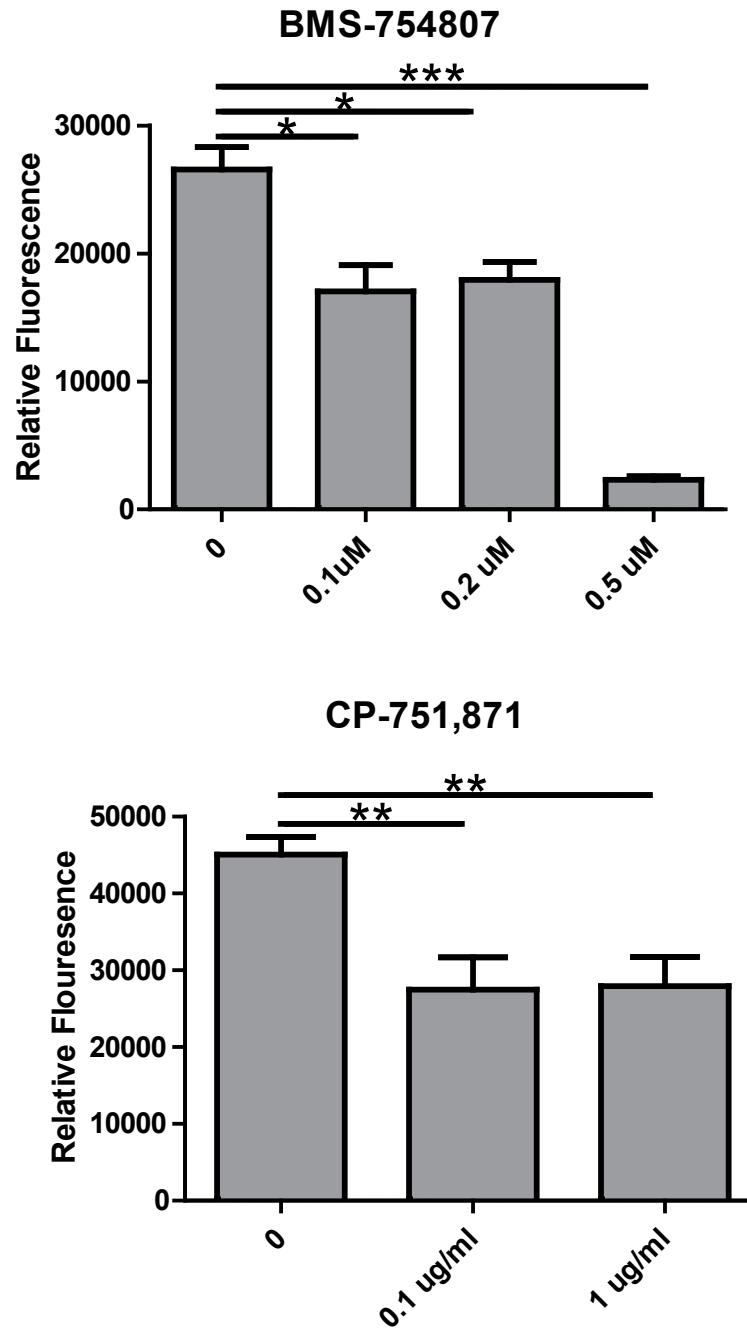
Figure 3.15 Sensitivity of patient T-ALL cells to IGF1R targeted therapy

Cell growth assay performed on xenograft-expanded patient T-ALL samples. Human T-ALL cells were plated on top of an MS5-DL1 feeder layer in WIT-L medium. Cells were treated with a titration of doses for either BMS-754807 or CP-751,871 Ab and then cultured for 66 hours (BMS) or 72 hours (CP Ab). Cells were then assayed for growth using a resazurin reduction assay to determine sensitivity to the drug. *, p<0.05; **, p<0.01; ***, p<0.001.
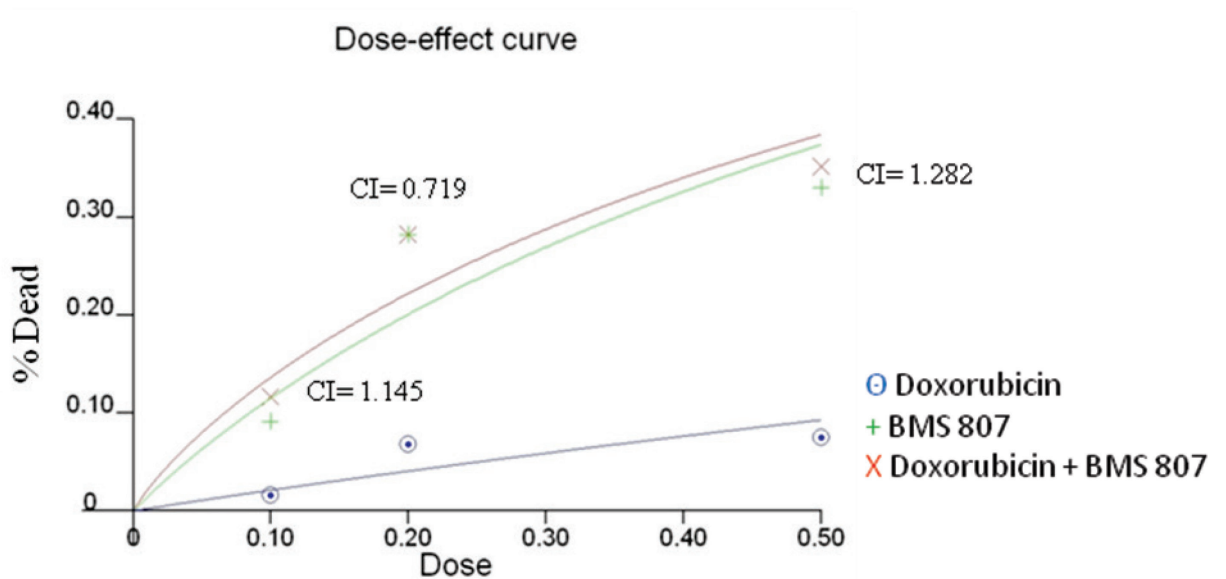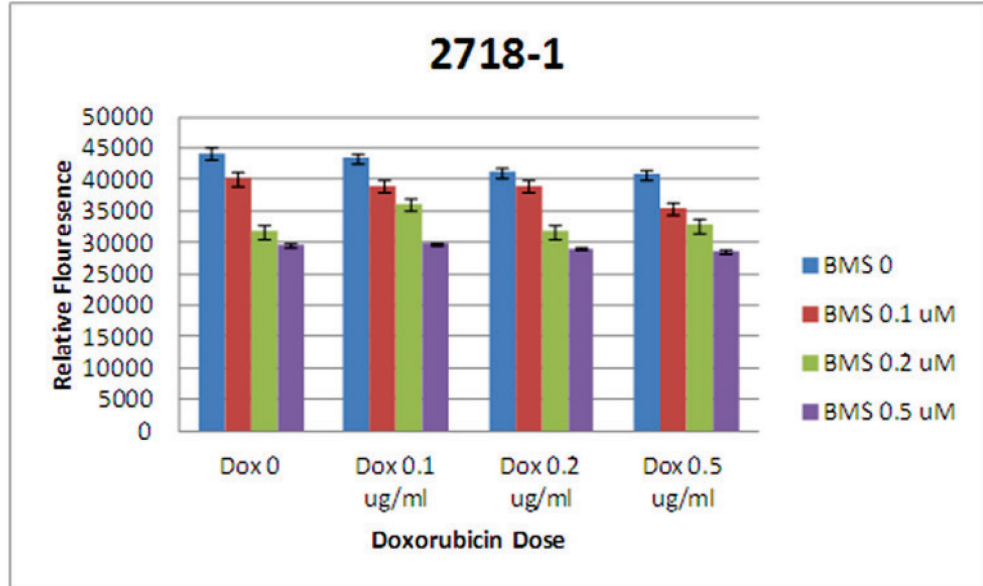
Figure 3.16 Possible synergism between doxorubicin and BMS-754807

Cell growth assay performed on xenograft-expanded patient T-ALL samples. Human T-ALL cells were plated on top of an MS5-DL1 feeder layer in WIT-L medium. Cells were treated with a titration of doses for Doxorubicin and BMS-754807 both alone and in combination. Cells were then assayed for growth using a resazurin reduction assay 66 hours later. Synergy was tested using the CalcuSyn software to calculate the combination index (CI). *CI<1, synergism; CI=1, additive effect; CI>1, antagonism.*

3.3.4 Borrelidin induced apoptosis in T-ALL

In collaboration with Dr. Chris Ong at the University of British Columbia we tested the ability of a novel small molecule, borrelidin, to induce apoptosis in patient leukemia samples. Borrelidin is a small molecule nitrile-containing macrolide inhibitor of threonyl-tRNA synthetase. It mediates inhibition of aminoacyl-tRNA synthesis and leads to an induction in the levels of uncharged tRNA, nutritional stress and ultimately inhibition of protein synthesis (Habibi et al., 2011). Nutrient deprivation is especially lethal to malignant cells in comparison with normal cells, because they need high levels of nutrients to maintain their increased proliferation rate. The study by D. Habibi and C. Ong used T-ALL cell lines, Jurkat and CEM, to show that treatment with borrelidin inhibits the proliferation of malignant ALL cells even at very low concentrations, with an IC_{50} of 50 ng/ml. Furthermore the drug showed a much greater effect on the malignant cells as compared to primary fibroblasts, which were relatively resistant to the suppressive effects of borrelidin with an IC_{50} of 400 ng/ml. They also reported that the borrelidin treatment increased the level of apoptosis, caused G_1 arrest, activated the general control nonderepressible-2 (GCN2) kinase stress response pathway and induced expression of CHOP protein, thereby indicating the mechanism through which the drug acts. In order to verify the findings they observed in T-ALL cell lines we collaborated to test the effects of borrelidin on primary human T-ALL cells using the WIT-L culture model. Cells were treated with a titration of borrelidin doses and cultured for two days before analysis for viability. The patient T-ALL samples tested were potently and significantly sensitive to borrelidin with an IC_{50} of 60 ng/ml (Figure 3.17). The sensitivity of both the T-ALL cell line and the primary human T-ALL cells in comparison with normal human fibroblasts indicates that this drug may be useful as a

potential therapeutic. Borrelidin is effective in killing T-ALL cells at a dose 8 times lower than that necessary to harm normal cells, providing a therapeutic window of drug dosing that will need to be further tested, but is encouraging for the potential use of this drug in patient treatment. This collaboration served to emphasize the utility of our culture model to test drug sensitivity in primary patient cells and further validate observations seen in cell line models.
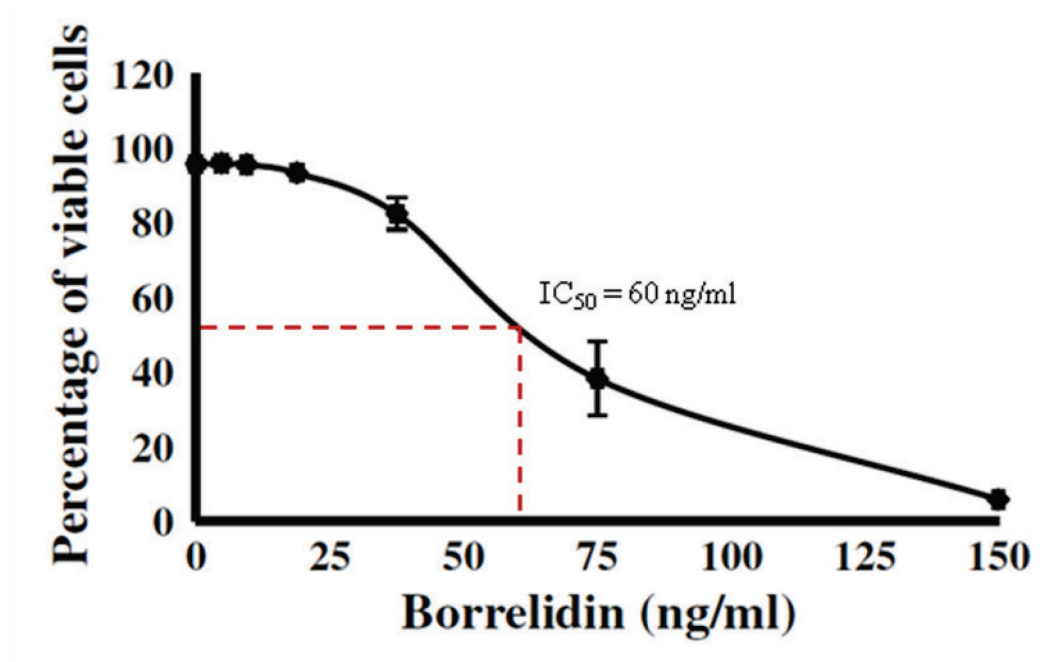
Figure 3.17 Borrelidin treatment results in loss of viability in patient T-ALL samples

Cell viability assay on xenograft expanded human T-ALL cells. T-ALL cells were seeded on top of an irradiated MS5-DL1 stromal feeder layer and treated with a titration of borrelidin doses. After 48 hours they were assayed for viability using PI with gating to exclude the feeder cells based on size.

3.3.5 TYK2-STAT1 pathway addiction in T-ALL

In collaboration with Dr. Jeffery Tyner at the Oregon Health and Science University Knight Cancer Institute and Dr. Takaomi Sanda at the Dana-Farber Cancer Institute we used patient T-ALL samples to help validate a reported TYK2-STAT1 pathway addiction in T-ALL. In the initial study Dr. Tyner and Dr. Sanda used an RNA interference (RNAi) screen on the entire tyrosine kinome of a patient T-ALL sample and were able to identify the novel therapeutic target tyrosine kinase 2 (TYK2). TYK2 is a non-receptor tyrosine kinase that is a member of the Janus kinase (JAK) family. It functions by signaling downstream of a variety of non-kinase cytokine receptors, which results in the phosphorylation and activation of many downstream substrates. They showed that in approximately 90% of T-ALL cell lines the cells are dependent on the TYK2-STAT1 signaling pathway for survival and that JAK/TYK2 kinase inhibitors induce T-ALL cell death. They also reported that the TYK2-STAT1 pathway found in TYK2-dependent T-ALL cells differs from canonical type-I interferon pathways in that JAK1 and STAT2 are not phosphorylated in these samples. After numerous studies in T-ALL cell lines to elucidate this pathway and its mechanism in the disease, Dr. Tyner and Dr. Sanda collaborated with us to test the effects of shRNA knockdown of TYK2 in primary patient samples. This collaboration was necessary because freshly thawed cryopreserved patient samples, both primary and xenograft expanded, are not efficiently transduced by siRNA. We cultured xenograft-expanded patient cells in our WIT-L growth system in order to get the cells back in cycle after thaw and made a panel of viable, growing primary samples with which they was able to effectively compare the effects of TYK2 siRNA knockdown to that seen in T-ALL cell lines. In the 19 T-ALL cell lines tested 17 were shown to be TYK2-dependent and 5 out of 8 primary samples tested were TYK2-

dependent (Figure 3.18), implicating that the TYK2-STAT1 pathway could be important in the pathogenesis of T-ALL. This collaboration is another example of how the WIT-L culture system provides a valuable tool for gaining insight into the biology of patient T-ALL cells and helps us to find and validate novel therapeutic targets.
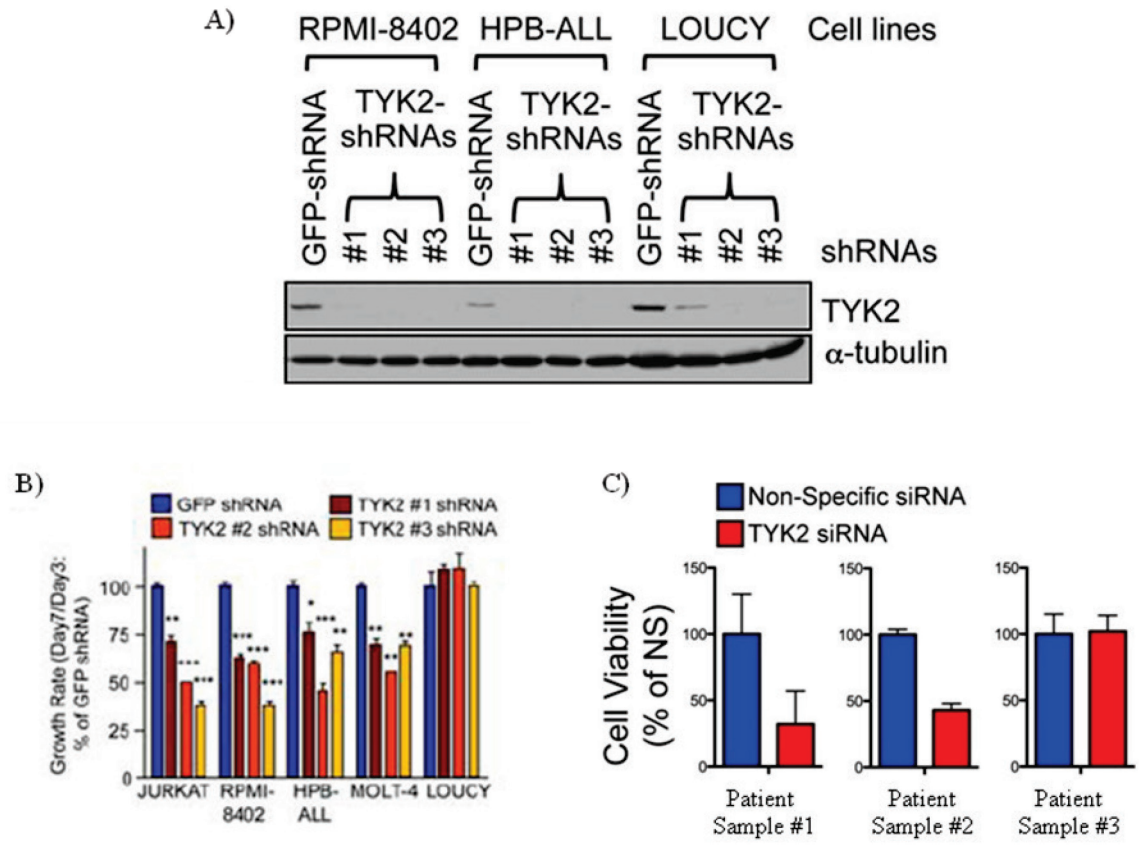
Figure 3.18 T-ALL cell lines and patient samples both exhibit TYK2-dependency

Validation of shRNA constructs. Lentiviral vectors carrying shRNAs targeting GFP (control) and TYK2 were infected into three T-ALL cell lines, RPMI-8402, HPB-ALL, and LOUCY to confirm equivalent knock-down in the cells lines (A). The three TYK-2 targeting shRNAs, as well as the control shRNA targeting GFP, were delivered by lentivirus into five T-ALL cell lines, JURKAT, RPMI-8402, HPB-ALL, MOLT-4 and LOUCY. Growth rate (day7/day3) was assessed for each TYK2 shRNA relative to control GFP shRNA in each cell line and is reported as the mean ± s.e.m. or triplicate experiments (B). Xenograft expanded patient T-ALL cells were cultured *in vitro* for 3-4 days and then washed and re-suspended in siPORT buffer with non-specific or TYK2 siRNA. Cells were then electroporated and transferred to culture medium. Quadruplicate wells were cultured for 4 days and then viability was assessed by MTS assay. All values are normalized to the average of the wells treated with non-specific siRNA (C). *$P<0.05$, ** $P<0.01$, *** $P<0.001$.

Chapter 4

Discussion

4.1 Efficacy of the WIT-L Co-culture Model

We have described a culture model for the growth of human T-ALL blasts. This model will allow us to gain insight into T-ALL biology through several different avenues. Here we showed its utility for the direct treatment of cells with common and novel therapeutics to determine sensitivity, for validation of novel signaling pathways and molecular mechanisms important to the initiation and maintenance of the disease, and as a platform for studying factors that can support self renewal activity.

4.1.1 Comparison with other culture systems from the literature

The model described here showed that we could expand primary patient T-ALL blasts *in vitro* for around two weeks. This short-term expansion allows a window of time to do functional and drug studies of T-ALL blasts that are directly representative of human biology. My first objective in this research was to improve upon existing *in vitro* culture models for patient T-ALL blasts and to create a reproducible system for blast expansion. We have succeeded in this aim by reporting a reproducible system using a defined, serum-free media that performed better in a head to head comparison with a previously reported culture model (Armstrong et al., 2009). While we only did head to head comparison with one other reported model, the amount of growth seen in the WIT-L co-culture system is better than or comparable to the reported growth from other systems reviewed (Cox et al., 2007; Chiu et

al., 2010). However, when one chooses a culture model for experiments, the best model may vary depending on the questions being asked and the assays being used.

4.1.2 Features of the culture model

Once we had shown that the model supported superior growth of patient T-ALL blasts as compared to a previously reported model, we furthered characterized the necessary components of the system and the relative contributions of each. We analyzed the cultures by flow cytometry immunophenotyping to ensure that the expanding cell population was indeed T-ALL blasts and to track any changes in the expression levels of various cell surface markers. This showed that the T-ALL blasts did expand preferentially to any other hematopoietic cells that may have been present in the culture from the initial sample. There were minor changes in cell surface receptor expression, but none of significance in the bulk cell population. We also used manual counts as well as cell cycle analysis to confirm that the cells were actually cycling and proliferating. Using an activated caspase-3 assay at multiple time points while the cells were *in vitro*, we tested the level of apoptosis in the culture. This showed that there were negligible levels of apoptosis present and this was not a major mechanism through which the cultures eventually died, suggesting the cause of death was most likely necrosis, senescence or autophagy, although more work would need to be done to show this conclusively.

4.1.3 Essential components of the model

With our cytokine/growth factor depletion experiments we further showed that human T-ALL cells are dependent on the addition of IL-7, and to a lesser extent IL-2, for growth in

this system. In order to more fully elucidate the roles of other cytokines/growth factors in supporting sustained human T-ALL blasts *in vitro* we plan to do additional experiments of this nature. We have hypothesized that addition or subtraction of various cytokines/growth factors such as Flt3L may have an effect on the length of proliferation *in vitro*. As our model incorporates a stromal feeder layer genetically engineered to express the Notch ligand DL1, we also tested the relative importance of this pathway stimulation. We found that Notch receptor stimulation with DL1 ligand alone without the presence of the feeder cells was not enough to sustain patient T-ALL cell growth. Furthermore, by using conditioned media made off of the stromal feeder cells, we were able to determine that the growth support the feeders are providing for the T-ALL cells is not a soluble factor and show that the patient T-ALL blasts require direct contact with the stromal feeder layer in order to proliferate in this culture model. This supports the finding of the group that established the MS5 stromal cell line, as they found that conditioned media alone was not sufficient to support hematopoietic cell growth (Itoh et al., 1989) and cell-cell contact in the co-culture is necessary.

4.1.4 Application of the culture model

Having tested and characterized the culture model we next set to answer the second aim of this study, which was to apply the culture model to directly answer biological and therapeutic questions using patient T-ALL blasts. The first studies we did involved testing the sensitivity of the patient blasts to standard chemotherapeutic reagents. We also used a dual IGF1R/IR inhibitor as a novel therapeutic. We tested its efficacy both alone and in combination with standard chemotherapeutics. In order to additionally and more specifically target the IGF1R signaling pathway we also tested a monoclonal antibody directed to IGF1R.

A further benefit of the antibody, as opposed to the inhibitor, for testing the sensitivity of patient T-ALL cells to inhibition of this pathway is that the antibody should have no effect on the feeder cells. These studies showed the relative sensitivity of the cells to the different treatments, a possible weak synergism between a novel therapeutic targeting the IGF1R pathway and a standard chemotherapeutic, and highlighted the utility of this model to test novel drugs on primary patient cells.

Aside from use as a drug sensitivity testing tool we also demonstrate that this culture model can be used as a platform to study patient T-ALL cell signaling pathways in a biologically relevant context. Our experiments using the IGF1R inhibitors were a combination of these two applications. The importance of the IGF1R pathway and its mechanism of action in T-ALL has been studied in our lab in mouse models, cell lines and some patient samples. The WIT-L co-culture system allowed us to test the effects of inhibiting IGF1R signaling on human T-ALL cell growth and viability and we found it to be very effective in killing them, adding further support to the body of evidence showing this pathway as important in T-ALL biology.

Furthermore, as T-ALL is a relatively rare disease and primary patient samples can be difficult to obtain it is unrealistic experimentally to make broad use of the samples. However, culture *in vitro* in our system allows these samples to be available for use to validate previously worked out mechanisms and pathways in a model biologically relevant to human disease. This is exemplified by our collaboration with Dr. Tyner presented in Chapter 3. After having discovered a novel signaling pathway that is important in T-ALL and doing many studies to further understand the pathway and its importance in cell lines, we

collaborated by contributing viable, cycling human T-ALL blasts which could be used experimentally to validate those findings.

As shown in section 3.3.1, this culture model can also be used as a tool to screen for agents that support long-term growth *in vitro*. We have begun preliminary investigations into this very interesting topic with the addition of the small molecule SR1 to our cultures. Although the addition of SR1 did not increase the longevity of the culture and its addition alone is not able to support long-term propagation of bulk patient T-ALL blasts in this model, interestingly we did find that the addition of SR1 to the cultures increased the magnitude of expansion in all cases. While this is not the effect we expected, it is an interesting phenomenon that we would like to follow up on elucidate the mechanism through which the molecule is causing an increase in bulk expansion. While this preliminary experiment did not yield a method to promote long-term expansion, we are still very interested in screening more agents, singly and in combination with each other. This will help us better understand the maintenance of leukemia initiating cells in culture which will in turn give us further insight into their biology and aid in the development of future therapeutics.

4.1.5 Limitations of the model

However, a major limitation of this model is the presence of the stromal feeder layer in the culture. This poses an obstacle to drug studies because while we can attempt to minimize the effect of the drug on the feeder layer alone we cannot determine the effect the treatment has on the interaction between the T-ALL cells and the feeders. As we have shown that the T-ALL cells are dependent on the feeder layer for growth in this model, it is difficult to conclusively state the efficacy of the drug alone because the death observed may be due to

a disrupted T-ALL cell-feeder layer interaction and not from the drug working on the T-ALL cells alone. One other complication caused by the presence of feeders in the culture for drug efficacy studies is the well reported effect of cell adhesion-mediated drug resistance (CAM-DR) (Damiano et al., 2001; Hazlehurst and Dalton, 2001; Nefedova et al., 2003). It has been reported for both solid tumors and hematopoietic malignancies that cell-cell and cell-matrix contact can result in the activation of signaling pathways that directly influence cell growth and survival and can initiate anti-apoptotic pathways. These anti-apoptotic pathways can protect the malignant cell and cause resistance to cytotoxic agents, which is referred to as CAM-DR. For the hematopoietic malignancies it has been shown that adhesion of a tumor cell to the single cell-matrix factor fibronectin alone is enough to protect the cell from the apoptotic stimuli (Hazlehurst and Dalton, 2001). We know that while our media is serum free so no fibronectin is present in the culture from that source, that the MS5 feeder cells produce extracellular matrix proteins including fibronectin (Itoh et al., 1989). So while the adhesion of the T-ALL cells to the feeder layer undoubtedly helps them to grow and proliferate *in vitro* it confounds the interpretation of drug efficacy as seen in this model. Interestingly we still saw dramatic death response to several of the drugs we used. However, while we are working towards a feeder-free culture model, the presence of the feeders may not be a problem for some experiments. When the leukemia cells are still in the human body they are living in a stromal environment, and any therapy given to the patient will have to kill the leukemic cells in this environment. In this respect the presence of the stromal feeder layer in the *in vitro* model can serve to recapitulate the environment cells would be in when in the human body.

Another limitation to this work is the use of the resazurin reduction assay as a method to measure cell growth. As it is based on a fluorescent signal produced by metabolically

active cells it can only be used as a relative measure of growth, not an absolute measure. This allows us to compare growth intra-experiment, but makes it difficult to make comparisons between different experiments. It is also not possible to do time course experiments with this assay as it can only be used as a static cell counter. However, it is cheap, easy to do in a high throughput manner and relatively simple which makes it good primary screening tool. We validate our resazurin reduction assay growth measures with manual counts by hemocytometer. For some samples we also assessed viability by PI and cell cycle status by BrdU. We could also validate the resazurin reduction assay using the cell division tracer, Carboxyfluorescein diacetate succinimidyl ester (CFSE). CFSE is able to diffuse into the cell across intact cell membranes and once inside the cell intracellular esterases cleave the acetate groups to yield a fluorescent molecule. Cell division can be measured in this manner as the successive halving of the fluorescence intensity of CFSE (Parish et al., 2009). While the resazurin reduction assay has its limitations, in combination with other assays to validate the findings, it is a useful cell growth and viability assay.

4.2 Future Directions

<u>4.2.1 Assessment of clonality in the culture</u>

We have shown that the T-ALL blasts in our culture model directly begin expanding *in vitro* without large amounts of death first; supporting the hypothesis that the growth observed in this culture model is bulk expansion, not outgrowth of a clone. In order to track and be able to definitively state the clonality of cells cultured in this system we would like to do bar coding studies. Cellular bar coding works by labeling retroviral vectors with random sequence tags, or barcodes. Once they integrate, each vector will introduce a unique, identifiable mark into the host cell genome. You can then sequence the cells and use the unique barcodes to track clonal progeny over time and sensitively detect both minor and major clones (Gerrits et al., 2010). This will enable us to label the primary patient sample with barcodes and then sequence the sample at different time points throughout the *in vitro* culture period to track the relative abundance of each clone. This should give us a clear answer of whether the observed growth is due to growth of many clones as we have hypothesized or if it is due to an outgrowth of a single clone.

<u>4.2.2 Test WIT-LO media</u>

In our cytokine withdrawal studies we found that the addition of IGF1 to our cultures provided no significant benefit, which was a surprise, as other data in our lab shows that pharmacologic inhibition or genetic deletion of IGF1R blocks growth and viability of T-ALL cells (Medyouf et al., 2011). We hypothesized that this lack of dependence of IGF1 for growth in our system may be due to the very high levels of insulin present in the WIT-L

media. In order to address this issue and for use in further experiments relating to the IGF1R pathway we have obtained an insulin-free formulation of the media called WIT-LO. We plan to test the relative contribution of supplemental IGF1 to primary human T-ALL blast growth in an insulin free context. We will also use this media in further tests with the IGF1R inhibitor BMS-754807 and monoclonal antibody CP-751,871 as well as any other function studies involving the IGF1R pathway.

4.2.3 Feeder-free culture models

To address the complication of interpreting certain results due to the presence of feeder cells in the culture we will direct future experiments towards the creation of a feeder-free culture model. We would also like to elucidate the factors provided by the stromal feeder layer essential for the support of patient T-ALL growth. To do this we will research further into the contribution of components such as soluble growth factors and cytokines secreted by the feeders which significantly contribute towards T-ALL cell growth, the relative contribution of fibronectin adhesion and DL1 ligand stimulation as well as the integrins crucial in the fibronectin-T-ALL cell interaction. Specific fibronectin-integrin interactions have been reported to be crucial in preventing apoptosis of malignant cells and thereby rendering them insensitive to cytotoxic agents in other hematopoietic malignancies (Lundell et al., 1996; de la Fuente et al., 1999) and we would like to add to this body of knowledge by elucidating the key interactions in a T-ALL specific context. Once we have optimized this system we posit that it can be used in the clinic as a tool to facilitate therapy regimen design. The immediate growth of patient cells *in vitro* under our culture conditions allows for

prospective testing of standard and novel compounds for therapeutic efficacy on patients'

own tumor cells and enables the implementation of personalized medicine approaches.

<u>4.2.4 Cancer stem cells</u>

The theory of cancer stem cells (CSCs) has grown in popularity in recent years and

the role of cancer stem cells has been reported in many different malignancies (Al-Hajj et al.,

2003; Singh et al., 2004; Hermann et al., 2007; O'Brien et al., 2007). The theory suggests that

a small subset of cancer cells may be responsible for the initiation and propagation of cancer.

In this model there would be a hierarchy of cells within the cancer (Figure 4.1), similar to the

hierarchy of cells seen in many normal tissue types. These CSCs would be able to self-renew

and give rise to all of the other cellular subsets in the hierarchy. Experimentally they would

be able to initiate a new cancer in a xenograft or mouse model. This model suggests that

relapse may be due the eradication of bulk tumor cells during treatment but not the CSC. The

CSC would then be able to reform the disease, creating a relapse within the patient. As

evidence is growing in support of the CSC model it is important to be able to study these

cells in order to design and develop novel therapies with which to specifically target CSCs.

The WIT-L culture model serves as a platform to test patient T-ALL blasts for the

conditions that will allow long-term propagation and in this manner gain insight into the

conditions necessary to support cells with self-renewal capacity. This will allow us to better

understanding of and access to these cells. *In vitro* culture allows us to quickly and easily test

prospective candidate genes through lentiviral transduction and knockdown for their ability

to support self-renewal. We can also test the cytokines and growth factors necessary to

support growth of the culture, and the sensitivity of these cells to various drugs, small

molecules and other compounds. Oxygen is an essential regulator of cellular metabolism, proliferation and survival and recent studies have reported a link between oxygen concentrations and CSCs (Heddleston et al., 2010). An *in vitro* culture system of patient T-ALL cells allows us to directly test and easily monitor the effect of hypoxic conditions on various cell fractions within the culture. Because this model uses patient cells we believe it is more directly biologically relevant to human disease than *in vitro* models using cell lines and will help gain insight in the field of human leukemia stem cell biology.
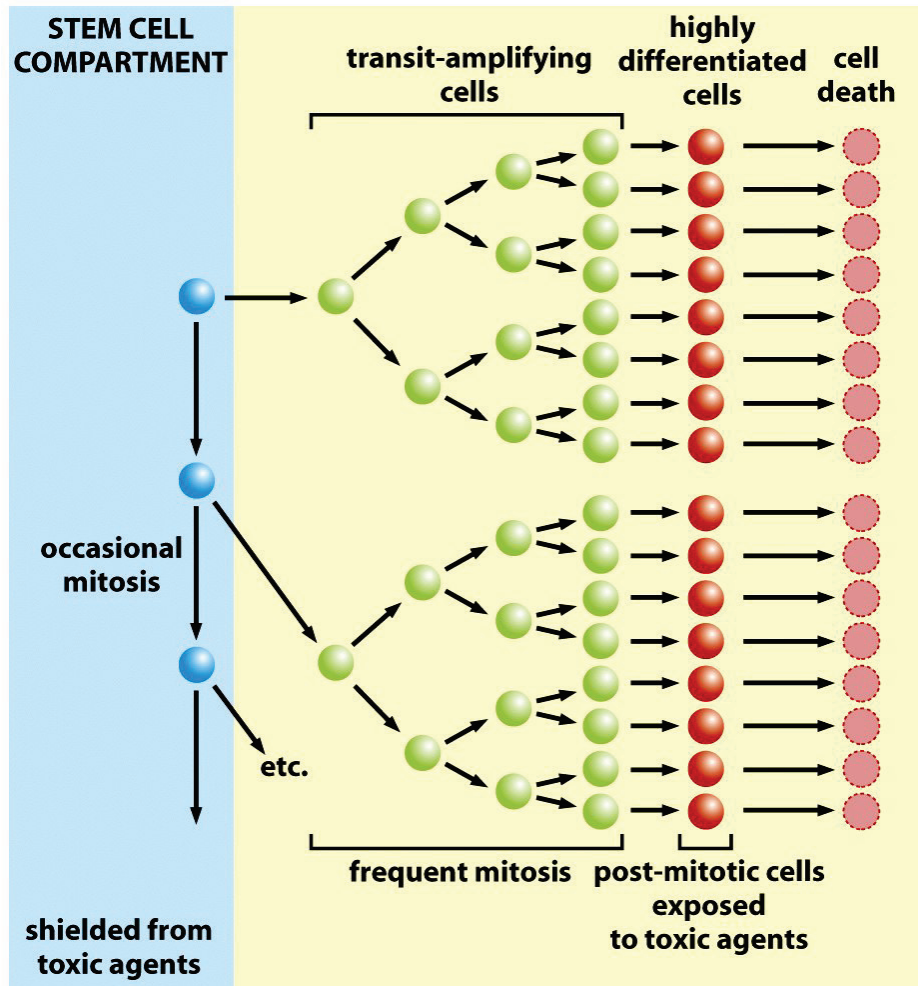
Figure 4.1 Cancer stem cell theory

     Cancer stem cell theory posits that tumor growth is driven by a rare subpopulation of cells that are less proliferative, able to self-renew and are resistant to apoptosis which shields them from cytotoxic agents. This population is shown in blue and the progenitor cells are shown in green and red. These progenitor cells are more differentiated, make up the bulk of the tumor mass and are more sensitive to cytotoxic agents. Figure from Robert A. Weinberg's textbook, The Biology of Cancer. ©Robert A. Weinberg, 2006, accessed January 2012.

Chapter 5

Concluding Chapter

The aim of this thesis was to develop and apply an *in vitro* culture model for primary human T-ALL cells. The results presented here show that a co-culture system with stromal feeder cells genetically engineered to express the Notch1 ligand, DL1, and a defined, serum-free medium allows for bulk expansion of primary patient T-ALL samples for 2-3 weeks. The use of the defined, serum-free medium will improve overall reproducibility of experimental results by eliminating the variation in medium composition due to differences between serum lots. We also report that in this model system the growth factor IL-7 and direct contact with the stromal feeder layer are necessary to support T-ALL cell growth.

We also demonstrate the utility of this culture model to facilitate ongoing efforts to identifying growth factors/cytokines required for maintenance of self-renewal activity in leukemia cells. This model also provides a platform for studying pathways critical for T-ALL pathogenesis and maintenance directly in human T-ALL cells. A major goal for this culture model was to be able to use it for prospective drug studies on a patient's own cells. As discussed in Chapter 4 there are some caveats for using this model for drug studies as the presence of the stromal feeder layer presents a complication in interpreting the data.

From a research prospective, this culture model facilitates gene manipulation through transduction with overexpression and knockdown viruses in primary T-ALL cells, enabling study of important pathways in this disease. It will also be useful for biochemical and molecular studies in human T-ALL cells, which will extend the range of investigations that

can be pursued, as we are not restricted to *in vivo* or cell line assays. From a clinical

perspective, the immediate growth of patient cells *in vitro* using this model will allow for the

first time prospective testing of novel compounds for therapeutic efficacy on patients' own

tumor cells and thus enable implementation of personalized medicine approaches.

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References

Adelman, D. M., Maltepe, E. and Simon, M. C. (1999) 'Multilineage embryonic hematopoiesis requires hypoxic ARNT activity', *Genes Dev* 13(19): 2478-83.

Aifantis, I., Raetz, E. and Buonamici, S. (2008) 'Molecular pathogenesis of T-cell leukaemia and lymphoma', *Nat Rev Immunol* 8(5): 380-90.

Al-Hajj, M., Wicha, M. S., Benito-Hernandez, A., Morrison, S. J. and Clarke, M. F. (2003) 'Prospective identification of tumorigenic breast cancer cells', *Proc Natl Acad Sci U S A* 100(7): 3983-8.

Alves, N. L., Richard-Le Goff, O., Huntington, N. D., Sousa, A. P., Ribeiro, V. S., Bordack, A., Vives, F. L., Peduto, L., Chidgey, A., Cumano, A. et al. (2009) 'Characterization of the thymic IL-7 niche in vivo', *Proc Natl Acad Sci U S A* 106(5): 1512-7.

Armstrong, F., Brunet de la Grange, P., Gerby, B., Rouyez, M. C., Calvo, J., Fontenay, M., Boissel, N., Dombret, H., Baruchel, A., Landman-Parker, J. et al. (2009) 'NOTCH is a key regulator of human T-cell acute leukemia initiating cell activity', *Blood* 113(8): 1730-40.

Arock, M., Hervatin, F., Guillosson, J. J., Mencia-Huerta, J. M. and Thierry, D. (1994) 'Differentiation of human mast cells from bone-marrow and cord-blood progenitor cells by factors produced by a mouse stromal cell line', *Ann N Y Acad Sci* 725: 59-68.

Artavanis-Tsakonas, S., Rand, M. D. and Lake, R. J. (1999) 'Notch signaling: cell fate control and signal integration in development', *Science* 284(5415): 770-6.

Aster, J. C., Pear, W. S. and Blacklow, S. C. (2008) 'Notch signaling in leukemia', *Annu Rev Pathol* 3: 587-613.

Aster, J. C., Xu, L., Karnell, F. G., Patriub, V., Pui, J. C. and Pear, W. S. (2000) 'Essential roles for ankyrin repeat and transactivation domains in induction of T-cell leukemia by notch1', *Mol Cell Biol* 20(20): 7505-15.

Avraham, H., Vannier, E., Cowley, S., Jiang, S. X., Chi, S., Dinarello, C. A., Zsebo, K. M. and Groopman, J. E. (1992) 'Effects of the stem cell factor, c-kit ligand, on human megakaryocytic cells', *Blood* 79(2): 365-71.

Barata, J. T., Cardoso, A. A. and Boussiotis, V. A. (2005) 'Interleukin-7 in T-cell acute lymphoblastic leukemia: an extrinsic factor supporting leukemogenesis?', *Leuk Lymphoma* 46(4): 483-95.

Barata, J. T., Keenan, T. D., Silva, A., Nadler, L. M., Boussiotis, V. A. and Cardoso, A. A. (2004a) 'Common gamma chain-signaling cytokines promote proliferation of T-cell acute lymphoblastic leukemia', *Haematologica* 89(12): 1459-67.

Barata, J. T., Silva, A., Brandao, J. G., Nadler, L. M., Cardoso, A. A. and Boussiotis, V. A. (2004b) 'Activation of PI3K is indispensable for interleukin 7-mediated viability, proliferation, glucose use, and growth of T cell acute lymphoblastic leukemia cells', *J Exp Med* 200(5): 659-69.

Benyoucef, S., Surinya, K. H., Hadaschik, D. and Siddle, K. (2007) 'Characterization of insulin/IGF hybrid receptors: contributions of the insulin receptor L2 and Fn1 domains and the alternatively spliced exon 11 sequence to ligand binding and receptor activation', *Biochem J* 403(3): 603-13.

Boitano, A. E., Wang, J., Romeo, R., Bouchez, L. C., Parker, A. E., Sutton, S. E., Walker, J. R., Flaveny, C. A., Perdew, G. H., Denison, M. S. et al. (2010) 'Aryl hydrocarbon receptor antagonists promote the expansion of human hematopoietic stem cells', *Science* 329(5997): 1345-8.

Bray, S. J. (2006) 'Notch signalling: a simple pathway becomes complex', *Nat Rev Mol Cell Biol* 7(9): 678-89.

Brennan, C., Momota, H., Hambardzumyan, D., Ozawa, T., Tandon, A., Pedraza, A. and Holland, E. (2009) 'Glioblastoma subclasses can be defined by activity among signal transduction pathways and associated genomic alterations', *PLoS One* 4(11): e7752.

Brou, C., Logeat, F., Gupta, N., Bessia, C., LeBail, O., Doedens, J. R., Cumano, A., Roux, P., Black, R. A. and Israel, A. (2000) 'A novel proteolytic cleavage involved in Notch signaling: the role of the disintegrin-metalloprotease TACE', *Mol Cell* 5(2): 207-16.

Broudy, V. C. (1997) 'Stem cell factor and hematopoiesis', *Blood* 90(4): 1345-64.

Chadwick, N., Zeef, L., Portillo, V., Fennessy, C., Warrander, F., Hoyle, S. and Buckle, A. M. (2009) 'Identification of novel Notch target genes in T cell leukaemia', *Mol Cancer* 8: 35.

Chan, S. M., Weng, A. P., Tibshirani, R., Aster, J. C. and Utz, P. J. (2007) 'Notch signals positively regulate activity of the mTOR pathway in T-cell acute lymphoblastic leukemia', *Blood* 110(1): 278-86.

Chiba, S. (2006) 'Notch signaling in stem cell systems', *Stem Cells* 24(11): 2437-47.

Chiu, P. P., Jiang, H. and Dick, J. E. (2010) 'Leukemia-initiating cells in human T-lymphoblastic leukemia exhibit glucocorticoid resistance', *Blood* 116(24): 5268-79.

Clappier, E., Gerby, B., Sigaux, F., Delord, M., Touzri, F., Hernandez, L., Ballerini, P., Baruchel, A., Pflumio, F. and Soulier, J. (2011) 'Clonal selection in xenografted human T cell acute lymphoblastic leukemia recapitulates gain of malignancy at relapse', *J Exp Med* 208(4): 653-61.

Cox, C. V., Evely, R. S., Oakhill, A., Pamphilon, D. H., Goulden, N. J. and Blair, A. (2004) 'Characterization of acute lymphoblastic leukemia progenitor cells', *Blood* 104(9): 2919-25.

Cox, C. V., Martin, H. M., Kearns, P. R., Virgo, P., Evely, R. S. and Blair, A. (2007) 'Characterization of a progenitor cell population in childhood T-cell acute lymphoblastic leukemia', *Blood* 109(2): 674-82.

D'Souza, B., Meloty-Kapella, L. and Weinmaster, G. (2010) 'Canonical and non-canonical Notch ligands', *Curr Top Dev Biol* 92: 73-129.

Dallas, M. H., Varnum-Finney, B., Martin, P. J. and Bernstein, I. D. (2007) 'Enhanced T-cell reconstitution by hematopoietic progenitors expanded ex vivo using the Notch ligand Delta1', *Blood* 109(8): 3579-87.

Damiano, J. S., Hazlehurst, L. A. and Dalton, W. S. (2001) 'Cell adhesion-mediated drug resistance (CAM-DR) protects the K562 chronic myelogenous leukemia cell line from apoptosis induced by BCR/ABL inhibition, cytotoxic drugs, and gamma-irradiation', *Leukemia* 15(8): 1232-9.

Dang, T. P., Eichenberger, S., Gonzalez, A., Olson, S. and Carbone, D. P. (2003) 'Constitutive activation of Notch3 inhibits terminal epithelial differentiation in lungs of transgenic mice', *Oncogene* 22(13): 1988-97.

Dang, T. P., Gazdar, A. F., Virmani, A. K., Sepetavec, T., Hande, K. R., Minna, J. D., Roberts, J. R. and Carbone, D. P. (2000) 'Chromosome 19 translocation, overexpression of Notch3, and human lung cancer', *J Natl Cancer Inst* 92(16): 1355-7.

De Keersmaecker, K., Marynen, P. and Cools, J. (2005) 'Genetic insights in the pathogenesis of T-cell acute lymphoblastic leukemia', *Haematologica* 90(8): 1116-27.

de la Fuente, M. T., Casanova, B., Garcia-Gila, M., Silva, A. and Garcia-Pardo, A. (1999) 'Fibronectin interaction with alpha4beta1 integrin prevents apoptosis in B cell chronic lymphocytic leukemia: correlation with Bcl-2 and Bax', *Leukemia* 13(2): 266-74.

De Meyts, P. (2004) 'Insulin and its receptor: structure, function and evolution', *Bioessays* 26(12): 1351-62.

Ellisen, L. W., Bird, J., West, D. C., Soreng, A. L., Reynolds, T. C., Smith, S. D. and Sklar, J. (1991) 'TAN-1, the human homolog of the Drosophila notch gene, is broken by chromosomal translocations in T lymphoblastic neoplasms', *Cell* 66(4): 649-61.

Fabbri, G., Rasi, S., Rossi, D., Trifonov, V., Khiabanian, H., Ma, J., Grunn, A., Fangazio, M., Capello, D., Monti, S. et al. (2011) 'Analysis of the chronic lymphocytic leukemia coding genome: role of NOTCH1 mutational activation', *J Exp Med* 208(7): 1389-401.

Faderl, S., O'Brien, S., Pui, C. H., Stock, W., Wetzler, M., Hoelzer, D. and Kantarjian, H. M. (2010) 'Adult acute lymphoblastic leukemia: concepts and strategies', *Cancer* 116(5): 1165-76.

Fehon, R. G., Kooh, P. J., Rebay, I., Regan, C. L., Xu, T., Muskavitch, M. A. and Artavanis-Tsakonas, S. (1990) 'Molecular interactions between the protein products of the neurogenic loci Notch and Delta, two EGF-homologous genes in Drosophila', *Cell* 61(3): 523-34.

Felli, M. P., Maroder, M., Mitsiadis, T. A., Campese, A. F., Bellavia, D., Vacca, A., Mann, R. S., Frati, L., Lendahl, U., Gulino, A. et al. (1999) 'Expression pattern of notch1, 2 and 3 and Jagged1 and 2 in lymphoid and stromal thymus components: distinct ligand-receptor interactions in intrathymic T cell development', *Int Immunol* 11(7): 1017-25.

Ferrando, A. A. (2009) 'The role of NOTCH1 signaling in T-ALL', *Hematology Am Soc Hematol Educ Program*: 353-61.

Ferrari-Toninelli, G., Bonini, S. A., Uberti, D., Buizza, L., Bettinsoli, P., Poliani, P. L., Facchetti, F. and Memo, M. (2010) 'Targeting Notch pathway induces growth inhibition and differentiation of neuroblastoma cells', *Neuro Oncol* 12(12): 1231-43.

Fortini, M. E. (2002) 'Gamma-secretase-mediated proteolysis in cell-surface-receptor signalling', *Nat Rev Mol Cell Biol* 3(9): 673-84.

Frese, K. K. and Tuveson, D. A. (2007) 'Maximizing mouse cancer models', *Nat Rev Cancer* 7(9): 645-58.

Fryer, C. J., Lamar, E., Turbachova, I., Kintner, C. and Jones, K. A. (2002) 'Mastermind mediates chromatin-specific transcription and turnover of the Notch enhancer complex', *Genes Dev* 16(11): 1397-411.

Fryer, C. J., White, J. B. and Jones, K. A. (2004) 'Mastermind recruits CycC:CDK8 to phosphorylate the Notch ICD and coordinate activation with turnover', *Mol Cell* 16(4): 509-20.

Gerrits, A., Dykstra, B., Kalmykowa, O. J., Klauke, K., Verovskaya, E., Broekhuis, M. J., de Haan, G. and Bystrykh, L. V. (2010) 'Cellular barcoding tool for clonal analysis in the hematopoietic system', *Blood* 115(13): 2610-8.

Goldstone, A. H., Richards, S. M., Lazarus, H. M., Tallman, M. S., Buck, G., Fielding, A. K., Burnett, A. K., Chopra, R., Wiernik, P. H., Foroni, L. et al. (2008) 'In adults with standard-risk acute lymphoblastic leukemia, the greatest benefit is achieved from a matched sibling allogeneic transplantation in first complete remission, and an autologous transplantation is less effective than conventional consolidation/maintenance chemotherapy in all patients: final results of the International ALL Trial (MRC UKALL XII/ECOG E2993)', *Blood* 111(4): 1827-33.

Grabher, C., von Boehmer, H. and Look, A. T. (2006) 'Notch 1 activation in the molecular pathogenesis of T-cell acute lymphoblastic leukaemia', *Nat Rev Cancer* 6(5): 347-59.

Greshock, J., Nathanson, K., Martin, A. M., Zhang, L., Coukos, G., Weber, B. L. and Zaks, T. Z. (2007) 'Cancer cell lines as genetic models of their parent histology: analyses based on array comparative genomic hybridization', *Cancer Res* 67(8): 3594-600.

Gu, L., Zhou, C., Liu, H., Gao, J., Li, Q., Mu, D. and Ma, Z. (2010) 'Rapamycin sensitizes T-ALL cells to dexamethasone-induced apoptosis', *J Exp Clin Cancer Res* 29: 150.

Habibi, D., Ogloff, N., Jalili, R. B., Yost, A., Weng, A. P., Ghahary, A. and Ong, C. J. (2011) 'Borrelidin, a small molecule nitrile-containing macrolide inhibitor of threonyl-tRNA synthetase, is a potent inducer of apoptosis in acute lymphoblastic leukemia', *Invest New Drugs*.

Hainaud, P., Contreres, J. O., Villemain, A., Liu, L. X., Plouet, J., Tobelem, G. and Dupuy, E. (2006) 'The role of the vascular endothelial growth factor-Delta-like 4 ligand/Notch4-ephrin B2 cascade in tumor vessel remodeling and endothelial cell functions', *Cancer Res* 66(17): 8501-10.

Haines, N. and Irvine, K. D. (2003) 'Glycosylation regulates Notch signalling', *Nat Rev Mol Cell Biol* 4(10): 786-97.

Hankinson, O. (1995) 'The aryl hydrocarbon receptor complex', *Annu Rev Pharmacol Toxicol* 35: 307-40.

Hazlehurst, L. A. and Dalton, W. S. (2001) 'Mechanisms associated with cell adhesion mediated drug resistance (CAM-DR) in hematopoietic malignancies', *Cancer Metastasis Rev* 20(1-2): 43-50.

Heddleston, J. M., Li, Z., Lathia, J. D., Bao, S., Hjelmeland, A. B. and Rich, J. N. (2010) 'Hypoxia inducible factors in cancer stem cells', *Br J Cancer* 102(5): 789-95.

Hermann, P. C., Huber, S. L., Herrler, T., Aicher, A., Ellwart, J. W., Guba, M., Bruns, C. J. and Heeschen, C. (2007) 'Distinct populations of cancer stem cells determine tumor growth and metastatic activity in human pancreatic cancer', *Cell Stem Cell* 1(3): 313-23.

Hernandez, C. P., Morrow, K., Lopez-Barcons, L. A., Zabaleta, J., Sierra, R., Velasco, C., Cole, J. and Rodriguez, P. C. (2010) 'Pegylated arginase I: a potential therapeutic approach in T-ALL', *Blood* 115(25): 5214-21.

Hozumi, K., Mailhos, C., Negishi, N., Hirano, K., Yahata, T., Ando, K., Zuklys, S., Hollander, G. A., Shima, D. T. and Habu, S. (2008) 'Delta-like 4 is indispensable in thymic environment specific for T cell development', *J Exp Med* 205(11): 2507-13.

Ince, T. A., Richardson, A. L., Bell, G. W., Saitoh, M., Godar, S., Karnoub, A. E., Iglehart, J. D. and Weinberg, R. A. (2007) 'Transformation of different human breast epithelial cell types leads to distinct tumor phenotypes', *Cancer Cell* 12(2): 160-70.

Itoh, K., Tezuka, H., Sakoda, H., Konno, M., Nagata, K., Uchiyama, T., Uchino, H. and Mori, K. J. (1989) 'Reproducible establishment of hemopoietic supportive stromal cell lines from murine bone marrow', *Exp Hematol* 17(2): 145-53.

Jemal, A., Siegel, R., Ward, E., Murray, T., Xu, J., Smigal, C. and Thun, M. J. (2006) 'Cancer statistics, 2006', *CA Cancer J Clin* 56(2): 106-30.

Karawajew, L., Ruppert, V., Wuchter, C., Kosser, A., Schrappe, M., Dorken, B. and Ludwig, W. D. (2000) 'Inhibition of in vitro spontaneous apoptosis by IL-7 correlates with bcl-2 up-regulation, cortical/mature immunophenotype, and better early cytoreduction of childhood T-cell acute lymphoblastic leukemia', *Blood* 96(1): 297-306.

Kaspers, G. J., Pieters, R., Klumper, E., De Waal, F. C. and Veerman, A. J. (1994) 'Glucocorticoid resistance in childhood leukemia', *Leuk Lymphoma* 13(3-4): 187-201.

Kim, H. P., Imbert, J. and Leonard, W. J. (2006) 'Both integrated and differential regulation of components of the IL-2/IL-2 receptor system', *Cytokine Growth Factor Rev* 17(5): 349-66.

Klinakis, A., Lobry, C., Abdel-Wahab, O., Oh, P., Haeno, H., Buonamici, S., van De Walle, I., Cathelin, S., Trimarchi, T., Araldi, E. et al. (2011) 'A novel tumour-suppressor function for the Notch pathway in myeloid leukaemia', *Nature* 473(7346): 230-3.

Koch, U., Fiorini, E., Benedito, R., Besseyrias, V., Schuster-Gossler, K., Pierres, M., Manley, N. R., Duarte, A., Macdonald, H. R. and Radtke, F. (2008) 'Delta-like 4 is the essential, nonredundant ligand for Notch1 during thymic T cell lineage commitment', *J Exp Med* 205(11): 2515-23.

Koch, U. and Radtke, F. (2011) 'Notch in T-ALL: new players in a complex disease', *Trends Immunol* 32(9): 434-42.

Kridel, R., Meissner, B., Rogic, S., Boyle, M., Telenius, A., Woolcock, B., Gunawardana, J., Jenkins, C., Cochrane, C., Ben-Neriah, S. et al. (2012) 'Whole transcriptome sequencing reveals recurrent NOTCH1 mutations in mantle cell lymphoma', *Blood*.

Kumano, K., Chiba, S., Kunisato, A., Sata, M., Saito, T., Nakagami-Yamaguchi, E., Yamaguchi, T., Masuda, S., Shimizu, K., Takahashi, T. et al. (2003) 'Notch1 but not Notch2 is essential for generating hematopoietic stem cells from endothelial cells', *Immunity* 18(5): 699-711.

Le Borgne, R., Bardin, A. and Schweisguth, F. (2005) 'The roles of receptor and ligand endocytosis in regulating Notch signaling', *Development* 132(8): 1751-62.

Leonard, W. J., Depper, J. M., Crabtree, G. R., Rudikoff, S., Pumphrey, J., Robb, R. J., Kronke, M., Svetlik, P. B., Peffer, N. J., Waldmann, T. A. et al. (1984) 'Molecular cloning and expression of cDNAs for the human interleukin-2 receptor', *Nature* 311(5987): 626-31.

LeRoith, D. (2000) 'Insulin-like growth factor I receptor signaling--overlapping or redundant pathways?', *Endocrinology* 141(4): 1287-8.

Lin, J. X. and Leonard, W. J. (2000) 'The role of Stat5a and Stat5b in signaling by IL-2 family cytokines', *Oncogene* 19(21): 2566-76.

Lipshultz, S. E., Scully, R. E., Lipsitz, S. R., Sallan, S. E., Silverman, L. B., Miller, T. L., Barry, E. V., Asselin, B. L., Athale, U., Clavell, L. A. et al. (2010) 'Assessment of dexrazoxane as a cardioprotectant in doxorubicin-treated children with high-risk acute lymphoblastic leukaemia: long-term follow-up of a prospective, randomised, multicentre trial', *Lancet Oncol* 11(10): 950-61.

Logeat, F., Bessia, C., Brou, C., LeBail, O., Jarriault, S., Seidah, N. G. and Israel, A. (1998) 'The Notch1 receptor is cleaved constitutively by a furin-like convertase', *Proc Natl Acad Sci U S A* 95(14): 8108-12.

Lowenberg, B. and Touw, I. P. (1986) 'Interleukin 2: its role in the proliferation of neoplastic T and B cells', *J Pathol* 149(1): 15-21.

Lundell, B. I., McCarthy, J. B., Kovach, N. L. and Verfaillie, C. M. (1996) 'Activation-dependent alpha5beta1 integrin-mediated adhesion to fibronectin decreases proliferation of chronic myelogenous leukemia progenitors and K562 cells', *Blood* 87(6): 2450-8.

Ma, F., Manabe, A., Wang, D., Ito, M., Kikuchi, A., Wada, M., Ohara, A., Hosoya, R., Asano, S. and Tsuji, K. (2002) 'Growth of human T cell acute lymphoblastic leukemia lymphoblasts in NOD/SCID mouse fetal thymus organ culture', *Leukemia* 16(8): 1541-8.

Makrynikola, V., Kabral, A. and Bradstock, K. (1991) 'Effects of interleukin 7 on the growth of clonogenic cells in T-cell acute lymphoblastic leukaemia', *Leuk Res* 15(10): 879-82.

Malecki, M. J., Sanchez-Irizarry, C., Mitchell, J. L., Histen, G., Xu, M. L., Aster, J. C. and Blacklow, S. C. (2006) 'Leukemia-associated mutations within the NOTCH1 heterodimerization domain fall into at least two distinct mechanistic classes', *Mol Cell Biol* 26(12): 4642-51.

Medyouf, H., Gao, X., Armstrong, F., Gusscott, S., Liu, Q., Gedman, A. L., Matherly, L. H., Schultz, K. R., Pflumio, F., You, M. J. et al. (2010) 'Acute T-cell leukemias remain dependent on Notch signaling despite PTEN and INK4A/ARF loss', *Blood* 115(6): 1175-84.

Medyouf, H., Gusscott, S., Wang, H., Tseng, J. C., Wai, C., Nemirovsky, O., Trumpp, A., Pflumio, F., Carboni, J., Gottardis, M. et al. (2011) 'High-level IGF1R expression is required for leukemia-initiating cell activity in T-ALL and is supported by Notch signaling', *J Exp Med* 208(9): 1809-22.

Mirandola, L., Chiriva-Internati, M., Montagna, D., Locatelli, F., Zecca, M., Ranzani, M., Basile, A., Locati, M., Cobos, E., Kast, W. M. et al. (2011) 'Notch1 regulates chemotaxis and proliferation by controlling the CC-chemokine receptors 5 and 9 in T cell acute lymphoblastic leukaemia', *J Pathol*.

Miyamoto, Y., Maitra, A., Ghosh, B., Zechner, U., Argani, P., Iacobuzio-Donahue, C. A., Sriuranpong, V., Iso, T., Meszoely, I. M., Wolfe, M. S. et al. (2003) 'Notch mediates TGF alpha-induced changes in epithelial differentiation during pancreatic tumorigenesis', *Cancer Cell* 3(6): 565-76.

Mohr, O. L. (1919) 'Character Changes Caused by Mutation of an Entire Region of a Chromosome in Drosophila', *Genetics* 4(3): 275-82.

Mohtashami, M., Shah, D. K., Nakase, H., Kianizad, K., Petrie, H. T. and Zuniga-Pflucker, J. C. (2010) 'Direct comparison of Dll1- and Dll4-mediated Notch activation levels shows differential lymphomyeloid lineage commitment outcomes', *J Immunol* 185(2): 867-76.

Mohtashami, M. and Zuniga-Pflucker, J. C. (2006) 'Three-dimensional architecture of the thymus is required to maintain delta-like expression necessary for inducing T cell development', *J Immunol* 176(2): 730-4.

Morgan, D. A., Ruscetti, F. W. and Gallo, R. (1976) 'Selective in vitro growth of T lymphocytes from normal human bone marrows', *Science* 193(4257): 1007-8.

Namen, A. E., Lupton, S., Hjerrild, K., Wignall, J., Mochizuki, D. Y., Schmierer, A., Mosley, B., March, C. J., Urdal, D. and Gillis, S. (1988) 'Stimulation of B-cell progenitors by cloned murine interleukin-7', *Nature* 333(6173): 571-3.

Nefedova, Y., Landowski, T. H. and Dalton, W. S. (2003) 'Bone marrow stromal-derived soluble factors and direct cell contact contribute to de novo drug resistance of myeloma cells by distinct mechanisms', *Leukemia* 17(6): 1175-82.

Nguyen, B. C., Lefort, K., Mandinova, A., Antonini, D., Devgan, V., Della Gatta, G., Koster, M. I., Zhang, Z., Wang, J., Tommasi di Vignano, A. et al. (2006) 'Cross-

regulation between Notch and p63 in keratinocyte commitment to differentiation', *Genes Dev* 20(8): 1028-42.

Nishino, T., Hisha, H., Nishino, N., Adachi, M. and Ikehara, S. (1995) 'Hepatocyte growth factor as a hematopoietic regulator', *Blood* 85(11): 3093-100.

Nunez, G., Benedict, M. A., Hu, Y. and Inohara, N. (1998) 'Caspases: the proteases of the apoptotic pathway', *Oncogene* 17(25): 3237-45.

O'Brien, C. A., Pollett, A., Gallinger, S. and Dick, J. E. (2007) 'A human colon cancer cell capable of initiating tumour growth in immunodeficient mice', *Nature* 445(7123): 106-10.

O'Connor, R., Cesano, A., Lange, B., Finan, J., Nowell, P. C., Clark, S. C., Raimondi, S. C., Rovera, G. and Santoli, D. (1991) 'Growth factor requirements of childhood acute T-lymphoblastic leukemia: correlation between presence of chromosomal abnormalities and ability to grow permanently in vitro', *Blood* 77(7): 1534-45.

O'Neil, J., Grim, J., Strack, P., Rao, S., Tibbitts, D., Winter, C., Hardwick, J., Welcker, M., Meijerink, J. P., Pieters, R. et al. (2007) 'FBW7 mutations in leukemic cells mediate NOTCH pathway activation and resistance to gamma-secretase inhibitors', *J Exp Med* 204(8): 1813-24.

Oberg, C., Li, J., Pauley, A., Wolf, E., Gurney, M. and Lendahl, U. (2001) 'The Notch intracellular domain is ubiquitinated and negatively regulated by the mammalian Sel-10 homolog', *J Biol Chem* 276(38): 35847-53.

Palomero, T., Sulis, M. L., Cortina, M., Real, P. J., Barnes, K., Ciofani, M., Caparros, E., Buteau, J., Brown, K., Perkins, S. L. et al. (2007) 'Mutational loss of PTEN induces resistance to NOTCH1 inhibition in T-cell leukemia', *Nat Med* 13(10): 1203-10.

Papayannopoulou, T., Brice, M., Broudy, V. C. and Zsebo, K. M. (1991) 'Isolation of c-kit receptor-expressing cells from bone marrow, peripheral blood, and fetal liver: functional properties and composite antigenic profile', *Blood* 78(6): 1403-12.

Parish, C. R., Glidden, M. H., Quah, B. J. and Warren, H. S. (2009) 'Use of the intracellular fluorescent dye CFSE to monitor lymphocyte migration and proliferation', *Curr Protoc Immunol* Chapter 4: Unit4 9.

Park, J. T., Li, M., Nakayama, K., Mao, T. L., Davidson, B., Zhang, Z., Kurman, R. J., Eberhart, C. G., Shih Ie, M. and Wang, T. L. (2006) 'Notch3 gene amplification in ovarian cancer', *Cancer Res* 66(12): 6312-8.

Parks, A. L., Klueg, K. M., Stout, J. R. and Muskavitch, M. A. (2000) 'Ligand endocytosis drives receptor dissociation and activation in the Notch pathway', *Development* 127(7): 1373-85.

Pear, W. S. and Aster, J. C. (2004) 'T cell acute lymphoblastic leukemia/lymphoma: a human cancer commonly associated with aberrant NOTCH1 signaling', *Curr Opin Hematol* 11(6): 426-33.

Pollak, M. (2008) 'Insulin and insulin-like growth factor signalling in neoplasia', *Nat Rev Cancer* 8(12): 915-28.

Proweller, A., Tu, L., Lepore, J. J., Cheng, L., Lu, M. M., Seykora, J., Millar, S. E., Pear, W. S. and Parmacek, M. S. (2006) 'Impaired notch signaling promotes de novo squamous cell carcinoma formation', *Cancer Res* 66(15): 7438-44.

Puente, X. S., Pinyol, M., Quesada, V., Conde, L., Ordonez, G. R., Villamor, N., Escaramis, G., Jares, P., Bea, S., Gonzalez-Diaz, M. et al. (2011) 'Whole-genome sequencing identifies recurrent mutations in chronic lymphocytic leukaemia', *Nature* 475(7354): 101-5.

Pui, C. H. (2006) 'Central nervous system disease in acute lymphoblastic leukemia: prophylaxis and treatment', *Hematology Am Soc Hematol Educ Program*: 142-6.

Pui, C. H. and Evans, W. E. (2006) 'Treatment of acute lymphoblastic leukemia', *N Engl J Med* 354(2): 166-78.

Radtke, F., Wilson, A., Mancini, S. J. and MacDonald, H. R. (2004) 'Notch regulation of lymphocyte development and function', *Nat Immunol* 5(3): 247-53.

Raimondi, S. C. (1993) 'Current status of cytogenetic research in childhood acute lymphoblastic leukemia', *Blood* 81(9): 2237-51.

Rangarajan, A., Talora, C., Okuyama, R., Nicolas, M., Mammucari, C., Oh, H., Aster, J. C., Krishna, S., Metzger, D., Chambon, P. et al. (2001) 'Notch signaling is a direct determinant of keratinocyte growth arrest and entry into differentiation', *EMBO J* 20(13): 3427-36.

Reedijk, M., Odorcic, S., Chang, L., Zhang, H., Miller, N., McCready, D. R., Lockwood, G. and Egan, S. E. (2005) 'High-level coexpression of JAG1 and NOTCH1 is observed in human breast cancer and is associated with poor overall survival', *Cancer Res* 65(18): 8530-7.

Reizis, B. and Leder, P. (2002) 'Direct induction of T lymphocyte-specific gene expression by the mammalian Notch signaling pathway', *Genes Dev* 16(3): 295-300.

Rosati, E., Sabatini, R., Rampino, G., Tabilio, A., Di Ianni, M., Fettucciari, K., Bartoli, A., Coaccioli, S., Screpanti, I. and Marconi, P. (2009) 'Constitutively activated Notch signaling is involved in survival and apoptosis resistance of B-CLL cells', *Blood* 113(4): 856-65.

Saito, T., Chiba, S., Ichikawa, M., Kunisato, A., Asai, T., Shimizu, K., Yamaguchi, T., Yamamoto, G., Seo, S., Kumano, K. et al. (2003) 'Notch2 is preferentially expressed in mature B cells and indispensable for marginal zone B lineage development', *Immunity* 18(5): 675-85.

Santagata, S., Demichelis, F., Riva, A., Varambally, S., Hofer, M. D., Kutok, J. L., Kim, R., Tang, J., Montie, J. E., Chinnaiyan, A. M. et al. (2004) 'JAGGED1 expression is associated with prostate cancer metastasis and recurrence', *Cancer Res* 64(19): 6854-7.

Schmitt, T. M., Ciofani, M., Petrie, H. T. and Zuniga-Pflucker, J. C. (2004) 'Maintenance of T cell specification and differentiation requires recurrent notch receptor-ligand interactions', *J Exp Med* 200(4): 469-79.

Scupoli, M. T., Perbellini, O., Krampera, M., Vinante, F., Cioffi, F. and Pizzolo, G. (2007) 'Interleukin 7 requirement for survival of T-cell acute lymphoblastic leukemia and human thymocytes on bone marrow stroma', *Haematologica* 92(2): 264-6.

Scupoli, M. T., Vinante, F., Krampera, M., Vincenzi, C., Nadali, G., Zampieri, F., Ritter, M. A., Eren, E., Santini, F. and Pizzolo, G. (2003) 'Thymic epithelial cells promote survival of human T-cell acute lymphoblastic leukemia blasts: the role of interleukin-7', *Haematologica* 88(11): 1229-37.

Silverman, L. B., Gelber, R. D., Dalton, V. K., Asselin, B. L., Barr, R. D., Clavell, L. A., Hurwitz, C. A., Moghrabi, A., Samson, Y., Schorin, M. A. et al. (2001) 'Improved outcome for children with acute lymphoblastic leukemia: results of Dana-Farber Consortium Protocol 91-01', *Blood* 97(5): 1211-8.

Singh, S. K., Hawkins, C., Clarke, I. D., Squire, J. A., Bayani, J., Hide, T., Henkelman, R. M., Cusimano, M. D. and Dirks, P. B. (2004) 'Identification of human brain tumour initiating cells', *Nature* 432(7015): 396-401.

Styczynski, J., Pieters, R., Huismans, D. R., Schuurhuis, G. J., Wysocki, M. and Veerman, A. J. (2000) 'In vitro drug resistance profiles of adult versus childhood acute lymphoblastic leukaemia', *Br J Haematol* 110(4): 813-8.

Sutherland, H. J., Blair, A. and Zapf, R. W. (1996) 'Characterization of a hierarchy in human acute myeloid leukemia progenitor cells', *Blood* 87(11): 4754-61.

Suzuki, J., Fujita, J., Taniguchi, S., Sugimoto, K. and Mori, K. J. (1992) 'Characterization of murine hemopoietic-supportive (MS-1 and MS-5) and non-supportive (MS-K) cell lines', *Leukemia* 6(5): 452-8.

Swanson, H. I. (2002) 'DNA binding and protein interactions of the AHR/ARNT heterodimer that facilitate gene activation', *Chem Biol Interact* 141(1-2): 63-76.

Taniguchi, T., Matsui, H., Fujita, T., Takaoka, C., Kashima, N., Yoshimoto, R. and Hamuro, J. (1983) 'Structure and expression of a cloned cDNA for human interleukin-2', *Nature* 302(5906): 305-10.

Thompson, B. J., Buonamici, S., Sulis, M. L., Palomero, T., Vilimas, T., Basso, G., Ferrando, A. and Aifantis, I. (2007) 'The SCFFBW7 ubiquitin ligase complex as a tumor suppressor in T cell leukemia', *J Exp Med* 204(8): 1825-35.

Ullrich, A. and Schlessinger, J. (1990) 'Signal transduction by receptors with tyrosine kinase activity', *Cell* 61(2): 203-12.

Van Vlierberghe, P., Pieters, R., Beverloo, H. B. and Meijerink, J. P. (2008) 'Molecular-genetic insights in paediatric T-cell acute lymphoblastic leukaemia', *Br J Haematol* 143(2): 153-68.

Varnum-Finney, B., Brashem-Stein, C. and Bernstein, I. D. (2003) 'Combined effects of Notch signaling and cytokines induce a multiple log increase in precursors with lymphoid and myeloid reconstituting ability', *Blood* 101(5): 1784-9.

Varnum-Finney, B., Xu, L., Brashem-Stein, C., Nourigat, C., Flowers, D., Bakkour, S., Pear, W. S. and Bernstein, I. D. (2000) 'Pluripotent, cytokine-dependent, hematopoietic stem cells are immortalized by constitutive Notch1 signaling', *Nat Med* 6(11): 1278-81.

Wang, G. L. and Semenza, G. L. (1995) 'Purification and characterization of hypoxia-inducible factor 1', *J Biol Chem* 270(3): 1230-7.

Wang, N. J., Sanborn, Z., Arnett, K. L., Bayston, L. J., Liao, W., Proby, C. M., Leigh, I. M., Collisson, E. A., Gordon, P. B., Jakkula, L. et al. (2011) 'Loss-of-function mutations

in Notch receptors in cutaneous and lung squamous cell carcinoma', *Proc Natl Acad Sci U S A* 108(43): 17761-6.

Weng, A. P., Ferrando, A. A., Lee, W., Morris, J. P. t., Silverman, L. B., Sanchez-Irizarry, C., Blacklow, S. C., Look, A. T. and Aster, J. C. (2004) 'Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia', *Science* 306(5694): 269-71.

Weng, A. P., Millholland, J. M., Yashiro-Ohtani, Y., Arcangeli, M. L., Lau, A., Wai, C., Del Bianco, C., Rodriguez, C. G., Sai, H., Tobias, J. et al. (2006) 'c-Myc is an important direct target of Notch1 in T-cell acute lymphoblastic leukemia/lymphoma', *Genes Dev* 20(15): 2096-109.

Weng, A. P., Nam, Y., Wolfe, M. S., Pear, W. S., Griffin, J. D., Blacklow, S. C. and Aster, J. C. (2003) 'Growth suppression of pre-T acute lymphoblastic leukemia cells by inhibition of notch signaling', *Mol Cell Biol* 23(2): 655-64.

Wharton, K. A., Johansen, K. M., Xu, T. and Artavanis-Tsakonas, S. (1985) 'Nucleotide sequence from the neurogenic locus notch implies a gene product that shares homology with proteins containing EGF-like repeats', *Cell* 43(3 Pt 2): 567-81.

Wullschleger, S., Loewith, R. and Hall, M. N. (2006) 'TOR signaling in growth and metabolism', *Cell* 124(3): 471-84.

Xiang, J., Ouyang, Y., Cui, Y., Lin, F., Ren, J., Long, M., Chen, X., Wei, J. and Zhang, H. (2011) 'Silencing of Notch3 Using shRNA Driven by Survivin Promoter Inhibits Growth and Promotes Apoptosis of Human T-Cell Acute Lymphoblastic Leukemia Cells', *Clin Lymphoma Myeloma Leuk*.

Xu, X., Zhao, Y., Xu, M., Dai, Q., Meng, W., Yang, J. and Qin, R. (2011) 'Activation of Notch signal pathway is associated with a poorer prognosis in acute myeloid leukemia', *Med Oncol* 28 Suppl 1: S483-9.

Zage, P. E., Nolo, R., Fang, W., Stewart, J., Garcia-Manero, G. and Zweidler-McKay, P. A. (2011) 'Notch pathway activation induces neuroblastoma tumor cell growth arrest', *Pediatr Blood Cancer*.

Zhu, J. and Emerson, S. G. (2002) 'Hematopoietic cytokines, transcription factors and lineage commitment', *Oncogene* 21(21): 3295-313.

Zweidler-McKay, P. A., He, Y., Xu, L., Rodriguez, C. G., Karnell, F. G., Carpenter, A. C., Aster, J. C., Allman, D. and Pear, W. S. (2005) 'Notch signaling is a potent inducer of

growth arrest and apoptosis in a wide range of B-cell malignancies', *Blood* 106(12): 3898-906.