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Deepika Kaveri

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Deepika Kaveri. Study of the role of Wnt pathway in a murine model of T-ALL. Biochemistry, Molecular Biology. Université de Strasbourg, 2012. English. NNT : 2012STRAJ061 . tel-00912330

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**ÉCOLE DOCTORALE
DES SCIENCES DE LA VIE ET DE LA SANTE
IGBMC-CNRS(UMR7104) -INSERM(U964)**

THÈSE présentée par :
Deepika KAVERI

soutenue le : **21 Septembre 2012**

pour obtenir le grade de : **Docteur de l'université de Strasbourg**

Discipline/ Spécialité : Aspects Moléculaires et Cellulaires de la
Biologie

***Study of the role of the Wnt pathway
in a murine model of T-ALL***

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Acknowledgements

It would not have been possible to write this doctoral thesis without the help and support of the kind people around me, to only some of whom it is possible to give particular mention here.

I would like to thank;

Foremost, Philippe KASTNER and Susan CHAN for being amazing mentors. Thank you for having faith in me and recognising the scientific potential within me, for all the dinners and barbeques I have enjoyed so much.

Peggy, for being a guide and a friend for these four years. Thank you for being patient and for helping me grow as a scientist.

Professor Eric SO, Dr. Jacques GHYSDAEL and Dr. Hinrich GRONEMEYER for accepting, taking time to read and judge my doctoral thesis. A special thanks to Dr. GRONEMEYER for pleasant conversations which encouraged me.

Dr. GHYSDAEL, Dr. PFLUMIO and their lab members for being wonderful hosts and having active discussions during INCA meetings.

Patricia (the maharani), for being so lively and colourful! You're the best :) Thank you for all the yummy desserts, especially the poires aux vins!

Claudine, for teaching me how to use the LSR and the ARIA. Thank you for the amazing pain d'épices you made every year.

William MAGNANT, Alexandre VINCENT and Sylvie FALCONE, for the animal house help.

MacLean, Robin, Qi Cai and Anne-Solen, the past members of our lab; for being wonderful seniors.

The present lab members in our team with whom I have spent most enjoyable and memorable moments – Attila, Ale, Apos, Beate, Marie, Arnaud, Jérôme, Rose and Kate. I would also like to thank some other members who were a part of the lab – Ambroise, Dimitri, Roxanne, Michelle and Sophie, for bringing freshness to the lab.

ACKNOWLEDGEMENTS

Jérômou, Romarin and Kit-kat-Kate, for all the support, coffees, and other fun times we shared. Thank you. Little mommy will miss you all!!

Beate, for proof-reading my thesis, for all the pep talks and for making yummy baked goodies for JCs.

Shankar, for being more than ready to help me through these years and for all the encouragement.

Bernado, and his lab members – Iza, Ebe, Sara, Anne-Sophie and Léa, thank you for being the best neighbours!

Irene, Adrien, David, Thomas, Thibaud, Anne-So, Ghislain, Benjamin and Floriana for fun times and their help. I thank the SPB for accepting me as the control freak I am.

Izoo-bizooo, thank you so much for being an important part of my life in Strasbourg! Thank you for supporting me and helping me get through these years! I'll miss the pool-side talks and Castle-discussions! I'll miss you a lot Iza! xoxo

Ebe, thank you for being there for me; for the unlimited muffin-olive cake-tiramisu supply; for the smiles you brought...thank you! I'll miss you a lot! xoxo

Bee bee boo! Thank you for making everything around me comfortable, for all the dinners and lunches you cooked and baked for me, for being the best listener and thank you for being you!! xoxo

Panic, for being extremely encouraging and for inspiring me to pursue my doctoral study!

Sheenumama, thank you for being my moral support and for making this place home away from home.

I would like to thank all my cousins and friends scattered around the world, for keeping in touch with me through my stay in Strasbourg.

Lastly, I would like to thank my family – Mamma, Pappa and Chandrika for being my world and my grandparents, both Ajjas and Ajjis for their blessings.

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ABBREVIATIONS

Numbers

5-FU 5-Fluorouracil

A

ABC Adenosine triphosphate-binding transporter cassette

ALL Acute lymphoblastic leukaemia

AML Acute myelogenous leukaemia

APC Adenomatous polyposis coli

ATP Adenosine triphosphate

AXIN Axis Inhibitor

B

B-ALL B-cell acute lymphoblastic leukaemia

Bcl-2 B-cell lymphoma – 2

β -TRCP β -transduction-repeat-containing-protein

BCR-ABL Break point cluster-Ableson tyrosine kinase

BM Bone marrow

bp Base pair

C

CNA Copy number alterations

CBP Cyclic AMP-responsive-element-binding protein (CREB)-binding protein

CD Cluster of differentiation

CD44v CD44 variant isoforms

CFU Colony forming unit

ABBREVIATIONS

CGH	Comparative genomic hybridization
CIC	Cancer initiating cell
CK1	Caesin kinase 1
CLL	Chromic lymphoblastic leukaemia
CLP	Common lymphoid progenitor
CMP	Chronic myelogenous leukaemia
CMP	Common myeloid progenitor
CO	Cell of origin
CSC	Cancer stem cell
cTEC	Cortical thymic epithelial cells

D

DKK	Dickkopf
DMEM	Dulbecco's modified Eagle's medium
DN	Double negative
DNA	Deoxyribo nucleic acid
dn-MAML1	Dominant negative mastermind-like 1
dn-Tcf4	Dominant negative T-cell factor 4
DP	Double positive
Dsh	Dishevelled

E

EDTA	Ethylenediaminetetraacetate
EFS	Event-free survival
ELP	Early lymphoid progenitor
ETP	Early thymocyte progenitor

ABBREVIATIONS

F

- FACS Fluorescence-activated cell sorting
FBW7 F-box and WD repeat domain-containing 7
FCS Foetal calf serum
FISH Fluorescence in situ hybridization
FITC Fluorescein isothiocyanate
Fz Frizzled

G

- GC Glucocorticoid
GFP Green fluorescent protein
GMP Granulocyte monocyte progenitor
GPI Glycerophosphatidylinositol
Grg Groucho-related gene
GSK3 Glycogen synthase kinase 3

H

- HBS Hank's balanced salt solution
HCELL Haematopoietic cell E-/L-selectin ligand
HOX Homeobox
HSC Haematopoietic stem cell

I

- ICAT Catenin- β interacting protein 1
IF Intrafemoral
IMDM Iscove's modified Eagle's medium
IP Intraperitoneal
ISP Immature single positive

ABBREVIATIONS

IV Intravenous

L

LEF Lymphocyte enhancing factor

LIC Leukaemia-initiating cell

Lin Lineage

LMO LIM domain only

LRP Lipoprotein receptor-related proteins

LSC Leukaemia stem cell

LSK Lin-Sca-1⁺c-kit⁺

LT-HSC Long-term haematopoietic stem cell

M

Mcl-1 Myeloid leukaemia cell 1

MEP Megakaryocyte erythroid progenitor

MFI Mean fluorescence intensity

miRNA Micro Ribonucleic acid

MM Multiple myeloma

MPD Myeloproliferative disorder

MPP Multipotent progenitor

mTEC Medullary thymic epithelial cells

mTOR Mammalian target of rapamycin

N

NK Natural killer cells

NOD/ SCID Non-obese diabetic/ severe combined immunodeficiency

O

OD Optical density

ABBREVIATIONS

P

- PBS Phosphate buffer saline
- PDC Plasmacytoid dendritic cell
- PDK 3-phosphoinositide-dependent kinase
- PE Phycoerythrin
- PerCP Peridinin-chlorophyll proteins
- PI3K Phosphatidylinositol 3-kinase
- PIP2 Phosphatidylinositol-4,5, bisphosphate
- PIP3 Phosphatidylinositol-3,4,5 trisphosphate
- PS Penicillin-streptomycin
- PTEN Phosphatase and tensin homologue on chromosome 10
- pT α Pre-T-cell receptor alpha

R

- RAG Recombination activating gene
- RBC Red blood cells
- RNA Ribonucleic acid
- RPM Rotations per minute
- RPMI Roswell Park Memorial Institute medium
- RT Room temperature
- RTK Receptor tyrosine kinase
- RT-PCR Reverse transcriptase polymerase chain reaction
- RT-qPCR Reverse transcriptase- quantitative polymerase chain reaction

S

- Sca-1 Stem cell antigen 1

ABBREVIATIONS

SCFR Stem cell factor receptor
Scl Stem cell leukaemia
Ser Serine
sFRP Secreted Frizzled-related proteins
Shh Sonic hedgehog
SKY Spectral karyotyping
SLAM Signalling lymphocyte activation molecule
SP Single positive
Sp Side-population
Spl Spleen
ST-HSC Short-term haematopoietic stem cell

T

Tal-1 T-cell acute lymphoblastic leukaemia protein 1
T-ALL T-cell acute lymphoblastic leukaemia
Tcf T-cell factor
TCR T-cell receptor
Thr Threonine
Thy Thymus
TRITC Tetramethylrhodamine isothiocyanate

V

VEC VE-cadherin

W

WIF Wnt inhibitory factor
WT Wild type

Preface

The first year of my thesis work was focused on studying plasmacytoid dendritic cell development and differentiation in Ikaros-deficient mice (summary in appendix II). From the second year onwards, I switched to working on R26- β -cat mice leukaemogenesis and leukaemia stem cell activity. My thesis is a compilation of my work of the last three years.

I. INTRODUCTION

I.1 Haematopoiesis

All blood cells arise from haematopoietic stem cells (HSCs), which are one of the best characterized among stem cells. Stem cells are cells that can differentiate into other cell types and are self-renewing. Early cutting-edge transplantation studies showed that there exists a population of clonogenic bone marrow (BM) cells that could reconstitute all blood lineages when transferred into secondary hosts (Becker et al., 1963; Till and Mc, 1961; Wu et al., 1968). With advancement of clonal assays and flow cytometry, stem cell populations marked by Lin⁻Sca-1⁺c-kit⁺ (LSK), were isolated in the bone marrow (BM) of the mouse (Goodell et al., 1996; Osawa et al., 1996; Spangrude et al., 1988). Lineage (Lin) marks the cells that are committed to any of the haematopoietic cell types; stem cell antigen 1 (Sca-1), a glycerophosphatidylinositol (GPI)-linked surface protein and c-kit, a stem cell factor receptor (SCFR) are associated with maintaining stem cells functionality (Okada et al., 1992). Two classes of multipotent (a cell that can give rise to multiple cell types of a single lineage) stem cells namely, long-term HSCs (LT-HSCs) and short-term HSCs (ST-HSCs) were identified by *in vivo* limiting dilution assays of the clonogenic BM cells in mice (Morrison and Weissman, 1994; Osawa et al., 1996; Smith et al., 1991; Uchida and Weissman, 1992). Whereas LT-HSCs (LSK Thy-1^{lo}IL7R α ⁻Fli2⁻CD34^{-/lo}) were shown to self-renew for life, ST-HSCs (LSK Mac-1^{lo}Thy-1^{lo}IL7R α ⁻Fli2⁻CD34⁺) self-renewed for up to 8 weeks. LT-HSCs differentiate into ST-HSCs and ST-HSCs differentiate into differentiation-committed multipotent progenitors (MPPs) (Morrison et al., 1997a; Morrison and Weissman, 1994). The MPPs further differentiate into two oligolineage-restricted progenitors; the common lymphoid progenitors (CLPs) and the

I. INTRODUCTION

common myeloid progenitors (CMPs). CLPs are restricted to differentiate into T lymphocytes, B lymphocytes and natural killer (NK) cells (Kondo et al., 1997) while CMPs will differentiate into progenitors of myeloerythroid lineages; granulocyte-monocyte progenitors (GMPs) and megakaryocytic-erythroid progenitors (MEPs) (Fig 1.1). All these cell populations can be distinguished using cell surface markers (Akashi et al., 1999) (Table1.1).

I. INTRODUCTION

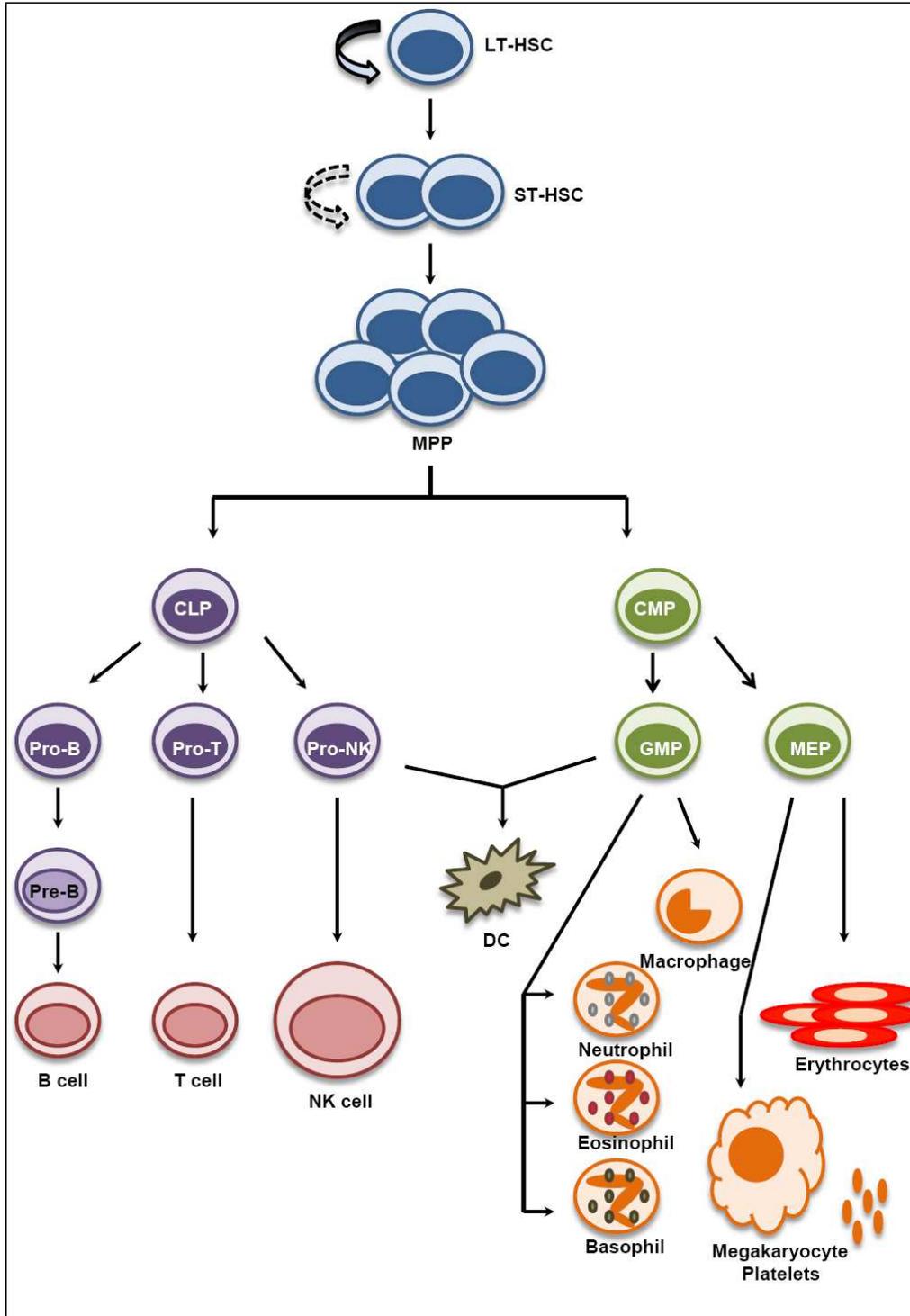


Figure 1.1. Simplified schematic representation of haematopoiesis. Adapted and modified from Larsson *et al* and Luc *et al* (Larsson and Karlsson, 2005; Luc *et al.*, 2008). LT-HSC, long term repopulating HSCs; ST-HSC, short term repopulating HSCs; MPP, multipotent progenitor; CMP, common myeloid progenitor; CLP, common lymphoid progenitor; GMP,

I. INTRODUCTION

granulocyte-monocyte progenitor and MEP, megakaryocyte-erythroid progenitor. Solid arrows indicate long-term self-renewal potential and dotted arrow indicates short-term self-renewal potential.

Table 1.1. Markers distinguishing progenitors of T-cell lineage.

LT-HSC	Lin ⁻ Sca-1 ⁺ c-kit ⁺ Thy-1 ^{lo} IL7R α Flk2 ⁻ CD34 ^{-/lo}
ST-HSC	Lin ⁻ Mac-1 ^{lo} Sca-1 ⁺ c-kit ⁺ Thy-1 ^{lo} IL7R α Flk2 ⁻ CD34 ⁺
MPP	Lin ⁻ Mac-1 ^{lo} Sca-1 ⁺ c-kit ⁺ Thy-1 ^{lo} IL7R α Flk2 ⁺ CD34 ⁺
CLP	Lin ⁻ Sca-1 ^{lo} c-kit ^{lo} Thy-1 ⁻ IL7R α ⁺ Flk2 ⁺ CD24 ^{lo} CD27 ⁺ CD34 ⁺ CD43 ⁺
ELP	Lin ⁻ Sca-1 ⁺ c-kit ⁺ FIt3 ⁺ IL7R α ⁻
ETP	Lin ⁻ Sca-1 ⁺ c-kit ⁺ CD44 ⁺ IL7R α ⁻
DN1	CD44 ⁺ CD25 ⁻ CD4 ⁻ CD8 ⁻
DN2	CD44 ⁺ CD25 ⁺ CD4 ⁻ CD8 ⁻
DN3	CD44 ⁻ CD25 ⁺ CD4 ⁻ CD8 ⁻
DN4	CD44 ⁻ CD25 ⁻ CD4 ⁻ CD8 ⁻
ISP	CD4 ⁻ CD8 ⁺ CD3 ^{lo}
DP	CD4 ⁺ CD8 ⁺ CD3 ^{lo}
SP	CD4 ⁺ CD3 ⁺ or CD8 ⁺ CD3 ⁺

I.I.1 Properties of HSCs

HSCs, like all stem cells, have certain properties that designate them as 'stem' cells.

These properties include:

- a. Self-renewal is defined as the capacity to undergo stochastic differentiation to produce daughter cells identical to the original cell. This property however, is restricted to the LT-HSCs for long term and ST-HSCs and MPPs for a short period (Morrison et al., 1997a; Morrison and Weissman, 1994).
- b. Quiescence is the property of stem cells to remain dormant or in G0 phase of the cell cycle for long duration. It has been reported that only 3-4% of the total HSCs are in S2/G2/M phases of the cell cycle at any given time. Rather than being

quiescent for long periods of time, HSCs were shown to divide regularly (Morrison et al., 1997b).

- c. Differentiation is the process of production of different cell types of a lineage by a single stem cell. For example, HSCs differentiate into myeloid cells, lymphocytes and erythrocytes (Morrison et al., 1997b).

I.1.2 T-cell development

Cell-mediated immunity is mainly regulated by T cells. Their development and maturation is a result of intricate interactions involving several pathways. CLPs are generally thought to be the progenitors for both B, and T cells. However, no studies have demonstrated the CLPs actually seed the thymus. The early progenitors are thought to migrate to the thymus and achieve complete thymic differentiation. But, no circulating CLPs have been detected in the blood (Bhandoola et al., 2003; Kawamoto et al., 1999; Ohmura et al., 1999; Petrie and Kincade, 2005). In fact, some other studies have shown that non-CLP progenitors from the BM can also differentiate into T-cells if provided with a thymic microenvironment. These cells, called early lymphoid progenitors (ELPs), were found by expressing GFP under the control of recombination activating gene 1 (RAG1) promoter. *RAG* genes are required exclusively for immunoglobulin and T-cell receptor (TCR) recombination and are thus considered as lymphoid-specific markers. Expression of GFP by ELPs in the BM indicated that lymphoid specification might start earlier than speculated (Igarashi et al., 2002). Because HSCs are known to circulate and the thymus is known to have a small population of cells with a LSK phenotype, it is thought that the HSCs themselves seed the thymus. However, this is highly controversial since the 'LSK' cells from the thymus fail to reconstitute irradiated recipient mice in transplantation assays (Benz and Bleul, 2005; Sambandam et al., 2005).

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The uncommitted early thymocyte progenitors (ETPs) enter the thymus, which provides a microenvironment favourable for differentiation and maturation of thymocytes. ETPs undergo a well-characterised sequence of events in different areas of the thymus. The thymus is subdivided into four sub-compartments namely: the subcapsular zone, the cortex, the medulla and the corticomedullary junction. The subcapsular zone is the outer most zone and is mainly composed of cortical thymic epithelial cells (cTECs); the cortex is the zone between subcapsular zone and medulla and is composed of cTECs, fibroblasts and macrophages; the medulla is the inner most area and is composed of medullary TECs (mTECs) and stromal network of dendritic cells (DCs) and the corticomedullary junction is the area that allows entry and exit of cells to and from the thymus (Schlenner *et al.*, 2010). Thymocyte development can be distinguished at different stages by the expression of the cell surface markers CD3, CD4, CD8, CD44, CD25, CD117 and c-kit (Table 1.1).

Primarily, the thymocytes are subdivided into double negative (DN), double positive (DP) and single positive (SP) cells with respect to the expression of the cell surface markers, CD4 and CD8. Immature thymocytes lacking the expression of cluster of differentiation 4 (CD4) and CD8, hence called double negative (DN), undergo T-cell commitment towards the $\alpha\beta$ TCR lineage by receptor rearrangement. The DN stage can be further subdivided into DN1, DN2, DN3 and DN4 stages based on CD44 and CD25 cell-surface marker expression. DN4 cells later acquire CD4 and CD8 markers and do not express CD3. Between DN4 and DP stages, an intermediary stage immature single positive (ISP), which are $CD4^-CD8^+CD3^-$ are also identified. The DP cells then mature into single positive (SP) $CD4^+CD3^+$ or $CD8^+CD3^+$ cells (Fig 1.2).

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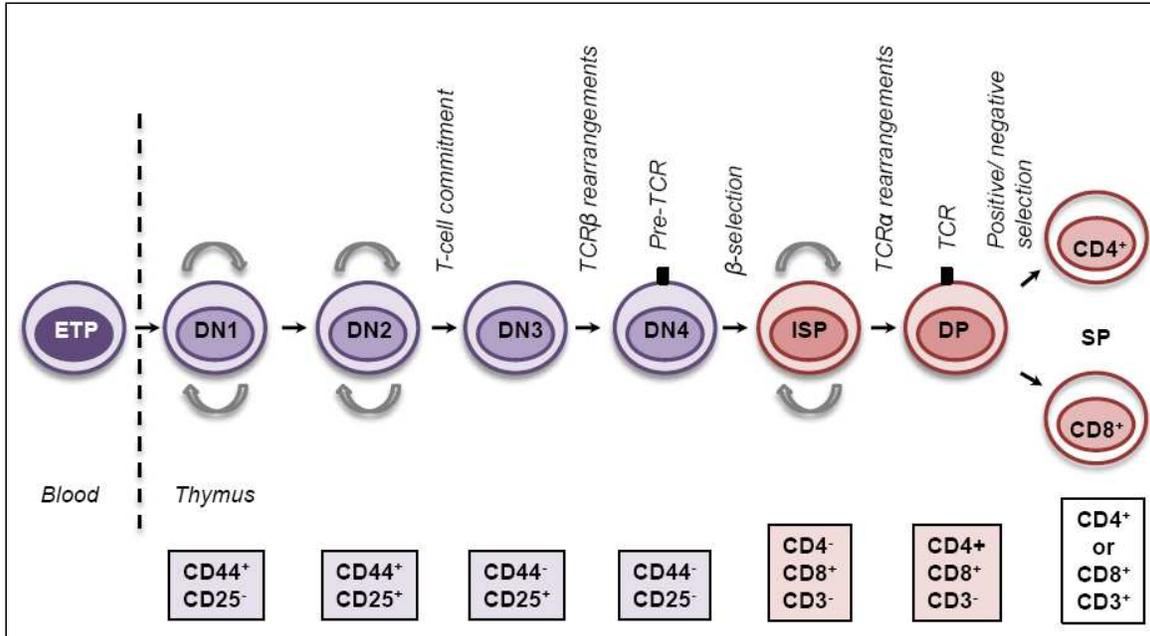


Figure 1.2. Schematic representation of T-cell development. Adapted and modified from Weerkamp *et al.* (Weerkamp *et al.*, 2006c) ETP, Early T-cell progenitor; DN1,2,3,4 double negative 1,2,3,4; ISP, Immature single positive; DP, double positive and SP, single positive. Arrows indicate proliferative stages in T-cell development. Boxes show markers used to distinguish different T-cell populations.

Progenitors entering the thymus undergo expansion at the DN1 and DN2 stages (Mori *et al.*, 2001). TCR β loci rearrangement starts at the DN3 stage and is completed at the DN4/ISP stage (Dik *et al.*, 2005). At the DN4 stage, the thymocytes undergo β -selection, a process in which the functionality of the TCR β is checked by the expression along with pre-TCR α (pT α) (Aifantis *et al.*, 1997). Signals from the pre-TCR complex initiate proliferation and pre-T-cells subsequently undergo TCR α rearrangement. This leads to the thymocytes becoming DP cells expressing TCR $\alpha\beta$ on the surface. These thymocytes are then tested for the recognition of self-MHC (major histocompatibility complex) molecules, a process called positive selection; and absence of self-reactive antigens, a process called negative selection (Kisielow and von Boehmer, 1995). These cells then

mature into SP CD4⁺ T helper cells or CD8⁺ cytotoxic cells and circulate as naive T cells (Surh and Sprent, 1994) (Fig 1.2).

I.II.2.1 Signals during T-cell development

There are many pathways involved in T-cell commitment and regulating development in the ETP's, including the Notch, the Wnt and the Sonic hedgehog (SHH) pathways. The involvement of Notch pathway in T-cell development has been extensively studied. Signalling via this pathway is shown to be indispensable for T-cell development by many laboratories (Koch *et al.*, 2008). The Wnt pathway has been shown to regulate early haematopoiesis and lymphopoiesis (Staal and Clevers, 2005; van de Wetering *et al.*, 2002a). More recently, the SHH pathway has been shown to be involved in T-cell development (Crompton *et al.*, 2007). Our study focuses exclusively on Wnt pathway in T-cell development.

I.II Canonical Wnt pathway

Wnt signalling is a complex process and at least three pathways have been reported to originate from the Wnt-Frizzled (Fz) complex, namely: the canonical Wnt pathway with β -catenin and T-cell factor (TCF)/ lymphocyte enhancing factor (LEF) complex, the non-canonical pathway involving calcium ions and the less understood planar-cell polarity pathway (Clevers, 2004; Kuhl, 2004; Malbon, 2004; Wang, 2004; Wang and Malbon, 2004). Overall, as many as ten signalling pathways are proposed to mediate Wnt responses (Staal et al., 2008). The canonical Wnt pathway is the most extensively studied and our study will also be confined to this division.

The canonical Wnt pathway events are mediated by the protein β -catenin encoded by the gene *Ctnnb1*. β -catenin is the vertebrate homologue of armadillo of *Drosophila melanogaster*. In the absence of Wnt ligands, β -catenin is a part of multiprotein destruction complex (Fig 1.3). This complex is comprised of two tumour suppressor genes, axis inhibitor (AXIN1 or AXIN2) and adenomatous polyposis coli (APC) together with glycogen synthase kinase 3 β (GSK3 β). The binding to AXIN and APC results in sequential phosphorylation of β -catenin by casein kinase 1 (CK1) and GSK3 β in the same order (Behrens et al., 1998) at Serine (Ser) 45, Threonine (Thr) 41, Ser37 and Ser33. An E3 ubiquitin ligase that contains β -transduction-repeat-containing-protein (β -TRCP) then recognises this complex. β -catenin is ubiquitinated and this leads to its proteosomal degradation. When Wnt proteins bind to the Fz receptors, a family of seven transmembrane receptors (Bhanot et al., 1996), they complex with low-density lipoprotein receptor-related proteins (LRP-5/6). It is necessary for both these receptors to be complexed to activate the canonical Wnt pathway (Pinson et al., 2000; Tamai et al., 2000; Wehrli et al., 2000). This results in dishevelled (Dsh)-mediated inhibition of GSK

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β activity. When GSK 3β does not bind to β -catenin, β -catenin can no longer be phosphorylated. This is the activated form of β -catenin. This results in the accumulation and translocation of β -catenin into the nucleus. Here, it replaces members of the transcriptional co-repressor family Groucho-related gene (GRG), to bind to TCF/ LEF1 transcription factors (Cavallo et al., 1998; Roose et al., 1998). This is followed by recruitment of cyclic AMP-responsive-element-binding protein (CREB)-binding protein (CBP) and a few other proteins facilitating the transcription of target genes (Barker et al., 2001). In the nucleus, several proteins compete with β -catenin to bind to TCF/ LEF1 to modulate the activity of the Wnt pathway. For example, Chibby has been shown to inhibit the binding of β -catenin to LEF1 and catenin β -interacting protein (ICAT) has been shown to inhibit the binding of β -catenin and TCF (Daniels and Weis, 2002; Takemaru et al., 2003) (Fig 1.3).

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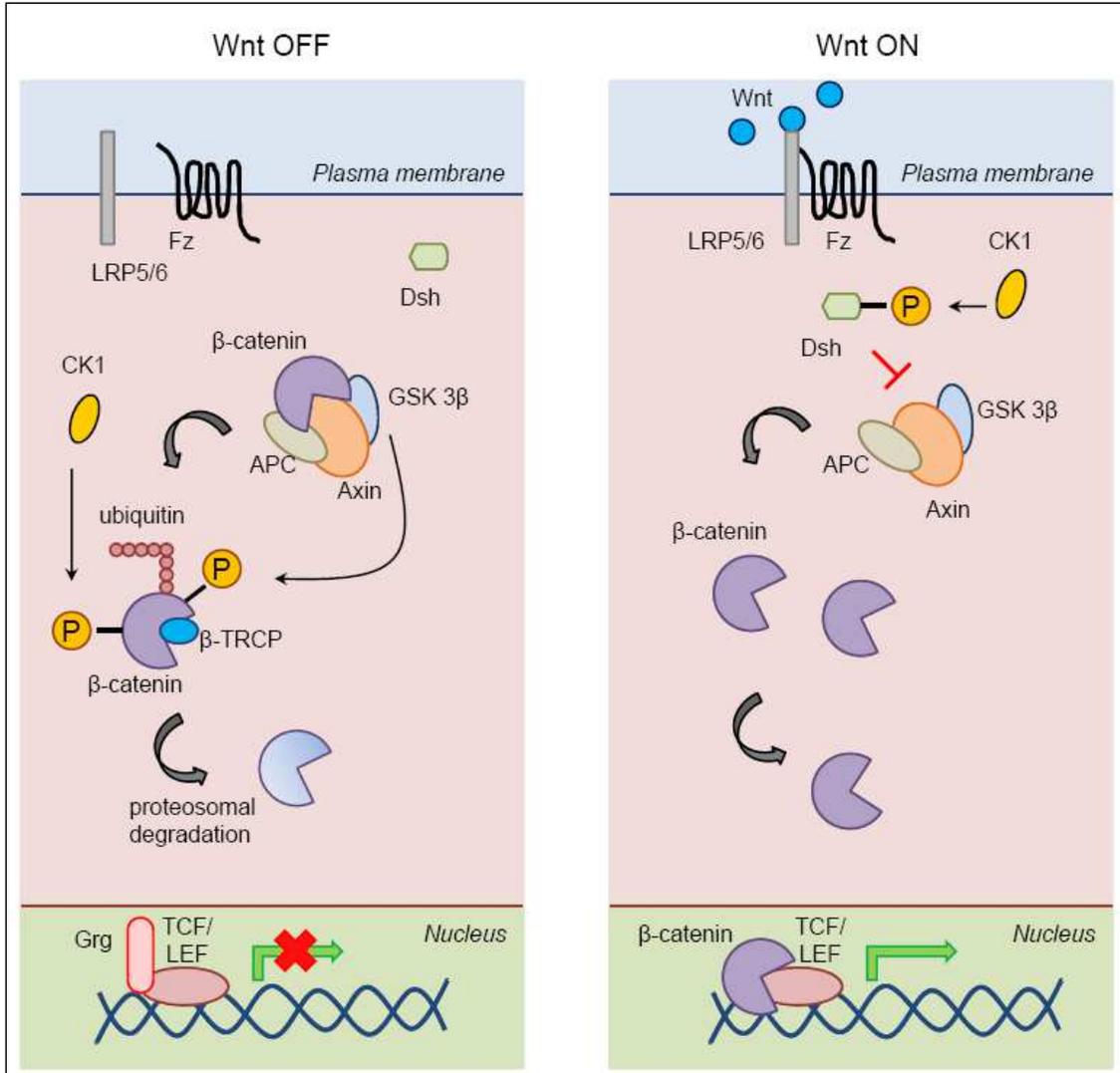


Figure 1.3. Canonical Wnt signalling pathway. Left panel, In the absence of Wnt ligands, β -catenin is in a complex consisting of axis inhibitor (AXIN), Adenomatous polyposis coli (APC) and glycogen synthase kinase 3 β (GSK3 β) in the cytoplasm. β -catenin is phosphorylated by casein kinase 1 (CK1) and GSK3 β . Phosphorylated β -catenin is recognised by β -transducin-repeat containing protein (β -TRCP) which ubiquitinates and degrades β -catenin by proteasome complex. Right panel, When Wnt binds to Frizzled receptor (Fz), it complexes with low-density lipoprotein-receptor related proteins 5/6 (LRP5/6) resulting in inactivation of GSK3 β via Dishevelled (Dsh). β -catenin accumulates in the cytoplasm and translocates into the nucleus and replaces transcriptional co-repressors like Groucho-related proteins (Grg) to bind to T-cell factor (Tcf) and lymphoid enhancing factor 1 (Lef1). This results in the expression of Wnt target genes.

I.II.1 Wnt signalling in HSCs

Most studies on Wnt signalling in HSCs show the crucial role of this pathway in haematopoiesis. Effects of Wnt pathway, both gain-of-function and loss-of-function approaches, have been recently reviewed by Luis TC *et al* (Luis *et al.*, 2011a). The majority of the studies show that Wnt signalling is required for the normal development and functioning of HSCs.

Constitutive activation of β -catenin using retroviruses in B-cell lymphoma 2 (Bcl2) - transgenic mice resulted in an increase in proliferation and self-renewal of HSCs in *in vivo* transplantation assays (Reya *et al.*, 2003). However, conditional over-expression of stabilized form of β -catenin led to a multilineage differentiation block and expansion of HSCs followed by their exhaustion (Kirstetter *et al.*, 2006; Scheller *et al.*, 2006). Studies like these, which are contradictory, have not allowed us to come to a precise conclusion regarding the effect of Wnt pathway in development and properties of HSCs. Wnts are thought to be present in controlled levels in different tissues. Therefore, the effect of this pathway is speculated to be dosage-dependent. When Wnt signals are slightly over normal (2-4 fold higher), HSCs are shown to reconstitute better. But, when Wnt signals are very high (>20 fold high), HSCs fail to reconstitute in new hosts (Luis *et al.*, 2011b). While the gain-of-function studies show the above mentioned effects, the loss-of-function studies show otherwise. Deletion of β -catenin by Mx-Cre showed no defects in HSC function (Cobas *et al.*, 2004; Staal and Sen, 2008). Two other reports demonstrated that loss of both β - and γ -catenin did not affect haematopoiesis (Jeannet *et al.*, 2008; Koch *et al.*, 2008). *In vivo* reporter assays showed that Wnt signals were still active in HSCs albeit the deletion. However, Wnt-3a-deficient mice, over-expression of Dickkopf1 (DKK1) in osteoblasts and deletion of β -catenin in haematopoietic system (by Vav-Cre),

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showed that Wnt signalling is necessary for normal HSC function (Fleming *et al.*, 2008; Luis *et al.*, 2009; Zhao *et al.*, 2007). These and several other studies show that complete absence of Wnt pathway is detrimental to HSC function while $\sim 1/4^{\text{th}}$ of the normal levels is sufficient to sustain normal functionality. Approximately two fold increase in Wnt activity can enhance HSC proliferation and functionality while very high levels of Wnt pathway can abolish HSC function (Luis *et al.*, 2011a; Luis *et al.*, 2011b) (Fig 1.4 and Table 2).

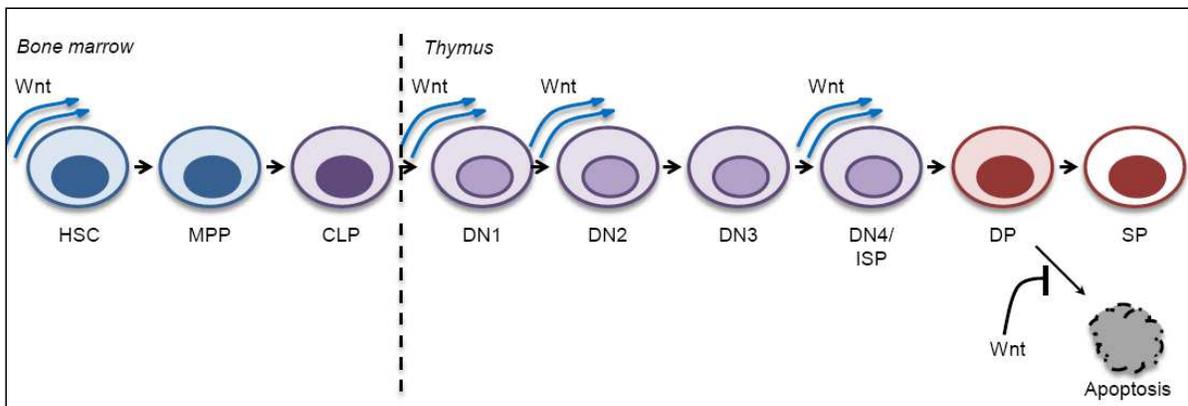


Figure 1.4. Wnt signalling in haematopoiesis and thymopoiesis. Adapted and modified from Staal and Clevers, 2005 (Staal and Clevers, 2005). A schematic representation of various cellular stages of HSC and T-cell development in bone marrow and thymus where Wnt/ β -catenin pathway plays a role. HSC, haematopoietic stem cells; MPP, multipotent progenitor; CMP, common myeloid progenitor; CLP, common lymphoid progenitor; ETP, Early T-cell progenitor; DN1,2,3,4 double negative 1,2,3,4; ISP, Intermediate single positive; DP, double positive and SP, single positive. Wnt signalling, depicted by blue arrows, shows proliferative and anti-apoptotic signals in this process.

I.II.2 Wnt signalling in the thymus

Wnt expression is seen mostly in thymic epithelium in some thymocytes. Fz is present mostly on the immature thymocytes (Balciunaite *et al.*, 2002; Staal *et al.*, 2001a; Weerkamp *et al.*, 2006b) and β -catenin mRNA is detectable in all subsets of thymocytes (Pongracz *et al.*, 2003). The Wnt pathway has been shown to be most active in the

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earlier phases (DN1-3) of T-cell development. The presence of members of Wnt/ β -catenin pathway in the thymocytes suggests that this pathway may be necessary through T-cell development. Numerous studies have contributed towards the understanding of the effects of the Wnt pathway in T-cell development. However, the precise role of this pathway in T-cell development remains to be discovered.

Thymocytes were shown to respond to Wnt signals by increased proliferation *in vitro* by Tcf-LacZ reporter construct (Staal *et al.*, 2001a) and retroviral transduction Wnt1 and Wnt4 (Weerkamp *et al.*, 2006b). Inhibiting the Wnt/ β -catenin pathway members showed a wide range of defects in T-cell development. Inhibiting Wnt pathway by using soluble Fz receptors resulted in a block in development at the DN-ISP stages. Mice deficient in both Wnt1 and Wnt4 show reduced thymic cellularity (Mulroy *et al.*, 2002). Tcf-1 exon 5 mutant mice (Tcf-1 ^{Δ V/ Δ V}) expressing low levels of truncated Tcf-1 protein, still show functionality of the protein and do not show major T-cell developmental defects. Tcf-1 exon 7 mutant mice (Tcf-1 ^{Δ VII/ Δ VII}) showed complete abolishment of DNA-binding activity of Tcf-1. This mutant is considered 'true' Tcf-1 knockout mice (Schilham *et al.*, 1998b) and regarded as Tcf-1-deficient mice. These mice show a developmental block at DN1, 2 and ISP stages when young, and a complete block at the DN1 stage as adults. Lef-1 knockouts however have normal lymphoid development presumably because Tcf-1 compensates for Lef-1 (van Genderen *et al.*, 1994). However, Lef-1/ Tcf1 ^{Δ V} double-deficient mice show a complete block in T-cell development at the ISP stage (Okamura *et al.*, 1998).

Only Tcf isoforms that contained β -catenin-binding domain could rescue thymic defect in Tcf-1 ^{Δ VII/ Δ VII} mice suggesting that Tcf-1- β -catenin binding is important for thymocyte development (Ioannidis *et al.*, 2001b; Staal *et al.*, 2001a). Mice over-expressing Axin

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exhibited reduced thymic cellularity (Hsu et al., 2001). Mice in which β -catenin was deleted after the DN3 stage, showed a block in T-cell development at DN4-ISP stages (Xu *et al.*, 2003b). Similarly, several studies have shown that β -catenin is indispensable for DN-DP transition (Pongracz et al., 2006; Verbeek et al., 1995). However, mice transplanted with HSCs in which β -catenin deletion was induced, displayed no thymic defect (Cobas et al., 2004).

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Table 1.2. List of all Wnt pathway mutations and their effects on haematopoiesis and thymopoiesis.

Wnt activity	Type of mutation in Wnt pathway	Effect	Reference
	β -cat ^{lox/lox} Mx-Cre	No defects in hematopoiesis	<i>Cobas, M; 2004</i>
	Dkk1-tg	Increased proliferation and impaired HSC self-renewal	<i>Fleming, H.E; 2008</i>
	Wnt3a ^{-/-}	Reduced HSC numbers and reconstitution potential	<i>Luis, T.C; 2009</i>
	β -cat ^{lox/lox} Vav-Cre	HSCs deficient in long-term maintenance and growth, results in CML and ALL	<i>Zhao, C; 2007</i>
	Apc ^{15lox} , Apc ^{15lox/1572T}	Increased proliferation of HSCs	<i>Luis, T.C; 2012</i>
	Apc ^{15lox/1638N} , Apc ^{15lox/15lox}	Impaired HSC self-renewal	<i>Luis, T.C; 2012</i>
	R26 ^{NC/NC} Mx1-Cre, Ctnnb1 ^{(Ex3)^{fl}} Mx1-Cre	Multilineage differentiation block of HSCs	<i>Kirstetter, P; 2006 and Scheller, M; 2006</i>
	Wnt1 ^{-/-} , Wnt4 ^{-/-}	Reduced thymic cellularity	<i>Mulroy, T; 2002</i>
	Wnt1 ^{-/-} Wnt4 ^{-/-}	Greater reduction in thymic cellularity	<i>Mulroy, T; 2002</i>
	Tcf-1 ^{ΔVI/ΔV}	No apparent deficiency in T-cell development	<i>Schilham, M.W; 1998</i>
	Tcf-1 ^{ΔVII/ΔVII}	Block at DN1 stage in adult mice	<i>Schilham, M.W; 1998</i>
	Lef-1 ^{-/-}	Normal T-cell development	<i>van Genderen, C; 1994</i>
	Lef-1 ^{-/-} Tcf-1 ^{ΔVI/ΔV}	Block at ISP stage	<i>Okamura, R. M; 1998</i>
	β -cat ^{lox/lox} Lck-Cre	Block at DN4-ISP stages	<i>Xu, Y; 2003</i>
	β -cat ^{lox/lox} Mx-Cre	Normal T-cell development	<i>Cobas, M; 2004</i>
	Axin ^{hi}	Increased apoptosis, reduced thymic cellularity	<i>Hsu, W; 2001</i>
	Tcf-LacZ-tg	Impaired T-cell development	<i>Staal, F.J; 2001</i>
	β -cat ^{ThyΔEx3}	Lack of $\alpha\beta$ TCR in mature T-cells, block at DP stage	<i>Guo, Z; 2007</i>

Haematopoiesis

Thymopoiesis

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Additionally, β -catenin is reported to regulate positive and negative selection of DP cells (Kovalovsky et al., 2009; Xu et al., 2009; Yu and Sen, 2007; Yu et al., 2007). Yu *et al* used transgenic β -cat^{lox/lox} Lck-Cre mice to show accelerated CD8⁺ SP cells production. In this model, the increased CD8⁺ cells were produced by enhanced IL-7 signals caused by β -catenin. In another model, Ctnnb ^{Δ ex3} Lck-Cre, in which β -catenin was stabilised by deletion of exon3, thymocytes showed enhanced negative selection (Kovalovsky et al., 2009). This result is consistent with the impaired negative selection in Tcf-1^{-/-} mice. Thus, subtle changes in β -catenin expression may affect the thymic outcome.

Although Wnt activity reduces after DP stage (Weerkamp *et al.*, 2006b; Xu *et al.*, 2003b), Wnt activity is reported to be critical for DP survival. Only thymocytes that express TCRs and interact with MHC molecules are able to survive and eventually develop into T-cells. Therefore, the life of a thymocyte is critical in shaping the T-cell repertoire. Longer the thymocytes can survive, greater are the chances to generate a TCR that will be positively selected. Tcf-1-deficient thymocytes undergo accelerated apoptosis which can be prevented by ectopic introduction of Bcl-2 (anti-apoptotic factor) (Ioannidis *et al.*, 2001b). β -catenin has been shown to enhance DP cell survival (Hossain *et al.*, 2008b; Xie *et al.*, 2005). Additionally, β -catenin-dependent survival was mediated by Bcl-x_L upregulation. This result is consistent with the Tcf-1^{-/-} study where Bcl-x_L level is greatly reduced (Ioannidis *et al.*, 2001b; Wang *et al.*). The study on Ctnnb ^{Δ ex3} Lck-Cre mice showed that stabilisation of β -catenin led to developmental block of DP-SP transition and malignant transformation. These studies suggest that Wnt signals may be involved in checking the mechanism of elimination of unwanted thymocytes during positive/ negative selection.

These studies (summed in Table 1.2) show that Wnt pathway is crucial at different stages during T-cell development. However, few other studies have demonstrated that β -catenin is dispensable for T-cell development. These and other results show that the precise role of Wnt pathway is still not fully understood.

I.II.3 Aberrant Wnt signalling and cancer

Wnt signalling plays a crucial role in the overall development of different cell types in several species. The importance of Wnt signalling in haematopoietic, nervous, digestive systems as well as mammary development have been most extensively studied. Mutations in this pathway at different levels are known to contribute to many cancers, including breast, brain, colon, oral cancers, melanomas, hepatocellular carcinoma, gastrointestinal tumours and haematological malignancies (Klaus and Birchmeier, 2008). In this study, we focus specifically on aberrant Wnt signalling in T-cell acute lymphoblastic leukaemia (T-ALL).

I.III T-cell acute lymphoblastic leukaemia

Acute lymphoblastic leukaemia (ALL) is a malignant disorder originating from a single-cell precursor of the B- or T-cell lineage. It is usually caused by blockade of the maturation and increase in proliferation. T-ALL account for 15-20% of all the ALL cases (Graux, 2011). ~75% of the paediatric and only 30-40% of the adult T-ALL undergo event-free survival (Kuiper et al., 2007; Van Vlierberghe et al., 2006). The precise pathogenetic events that lead to this leukaemia are not fully understood. In paediatric T-ALL, there are two infection-based models proposed for the occurrence of increased acute leukaemia cases (Pui et al., 2008). First one called Kinlen's population-mixing hypothesis postulates that childhood cases of ALL result from exposure of non-endemic pathogens to susceptible individuals after population-mixing (Kinlen, 2004). The second theory hypothesised by Greaves, is called the delayed-infection hypothesis. Greaves suggests that prenatally acquired pre-leukaemic clones make susceptible individuals. When exposed to common infections in early life, it predisposes the immune system of these individuals to aberrant pathological responses leading to uncontrolled lymphocyte growth or leukaemia (Greaves; Greaves, 2006) (Fig 1.5).

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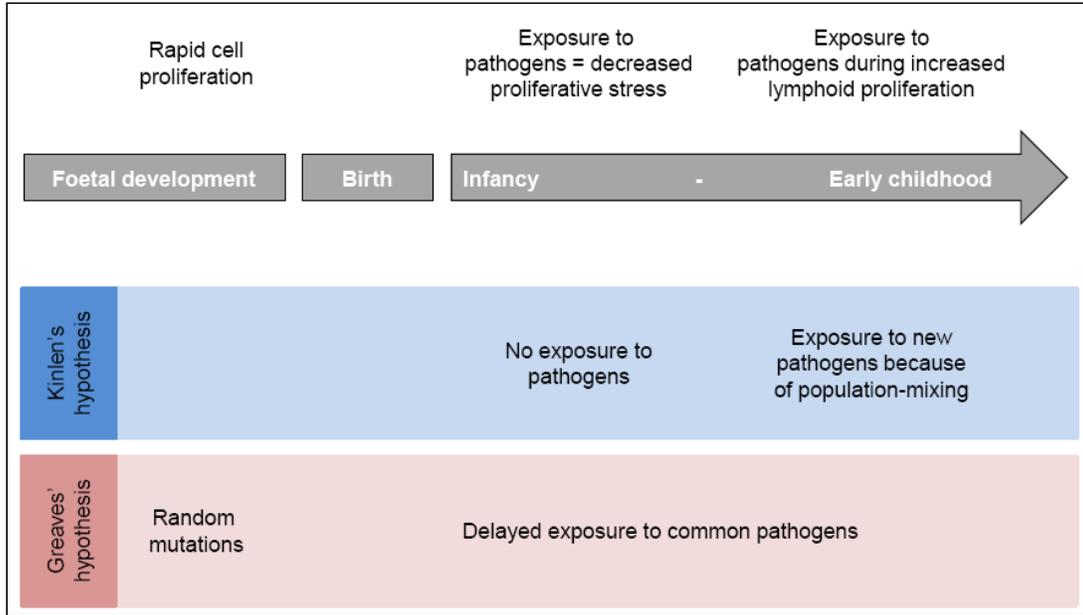


Figure 1.5. Infection-based models of leukaemia development. Adapted and modified from Ching-Hon Pui *et al.*, 2008 (Pui *et al.*, 2008).

Multiple genetic events affecting cellular processes resulting in developmental arrests in nearly all stages have been identified in T-ALL. Collective studies have shown that more than 50% of T-ALL cases exhibit mutations in the Notch pathway (Aster *et al.*, 2008). Notch pathway is reportedly active and necessary during the earlier stages of T-cell development (Sambandam *et al.*, 2005; Tan *et al.*, 2005). Studies on numerous animal models have revealed that mutations in this pathway result in T-cell leukaemias (Aster *et al.*, 2008). In addition to Notch1, several other mutations like FBW7 (Wu *et al.*, 2001), HOXA (Soulier *et al.*, 2005), TAL-1 (Mikkola *et al.*, 2003), MYB (Mucenski *et al.*, 1991), LMO1 and LMO2 (Curtis and McCormack) have also been reported.

Deregulation of the Wnt pathway associated with the phosphoinositide-3-kinase (PI3K) pathway in T-ALL has been reported in some animal models. Loss of the tumour suppressor phosphatase and tensin homologue deleted on chromosome 10 (PTEN) and

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Wnt signalling have been shown to co-operate in T-cell leukaemogenesis (Guo et al., 2008).

PTEN is an antagonist to class I phosphatidylinositol 3-kinase (PI3K) signalling. In response to external stimuli, PI3K is recruited to receptor tyrosine kinases (RTKs) or G-protein coupled receptors (GPCRs) at the membrane. Here, it phosphorylates phosphatidylinositol-4,5, bisphosphate (PIP₂) to generate phosphatidylinositol-3,4,5 triphosphate (PIP₃). PTEN is a lipid phosphatase that antagonizes this action by dephosphorylating PIP₃ to give PIP₂ (Fig 1.6), thus acting as a central regulator. The serine-threonine kinase AKT, downstream of PIP₃, is recruited to the membrane via PIP₃ binding. Further, it is activated by phosphorylation by 3-phosphoinositide-dependent kinase (PDK1) by mammalian target of Rapamycin complex (mTORC2) or by other kinases (Chalhoub and Baker, 2009).

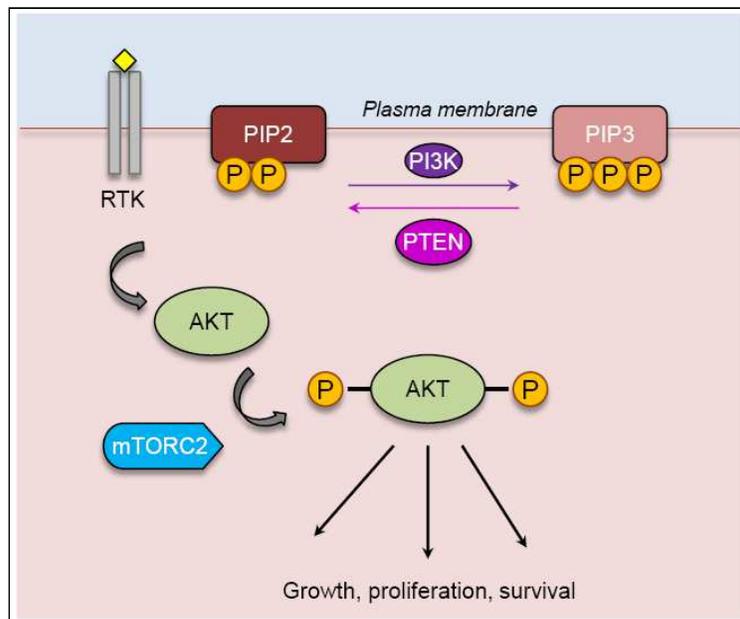


Figure 1.6. The phosphatidylinositol 3-kinase (PI3K) signalling pathway. Adapted and modified from Nader Chalhoub *et al*, 2009 (Chalhoub and Baker, 2009). Phosphatase and tensin homologue on chromosome 10 (PTEN) dephosphorylates phosphatidylinositol-4,5, bisphosphate (PIP₃) to generate phosphatidylinositol-3,4,5 triphosphate (PIP₂). Activated receptor

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tyrosine kinases (RTK) recruit and activate PI3K. PI3K recruits many proteins including Akt. Akt is activated by phosphorylation. Activated Akt may phosphorylate large range of substrates involved in cell growth, proliferation and survival.

Loss of PTEN has been reported to be involved in leukaemogenesis in several studies (Alimonti *et al.*, 2010; Guo *et al.*, 2008; Liu *et al.*, 2010). However, involvement of the Wnt pathway in T-ALL along with PTEN loss has been only recently reported. This study shows that mice with VE-cadherin-cre (VEC-Cre)-mediated *Pten* deletion developed two types of leukaemic blasts; 74% of blasts were T-ALL and the rest of the cases were AML (acute myeloid leukaemia). A rare c-Kit^{mid}CD3⁺Lin⁻ population, identified as leukaemia stem cells, showed high expression of unphosphorylated β -catenin. Moreover, by conditional ablation of one allele of β -catenin in *Pten*^{loxP/loxP} VEC-cre⁺ mice, a substantial decrease in the disease was noted (Guo *et al.*, 2008).

In the non-T-ALL context, PI3K pathway and Wnt pathway signalling is shown to cooperate to promote self-renewal and expansion in HSCs (Perry *et al.*, 2011). In this study, double mutant mice (bearing deletion of *Pten* (*Pten*^{loxP/loxP})) with conditional activation of β -catenin ((*Ctnnb1*^{tm1Mmt}) driven by tamoxifen-induced (stem cell leukaemia Scl-Cre-ER^T) exhibited expansion of LT-HSCs without extensive differentiation. Activation of β -catenin alone resulted in apoptosis of HSCs, while loss of PTEN enhanced HSC anti-apoptotic factors contributing to self-renewal and expansion. By bringing alteration to both these pathways, it was demonstrated that HSCs cannot function due to blocked differentiation. Therefore, these studies provided evidence that activation of either of these pathways alone is insufficient for HSC expansion, while cooperation between both drive expansion and self-renewal of HSCs.

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In addition to these reports, numerous studies have reported direct epigenetic deregulation of Wnt pathway in haematological malignancies. In some multiple myeloma (MM) cell lines, hypermethylation of secreted Frizzled-related proteins (sFRP) genes was a common event. Although, the epigenetic silencing of Wnt antagonist sFRP was detected in later stages of MM, the authors discuss its importance in disease progression (Chim et al., 2007; Jost et al., 2009). In AML patients, aberrant methylation of Wnt antagonists was associated with a decreased relapse-free survival (Valencia *et al.*, 2009b). Further, in B- and T-ALLs, epigenetic silencing of Wnt inhibitors like sFRP1, sFRP2, sFRP4, sFRP5, Wnt inhibitory factor-1 (WIF1) and Dkk3 has been associated with decreased EFS and poor prognosis (Moskalev et al.; Roman-Gomez et al., 2007).

Notably, no human T-ALL has been reported as 'Wnt-dependent' till date. However, an animal model of T-ALL that is β -catenin dependent has been reported. A study in which mice with Cre-mediated T-cell specific deletion of exon3 (containing phosphorylation site) of the gene encoding β -catenin (Ctnnb1), called Ctnnb1^{ex Δ 3}, showed T-cell developmental defects at the DP stage, leading to leukaemia. It was shown that β -catenin dependence of this leukaemia, accompanied by c-Myc upregulation, did not require Notch activation (Guo et al., 2007).

These and other accumulating data support a causative role of the Wnt pathway in haematological malignancies.

I.IV Cancer stem cell concepts

Tumour cells from any cancer, solid or not, exhibit heterogeneity in terms of morphology, phenotype, proliferation rates and response to treatments. It is also thought-out that such heterogeneity can exist within a tumour. Even though all cells of a tumour are clonal (arising from a single cell), several intrinsic and acquired pressures can generate differed responses resulting in heterogeneity among cells. This of course, is one of the several hypotheses that the present scientific community is considering. A second explanation could be that a tumour is likely the result of a mis-directed normal tissue development. There is increasing evidence suggesting the existence of subset of cells, termed cancer stem cells (CSCs) or cancer/ leukaemia-initiating cells (C/LICs), that are distinct from the rest of the tumour cells, but are responsible for tumour progression (Dalerba et al., 2007). The existence of CSCs was hypothesised as a result of very high relapse rates in many cancers. CSCs are notorious for their ability to survive conventional cytotoxic chemotherapies and radiotherapies. Research in CSC biology has recently gained importance as it strongly predicts that targeting CSCs might lead to higher event-free survival rates (Fig 1.7).

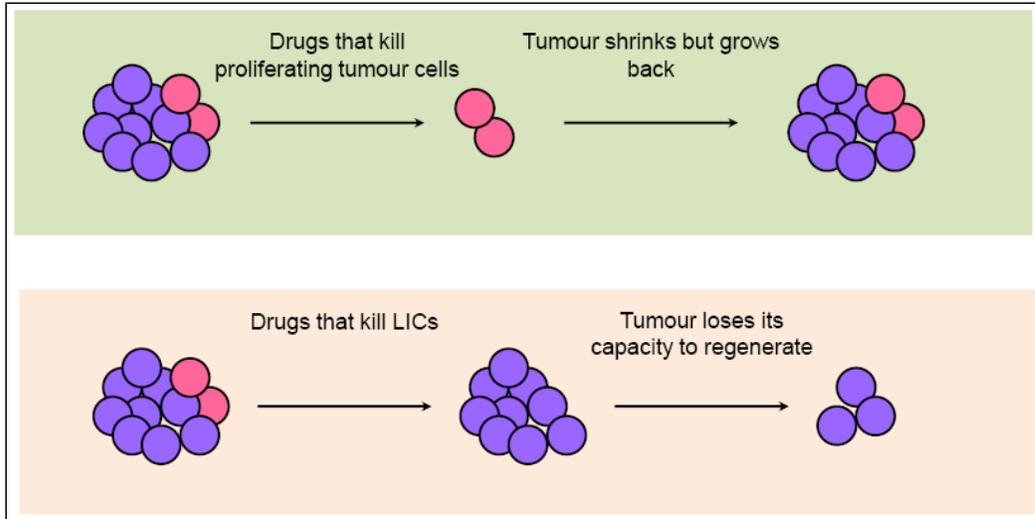


Figure 1.7. A schematic presentation of effects of conventional therapies used against cancers. Adapted and modified from Reya *et al*, 2001 (Reya *et al.*, 2001). Cancer stem cells (CSCs) will remain viable and re-establish tumours since most conventional drugs target proliferating cells. In contrast, if drugs target CSCs, the tumour will shrink and fail to re-establish.

I.IV.1 Tumour heterogeneity

Normal tissue undergoes continuous regeneration. Since cancer cells are known to proliferate at very high rate, initial pharmacogenomics aims were to design drugs to target cells undergoing neoplastic proliferation. In response to these drugs, the rate of relapse did not decrease significantly; preliminary hypotheses were that regular chemotherapies resulted in 'resistant' clones. But, if the drugs are designed to target neoplastic proliferation at the molecular level, the treatment should kill all cells excluding the possibility of emerging 'resistant' clones. With the advancement of transplantation of cancer cells, it was possible to determine potentials of single cells. Over the years, as the research advanced, it came to be known that the CSCs of haematological malignancies existed at a certain frequency varying vastly among tumours (Bruce and Van Der Gaag, 1963; Hewitt, 1958; Makino, 1956). These and several other studies suggested that a tumour can be considered hierarchical (Pierce *et al.*, 1960). Following

an era concentrated in studying cellular heterogeneity in tumours, it was first suggested by Clarkson and colleagues that CSCs are dormant and anti-proliferative agents will not destroy these cells (Clarkson, 1969). Clonogenic assays like colony-forming unit assays (CFU) were later developed for AMLs. These assays revealed that the cells within a single tumour bore different clonogenic potentials (Buick et al., 1977; Dicke et al., 1976; Griffin and Lowenberg, 1986; McCulloch, 1983; McCulloch et al., 1983; Metcalf et al., 1969; Moore et al., 1973). The current technology allows us to analyse tumour cells only in large numbers and not at a single cell level. This is one of our biggest limitations. These factors force us to treat tumours as if they were homogenous while we know from numerous studies that tumours are mostly heterogeneous (Dick, 2008).

[I.IV.1.1 Evidence for cancer stem cells](#)

AML is probably the most extensively studied leukaemia in the cancer stem cell biology. It was shown in 1960's that only 1-4% of the mouse myeloma cells could form spleen colonies when transplanted *in vivo* (Bergsagel and Valeriote, 1968; Bonnet and Dick, 1997; Bruce and Van Der Gaag, 1963), showing different clonogenic potentials within a tumour. Such differences in clonogenicity of leukaemic cells mirrored the clonogenicity of normal HSCs. But, it still left us with two possibilities: either all leukaemic cells can behave like LICs or that most leukaemic cells are unable to proliferate extensively and that only a subset of cells are consistently clonogenic. Furthermore, it has also been shown that solid tumours are typically heterogeneous and only a small proportion of cells are clonogenic (Fidler and Hart, 1982; Fidler and Kripke, 1977; Heppner, 1984). These findings led to the hypothesis of different models of heterogeneity in cancers.

I.IV.1.2 Models of heterogeneity

The two mutually exclusive models that explain tumour heterogeneity are the stochastic and the hierarchal models.

The stochastic model describes tumours as biologically homogeneous. Some intrinsic (e.g. signalling pathways) or extrinsic (e.g. microenvironment, niches) influences can alter normal cells at random with certain unpredictability. These transformed cells can regenerate tumours. Here, every single cell is susceptible to the intrinsic and extrinsic factors and the changes caused by these factors. The hierarchal model suggests that tumours develop primarily like normal tissue, with stem cells at top of the hierarchy. Here, leukaemia-initiating cells or LICs, capable of self-renewal, are distinct from the rest of the tumour cells (Fig 1.8).

Both these models suggest the existence of LICs, however, the key difference is that in the stochastic model, an LIC can arise randomly and any cell can become an LIC. In the hierarchal model, subsets of cells born distinctly are alone capable of regenerating tumours (Reya et al., 2001).

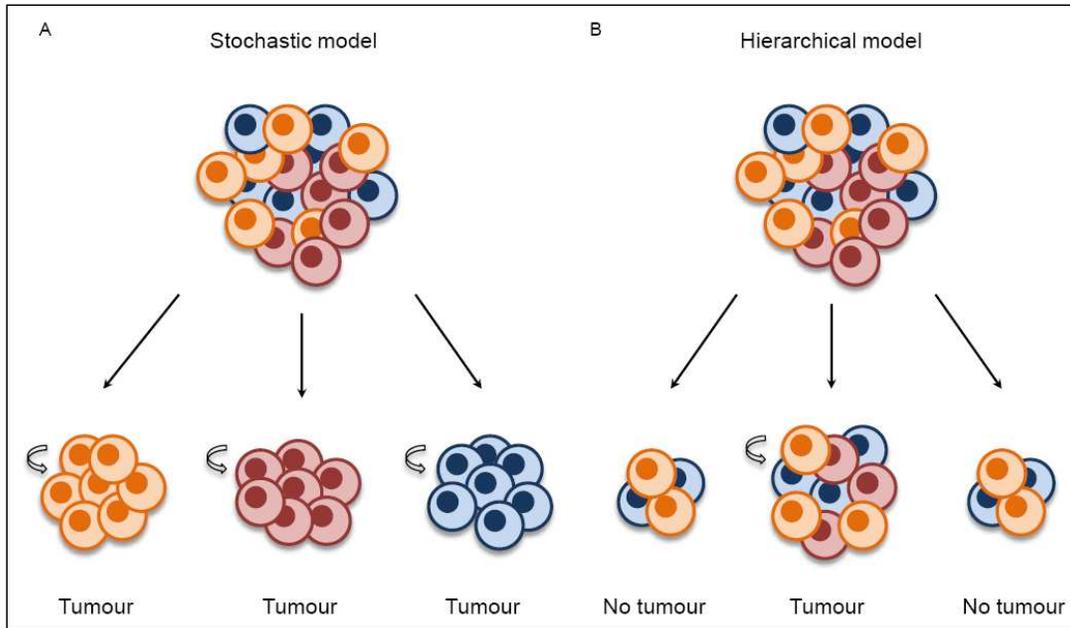


Figure 1.8. Models of tumour heterogeneity. Adapted and modified from Reya *et al*, 2001 (Reya *et al.*, 2001). Tumours are heterogeneous group of cells. A) Stochastic model states that every cell in a tumour can behave like a cancer stem cell (CSC) and have equal potential to re-establish tumours. B) Hierarchical model states that only a subset of cells can re-establish tumour and these are the CSCs. Arrows indicate self-renewal potential of the cells.

I.IV.2 Self-renewal and leukaemogenesis

Are the stem cells most prone to becoming CSCs? There are two reasons why the stem cells are 'targets' to become CSCs. Firstly, because the self-renewal machinery is already activated in stem cells, it would require fewer mutations to de-regulate a already activated self-renewal pathway than to start a *de novo* self-renewal pathway ectopically. Secondly, since the self-renewing process allows stem cells to persist for longer periods, these cells have a greater chance of accumulating mutations. Even progenitor cells have lower chances than stem cells of undergoing neoplastic transformation because they divide after a short period of time. If progenitors were to be CSCs, they have to first acquire long term self-renewal potential to gain mutations. Nonetheless, the progenitors

I. INTRODUCTION

still have greater chances of being CSCs than the differentiated cell types. Stem cells have the greatest probability to become CSCs, followed by progenitor cells and differentiated cells (Fig 1.9). Several studies have identified that the pathways deregulated in CSC formation are the ones that are mostly associated in maintaining self-renewal of normal stem cells. Signalling pathways like Notch, (Sonic hedgehog) Shh and Wnt associated with oncogenesis have been shown to be involved in self-renewal of stem cells (Taipale and Beachy, 2001). Notch1 activation by Jagged-1 ligand has been shown to increase the progenitor activity of HSCs *in vitro* and *in vivo*, showing that the Notch pathway is involved in self-renewal and multipotentiality (Karanu et al., 2000). Similar studies have been done on the Shh pathway involvement in self-renewal. HSCs were shown to exhibit increased self-renewal response upon Shh stimulation with other growth factors (Bhardwaj et al., 2001) (Table 1.3).

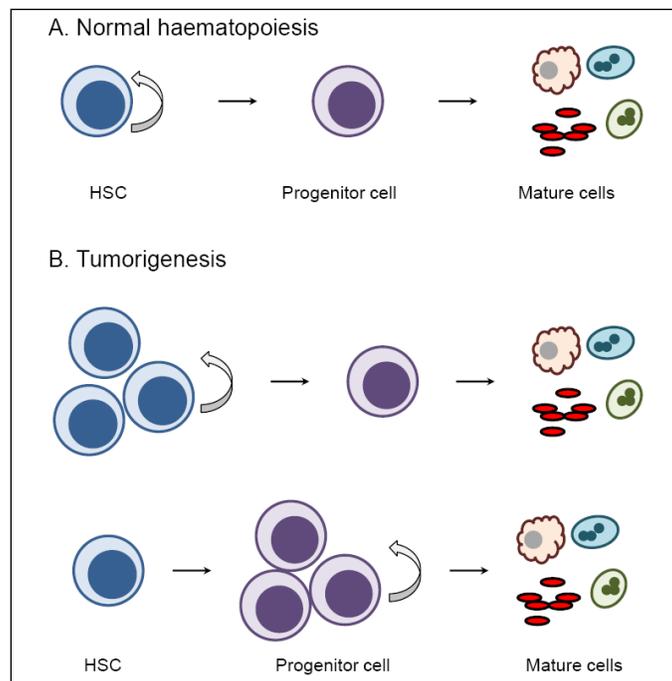


Figure 1.9. Comparison of self-renewability in normal haematopoiesis and leukaemic transformation. Adapted and modified from Reya *et al*, 2001 (Reya et al., 2001). A) Normal haematopoiesis sequence of events where HSCs differentiate into mature cell via

I. INTRODUCTION

intermediary progenitor cells and B) HSCs have self-renewal mechanisms activated making them the most likely targets of transformation. However, the progenitor cells can also be potential targets if the self-renewability is prolonged to the progenitor stage.

Table 1.3. Signalling pathways involved in self-renewal reported in tumorigenesis.

Adapted and modified from Reya *et al*, 2001 (Reya et al., 2001).

Pathway	Stem/ progenitor cell self-renewal	Tumorigenesis
Wnt/ β -cat	Haematopoietic, epidermal, neural and gut stem cells	Colon, epidermal, colorectal tumours and leukaemia
Shh	Haematopoietic, neural and germline stem cells	Medulloblastoma and basal cell carcinoma
Notch	Haematopoietic, neural and germline stem cells	Leukaemia and mammary tumours
PI3K	Haematopoietic stem cells	Leukaemia

The Wnt pathway is one of the most extensively studied pathways with regard to self-renewal, not just in HSCs, but also in neuronal, dermal and gut stem cells. As mentioned earlier, two-fold-increase in Wnt activity results in enhanced HSC proliferation and functionality (Reya and Clevers, 2005; Reya et al., 2001). Cumulatively, the Wnt pathway has been shown to be involved in HSC self-renewal albeit that the underlying molecular mechanisms need to be elucidated.

I.IV.3 Clonal evolution model

Clonal evolution of cancer was first suggested by Nowell and colleagues in 1976 (Nowell, 1976). This model follows the basic neo-Darwinian principle of evolution which talks about natural selection of the fittest variants. Clones which fail to repair acquired oncogenic mutations could compete amongst themselves to generate leukaemia. The

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mutation-endowed fitness can depict the clonal selection a cancer can have. Mel Greaves has suggested in a recent review that clones evolve through interactions of 'driver', 'passenger' and deleterious lesions. Some lesions can be affected by the microenvironment and increase the rate of other genetic changes (Greaves and Maley, 2012). Epigenetic variations affect the natural selection of clones. Collectively, the clonal evolution model suggests that clonal expansions compete to dominate a neoplasm.

Several studies suggest that there are several genetic subclones of LSCs that are within a complex architecture (Bonnet and Dick, 1997; Goardon et al.; Sirard et al., 1996) (Fig 1.10). With the advent of multicolour fluorescence *in situ* hybridisation (FISH), comparative genomic hybridisation (CGH) and spectral karyotyping (SKY), it has become easier to identify candidate genetic lesions that could be the oncogenetic event leading to leukaemia.

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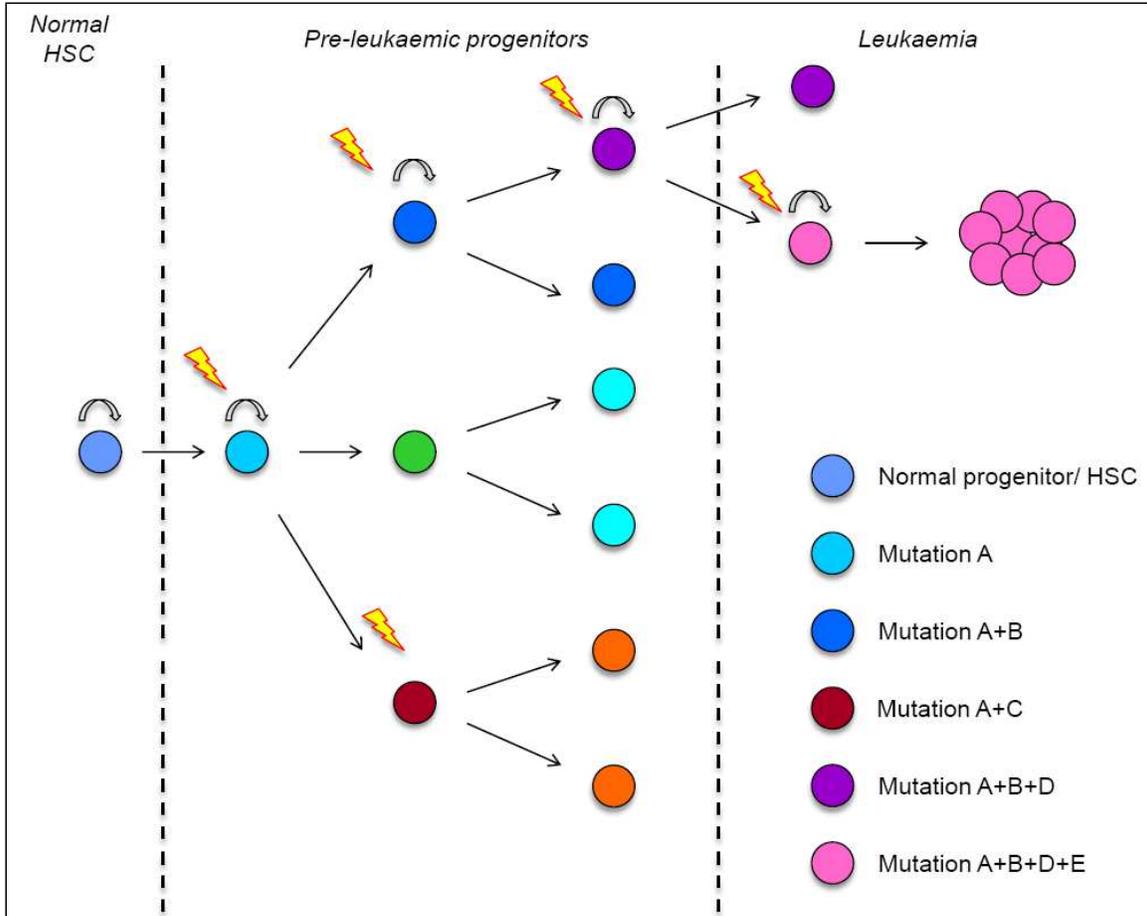


Figure 1.10. Schematic representation of clonal evolution of dominant leukaemic clones. Adapted and modified from Jan and Majeti, 2012 (Jan and Majeti, 2012). The model shows clonal heterogeneity in acute leukaemia. Several subclones exist in a single clonal architecture. There are many possibilities for the generation of several clones. Dominant clones can gain additional mutations from a single hit in the cell of origin leading to a leukaemic clone. Alternatively, some other clones can remain 'silent' as the dominant clone outcompetes.

One of the first studies to use genome-wide copy number alterations (CNA) described evolutionary relationships between pre-diagnostic and relapse clones (Mullighan et al., 2008). Amongst several other conclusions, this study showed that 52% of the relapse clones shared some, but not all CNA identified in the diagnostic clone demonstrating that relapsing is an event evolving from the diagnostic clone into new subclones. They also proved by PCR that a leukaemia can contain genetically diverse subclones and that

therapy allows selection of a dominant relapse clone. Several other studies followed this study and showed similar results (Kuster et al.; van Delft et al.; Yang et al., 2008).

It is difficult to find leukaemia-initiating mutations because they occur mostly in pre-leukaemic clones. Pre-leukaemic cells are clinically undetected and are outcompeted by their malignant counterparts. Some studies have used reverse-tracking strategies to identify pre-leukaemic clones which are both rare and important (Greaves, 2009). Such backtracking studies suggested that leukaemia-initiating events occurred *in utero* and this was identified by chromosomal translocations in blood samples of monozygotic twins (Greaves, 2003). Interestingly, this group identified one of these twins as 'healthy' and the other as 'leukaemic' (Hong et al., 2008). In paediatric ALL, a distinct population ($\text{Lin}^- \text{CD34}^+ \text{CD38}^-$), absent in the healthy twin's BM, was found to have leukaemic potential by xenograft assays (Castor et al., 2005). This led to the hypothesis of clonal evolution of pre-leukaemic sub-clones.

It is still not understood how many hits/ mutations are required to induce a leukaemic clone. Studies in AML have suggested that up to 10 mutations occur in a single clone (Jan and Majeti, 2012). Since most BM cells do not possess self-renewing capacity, the few cells that can self-renew are hypothesised to gain mutations serially, giving rise to a tree of sub-clones among which a dominant clone will survive to regenerate the leukaemia (Fig 1.10).

[I.IV.3.1 Cells of origin](#)

Many authors have recently pointed out that CSCs and cell of origin (CO) are often used interchangeably whereas they might not necessarily be the same entities. CO and CSCs refer to cancer-initiating and cancer-propagating cells, respectively. The CO, the different

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mutations acquired and differentiation potential of cancer cells are the factors that determine whether a cancer follows a CSC model. It is said that in most situations the CO and CSCs differ considerably (Visvader, 2011) (Fig 1.11).

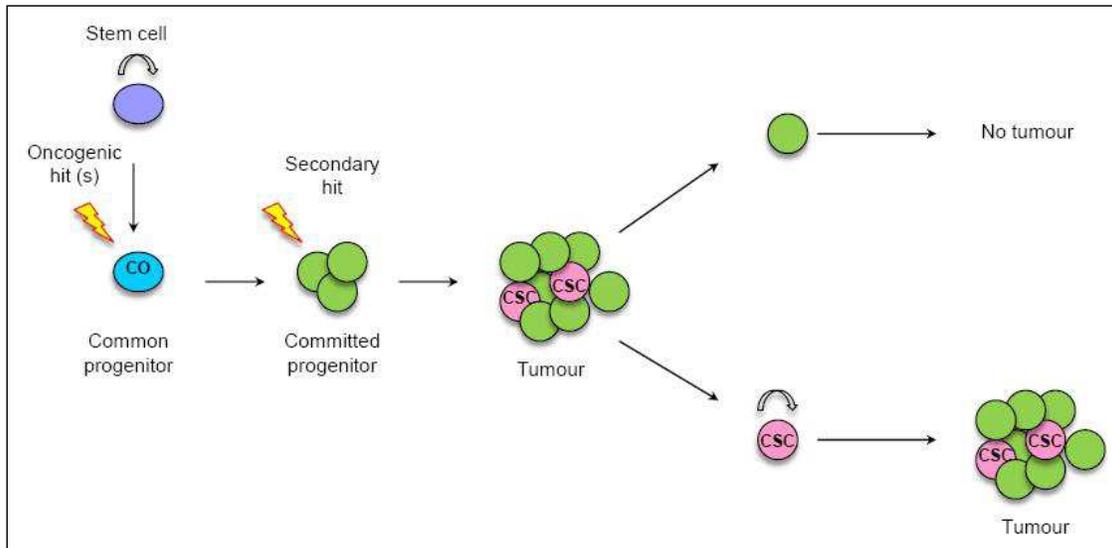


Figure 1.11. The cell of origin and cancer stem cells. Adapted and modified from Visvader *et al*, 2011 (Visvader, 2011). The cell of origin (CO) of a tumour can be an early common precursor and accumulation of oncogenic hits over the hierarchy may result in the emergence of cancer stem cells (CSCs). Thus, CO and CSCs, tumour-initiating and tumour-promoting cells are distinct.

There are two common methods that are used to identify the CO of any cancer: transgenic or conditionally targeted gene technology, and genetic alterations of cells *ex vivo*.

In haematological malignancies, both stem and committed progenitors have been associated as targets of transformation. In AML, it has been shown that the CO shares its phenotype with HSCs (Hope *et al.*, 2004) suggesting that primitive haematopoietic cells are likely to be CO. Studies in chronic myelogenous leukaemia (CML) have shown that BCR-ABL (an oncogene with break point cluster and Ableson tyrosine kinase gene translocation) expression in HSCs can induce myeloproliferative disorders (MPD) while

expression in committed progenitors could not (Huntly et al., 2004). Similarly, in a knock-in mouse model (MLL-AF9 mice), HSCs expressing MLL-AF9 fusion protein (one of the most common chromosomal translocations in AML) were transformed while GMPs transformed with the same fusion protein could not be transformed. However, if the GMPs could be efficiently transformed with higher doses of MLL-AF9 suggesting that oncogene dosage affects susceptibility of cells (Chen et al., 2008). While HSCs have been considered the most likely candidates as the targets of mutations, progenitors have been shown to have altered effects depending on the dose of the oncogene. However, in *Lmo2* over-expressing transgenic mice, it is the pre-leukaemic T-cell precursors that were identified as the CO (McCormack et al., 2010).

Although the relationship between CO and CSCs is not fully understood, cellular analyses of pre-neoplastic and neoplastic cells from different tumours may allow us to discover the link.

I.IV.4 Assays used to study cancer stem cell activity

There are two main technologic approaches that are widely used in LSC characterisation. First, is the fluorescence-activated cell sorting (FACS). Spangrude and colleagues were among the first ones to purify and isolate mouse HSCs (Spangrude et al., 1988) by depletion of several maturation marker-expressing cells, and sorting Sca-1 and c-kit positive cells (i.e. LSK population).

The next most commonly used method is the transplantation assay. To study LSC-activity, cancer cells, both of human or mouse origin, are transplanted into immunologically compromised mice. The xenotransplantation of human cancer cells into mice differs in a number of aspects. The microenvironment of human tissues

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(architecture and stromal cells) is very different from that of the mice (Kuperwasser et al., 2004). Autologous immune cells are shown to impair or promote cancer development (de Visser and Coussens, 2006; de Visser et al., 2006; Manz, 2007). Transplanted human cells result in xenogenic responses killing most of the human cells before they home and proliferate (Auchincloss, 1989). This is basically why cancer cells should be injected in a highly immuno-compromised environment to assay tumorigenic potential. Even the NOD/SCID mice have a weakened immunological barrier. Recently, the usage of NOD/SCID IL2R γ ^{null}, the most immunologically compromised system available, is recommended. However, some scientists argue that less immunologically compromised mice would be ‘better’ models, since they preserve some immune activity mimicking the actual patient’s condition (Magee *et al.*, 2012). No xenotransplantation assay can mimic the immune responses that occur in patients with their own tumours. Therefore, highly immuno-compromised mice (like NOD/SCID and lethally irradiated mice) are thought to be best systems (Eppert *et al.*, 2011; McDermott *et al.*, 2010; Quintana *et al.*, 2008).

Many studies have pointed out that xenotransplantation assays test the potential of cancer cells to develop a tumour and not their actual fate in the original tumour (Fig 1.12). Many environmental parameters, like hypoxia and immune responses, have the potential to slow a tumour progression. Having said this, it has not been proven if these factors are in fact involved in tumour regression. By using the transplantation assay, one can identify a subset of cells that have tumorigenic potential. However, we still do not have any means to prove that it is actually these cells that drive the disease progression *in situ*.

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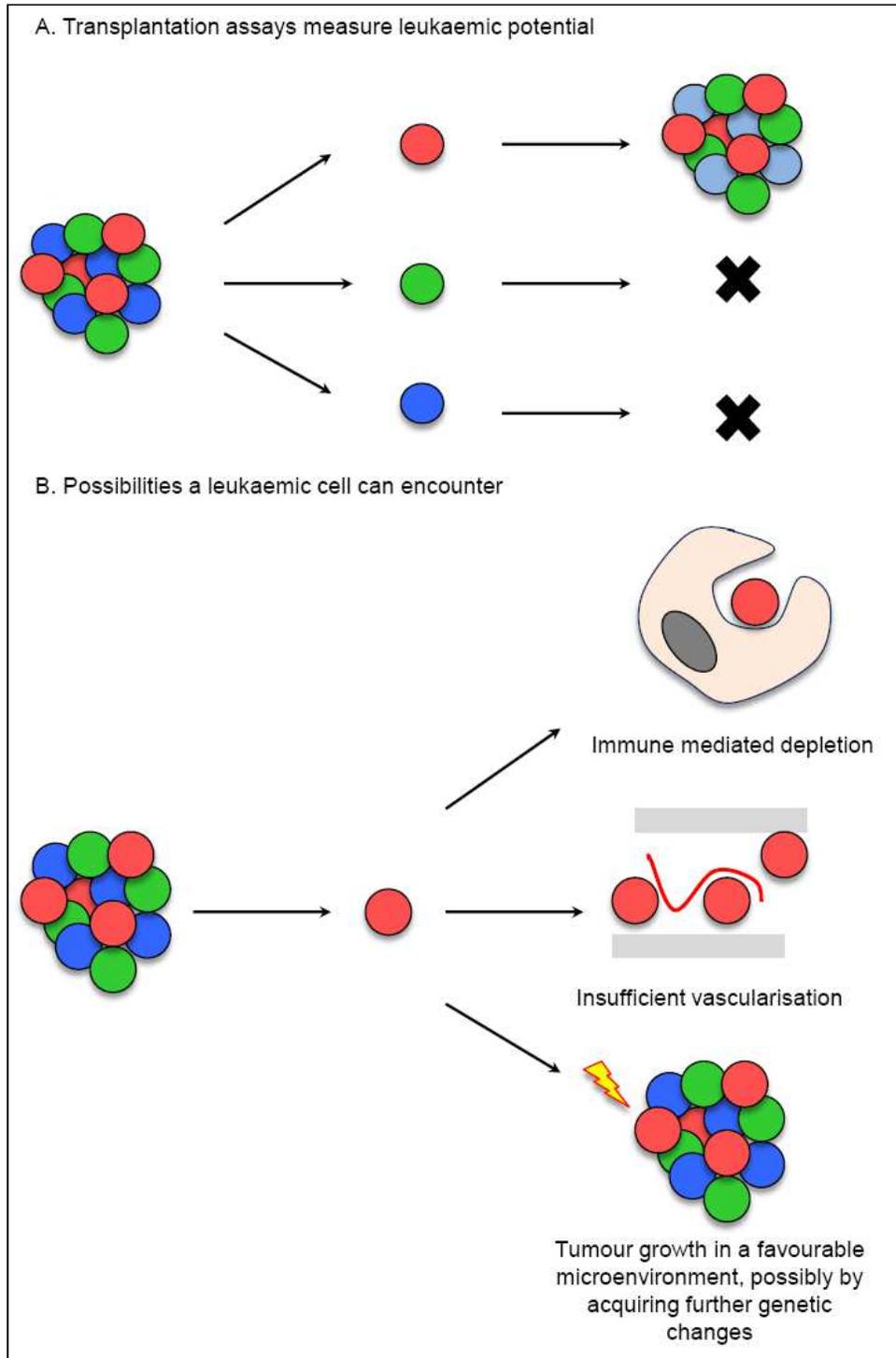


Figure 1.12. Fate versus potential of cancer stem cells. Adapted and modified from Magee *et al*, 2012 (Magee *et al.*, 2012). A) Transplantation assays address the potential of cancer cells to form tumours. Potential is defined as the ability of a cancer cell to regenerate tumours in a given microenvironment. B) Once transplanted, a leukaemic cell can encounter various physiological barriers which is defined as fate. Which cells are fated to contribute to tumour growth in a patient's actual microenvironment? Many cells might have potentials to

regenerate tumours. Transplantation assays only assess the capacity of cells to give rise to tumour and not the actual fate of the cell in a patient's microenvironment.

I.IV.5 Cancer stem cell controversies

The first mentions of CSCs were in the 1970's in leukaemia and the CSC biology has been ever since very highly debated. The recent hype of CSC and clonal evolution of cancer cells is not new. Since the advent of transplantation assays, the major debate has been of what we experiment is actually what happens in a patient's condition. While we are still pondering over this issue, there are several reviews and articles in which the eminent scientists of this field have given their opinions. Many issues are being discussed.

Are CSCs really rare? Numerous studies questioned the recognition of the hierarchical model of CSCs. Evidence of tumour heterogeneity was not found in several experimentally induced murine models (Kelly et al., 2007; Somerville and Cleary, 2006; Williams et al., 2007). These reports question the rarity of CSCs. They argue that the rarity of CSCs observed during transplantation assays might be a result of host resistance factors and absence of cross-species reactivity of cytokines and other microenvironmental parameters. The role of these parameters cannot be excluded, even though direct interactions have not been proven. Some studies have started co-implanting human stromal cells with solid tumours to mimic the human microenvironment as much as possible. It is important to note however, that several murine models are heterogeneous and contain rare and distinct CSCs (Kennedy et al., 2007).

Can the mouse data be 'adequate' to infer human conditions? In a study where human and murine leukaemic models were induced with the same MLL fusion protein, LSC frequencies were the same (Kennedy et al., 2007). The frequencies can vary widely

among cancers, irrespective of whether they are measured in xeno- or a syngeneic transplantation assay. In CSCs studies, it is more important to obtain the functional evidence of tumour heterogeneity. A broader range of human tumour studies will probably help us determine whether or not heterogeneity is the norm in cancer.

I.IV.6 Clinical implications and perspectives

Adult T-ALL patients have less than 40% long-term survival with the current treatments (Pui *et al.*, 2008; Savage, 2011). In these patients, a true leukaemic clone(s) survives the treatment and relapses. The relapsed individuals have a very low chance of survival despite the strong treatments that will follow.

What is the cellular basis of relapse? It is mysterious how clones responsible for relapse behave after treatment. And how are these clones linked to the pre-treatment disease? There are a few possibilities suggested by R. Majeti in a recent review (Jan and Majeti); i) a dominant leukaemic clone survives the treatment and persists after therapy, eventually expanding to a tumour, ii) some treatment-resistant clones can be 'selected' and may result in relapse after treatment, iii) treatment which targets DNA may contribute in genetic evolution of leukaemia resulting in a clone that can relapse and iv) drugs can act on pre-leukaemic cells to induce additional oncogenic mutations resulting in a more resistant leukaemic clone (Fig 1.13).

Currently, cancer cells are treated like entities that have unlimited proliferative potentials that can invade other organs and metastasize. A possibility is that only dissemination of CSCs and not all cells can regenerate a new tumour. The goal of therapies should be to eliminate the core of the tumours, the CSCs (Reya *et al.*, 2001). No doubt the current therapies can shrink metastatic tumours. However, the effects of these therapies are

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transient. This may be because the CSCs are said to acquire mutations as they evolve, making themselves resistant to treatment. It is speculated that CSCs are drug-resistant because they can resemble normal stem cells in expressing high levels of anti-apoptotic factors (Bouwens and De Blay, 1996; Domen et al., 1998; Feuerhake et al., 2000; Peters et al., 1998), or ATP-binding cassette (ABC) transporters (Terskikh et al., 2001; Zhou et al., 2001).

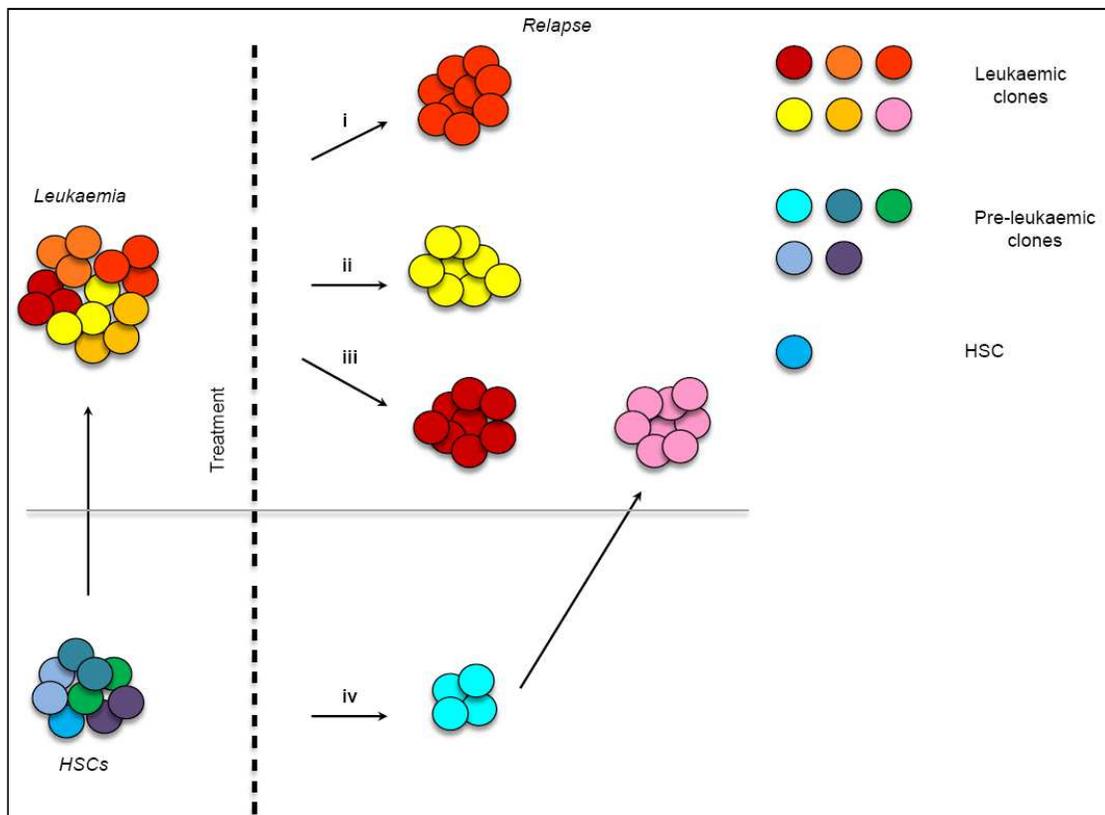


Figure 1.13. Clonal basis for relapse. Adapted and modified from M Jan and R Majeti, 2012 (Jan and Majeti, 2012). Different hypothesis for clonal basis of relapse; i) a predominant clone survives treatment resulting in relapse, ii) a rare resistant clone is 'selected' during the treatment process giving rise to relapse, iii) DNA-damaging therapeutic agents contributes to evolution of novel clones and iv) pre-leukaemic clones can acquire more oncogenic mutations and evolve into leukaemic relapsed clone.

I.V Aim of the study

T-ALL accounts for 15-20% of all the ALL cases (Weerkamp *et al.*, 2006b; Weerkamp *et al.*, 2006c). Given the high relapse rates and low EFS of T-ALL patients, identifying drugs that can destroy cancer cells of T-ALL has been in focus for decades. There is increasing evidence suggesting the existence of subset of cells, termed cancer or leukaemia stem cells (CSCs) as the root cause for relapse of tumours. Research in CSC biology has recently gained importance as it strongly predicts that targeting CSCs might lead to higher rate of EFS. It is known that most T-ALL (>50%) is a result of mutations in the Notch pathway. However, the molecular causes involved in the rest of the T-ALL are not well studied. On the other hand, Wnt pathway is shown to be indispensable for HSC proliferation and functioning. However, the role of this pathway in T-cell development and differentiation has been rather controversial (Cobas *et al.*, 2004; Xu *et al.*, 2003b). Role of Wnt pathway in HSC proliferation, self-renewal and evidence in T-cell differentiation suggested that it is likely for the Wnt pathway to be the underlying cause of some leukaemias.

Our project aims to study the role of Wnt signalling in T-cell development. To better understand the role of Wnt pathway in T-cell development, we generated the R26- β -cat mice expressing stabilised β -catenin protein under the control of CD4 promoter to obtain a T-cell specific Wnt activation. These mice acquired T-cell acute lymphoblastic leukaemia. We have attempted to understand and identify the factors that contribute to this leukaemia formation. Our studies provide evidence that this T-ALL is Notch-independent. Thus, R26- β -cat makes a good model to study Notch-independent T-ALL.

The main aims of this study are;

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- i. To identify the mechanisms and secondary genetic events that co-operate with Wnt pathway to induce this leukaemic progression.

Although controversial, there is accumulating evidence that Wnt pathway plays an important role in T-cell development. To delineate the role of this pathway in T-cell leukaemogenesis, we used the R26- β -cat mice. We hypothesised that several secondary genetic events may establish networks with high Wnt activity that contribute to this leukaemogenesis. Identification of the hidden molecular players that co-operate with Wnt pathway may help reveal new drug targets to regress this tumour and provide better prognosis in the long run.

- ii. To characterise the leukaemia stem cells (LSCs) of R26- β -cat tumours.

LSCs were previously reported in many T-ALL models including that caused by the loss of tumour suppressor PTEN (Guo *et al.*, 2008). High intracellular level of β -catenin was found as an accomplice for the progression of this leukaemia. Hence, we hypothesized that candidate LSC populations may also exist in R26- β -cat model. Given the necessity to identify such cells, we have attempted to characterise the LSCs of R26- β -cat thymic tumours.

I.VI The R26- β -cat model

The R26^{NC/NC} mouse line was generated by Peggy KIRSTETTER in the laboratory of Claus NERLOV at the European Molecular Biology Laboratory, Monterotondo, Italy (Kirstetter et al., 2006). This work has added to the understanding of the role of Wnt pathway in haematopoiesis. Primarily, they designed a mouse model that allowed conditional activation of β -catenin in the haematopoietic system.

The R26-*loxP*Neo/*loxP*- β -catS33Y mouse strain was generated by introducing loxP-flanked stop cassette (Angrand et al., 1999) upstream of a Myc-tagged β -catS33Y cDNA (Kolligs et al., 1999) into the mouse Rosa26 (R26) locus by RecE-RecT recombination (Awatramani et al., 2001). The β -catenin, containing a mis-sense mutation of tyrosine for serine at codon 33 (S33Y) was identified and isolated from a human colon cancer cell line (SW48) (Kolligs et al., 1999). Cre-mediated excision resulted in removal of stop cassette and expression of β -catS33Y from R26 promoter (Fig 1.14 A). In this study R26^{NC/NC} mice were crossed with Mx1-Cre transgenic line in order to express activated form of β -catenin after poly (I:C) induction in the haematopoietic system.

This work demonstrated that the activation of Wnt pathway resulted in over-expression of Wnt target genes in LSK cells. Poly(I:C) induced mice showed hematopoietic failure that included loss of myeloid lineage commitment at GMP stage, erythroid and lymphoid developmental defects and loss of stem cell self-renewal. Specifically, although T-cell subpopulations remained normal, DN cells were relatively fewer in number and showed a developmental block at DN1 stage. Additionally, ETP numbers were significantly reduced.

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Overall, activation of β -catenin in haematopoietic system resulted in multilineage differentiation block and specifically subtle developmental block in T-cells.

In order to investigate the more precise role of β -catenin in T-cell, development, we crossed the $R26^{NC/NC}$ mice with transgenic mice expressing Cre under CD4 promoter to achieve expression of activated β -catenin in T-cells (Fig 1.14 B).

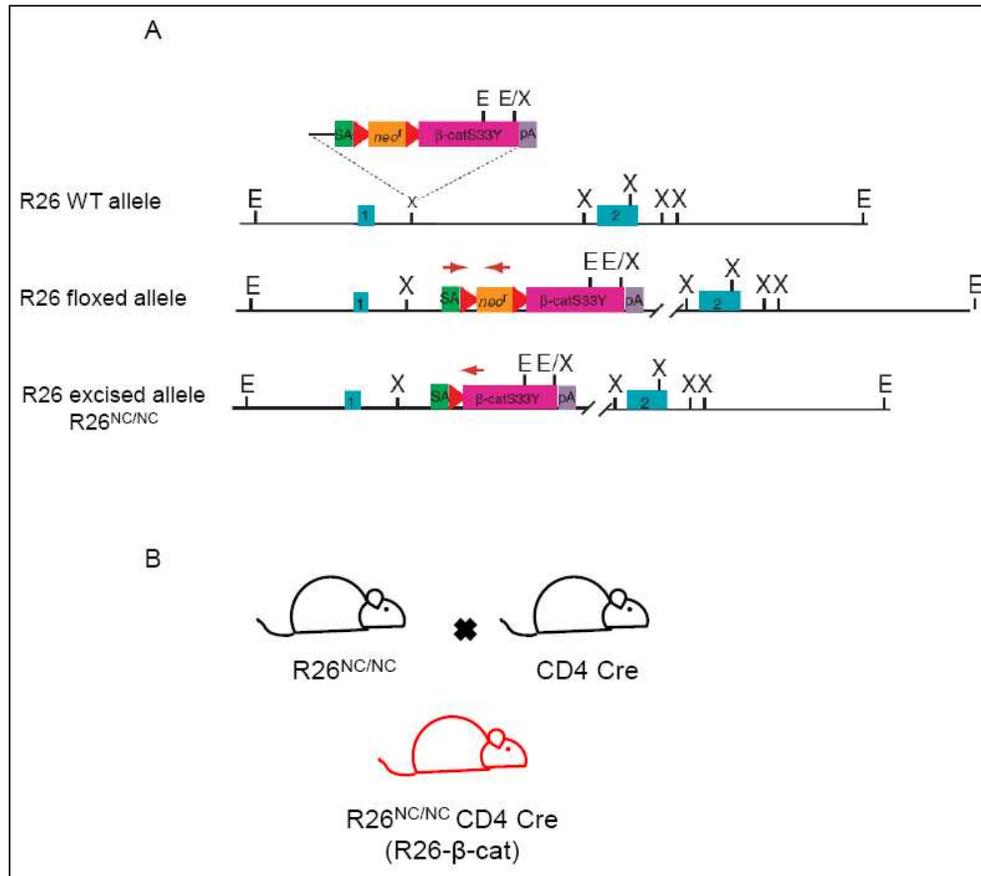


Figure 1.14. Generation of R26- β -cat mice (Kirstetter et al., 2006). A) Structure of R26 locus WT, R26-NC targeted and R26-LC recombined loci after excision of neo^r cassette. Restriction sites are marked by E (EcoRI) and X (XbaI). Red arrows indicated primer positions to verify neo -cassette excision and B) Mice $R26^{NC/NC}$ bred with CD4Cre to generate R26- β -cat model.

II. Overview of the work

T-cell acute lymphoblastic leukaemia (T-ALL) arises from malignant transformation of T-cells. T-ALL accounts for 15% of childhood and 20% of adult ALL cases. Greater than 50% of T-ALL is known to be caused by activation of the Notch pathway. However, some of the molecular players that cause the rest of the T-ALL are yet to be discovered. β -catenin, a master regulator of Wnt pathway, has been reported to be both dispensable and indispensable for T-cell development leaving its role rather controversial. We generated a mouse line, R26- β -cat, expressing a stabilized form of β -catenin under the control of the CD4 promoter. Stabilization of β -catenin resulted in T-cell developmental block at the DN4 and DP stages and prolonged survival of pre-leukemic DP cells, probably creating a favourable condition for acquisition of secondary genetic hits. Additionally, we detected no Notch activation in R26- β -cat tumours and R26- β -cat cell line proliferation was Notch-independent. Thus, the R26- β -cat mouse line serves as a good model to study Notch-independent T-ALL and may define a new sub-group of T-ALL. Furthermore, by inhibiting the Wnt pathway in R26- β -cat cell lines, we demonstrated that Wnt activity was required for the proliferation and expansion of these cells. Moreover, CD44, a Wnt target gene found upregulated in R26- β -cat tumours and pre-leukaemic cells, did not contribute to the leukaemogenesis or homing of R26- β -cat tumour cells. Comparative genomic hybridization (CGH) analysis R26- β -cat tumours revealed that all the R26- β -cat tumours showed persistent aberrations in the enhancer of $TCR\alpha/\delta$ and the 3' region of *Myc* locus. Moreover, we observed by RT-qPCR analysis that the expression of c-Myc was increased only at the leukaemic stages suggesting that *Myc* over-expression may contribute to this leukaemia. In addition, CGH analyses also

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showed that 50% of R26- β -cat tumours showed a deletion in PTEN (phosphatase and tensin homologue on chromosome 10). We demonstrated that loss of PTEN co-operates with the Wnt pathway and contributes to R26- β -cat leukemogenesis.

It is known that only 30-40% of the adult T-ALL undergoes event-free survival (EFS). Most conventional therapies for eradicating cancer aim to eliminate proliferating cells leaving behind the quiescent and drug-resistant cancer/ leukaemia stem cells (CSC or LSC). To increase EFS, it is important to target LSC in therapies. We have also attempted to understand the LSC activity of R26- β -cat tumours. To be able to identify LSCs of this tumour, we first established that these tumours were heterogeneous by limiting dilution assays. We demonstrated that the LSCs were contained in the CD4⁺CD8⁺ cells since these cells alone were capable of re-initiating tumours. Flow cytometry analyses revealed that R26- β -cat tumour cells were phenotypically different when compared to normal T-cells. Staining of R26- β -cat tumour cells by Hoechst dye showed that this tumour contains some side-population (Sp) cells, which are known to display LSC activity. To identify LSCs from the bulk tumour, we attempted to isolate drug-resistant LSCs by 5-fluorouracil (5-FU) treatment. 5-FU administration to R26- β -catenin mice revealed that R26- β -cat leukemic cells were not consistently enriched for cells expressing specific cell surface markers or Sp cells.

Collectively, we report a murine model that expresses a stabilised form of β -catenin in T-cells. Our project provides significant insight into i) the mechanisms that may be involved β -catenin-dependent T-ALL, ii) the underlying molecular mechanisms and iii) provide evidence that β -catenin may be a potential target for the therapies against cancer.

II. OVERVIEW OF THE WORK

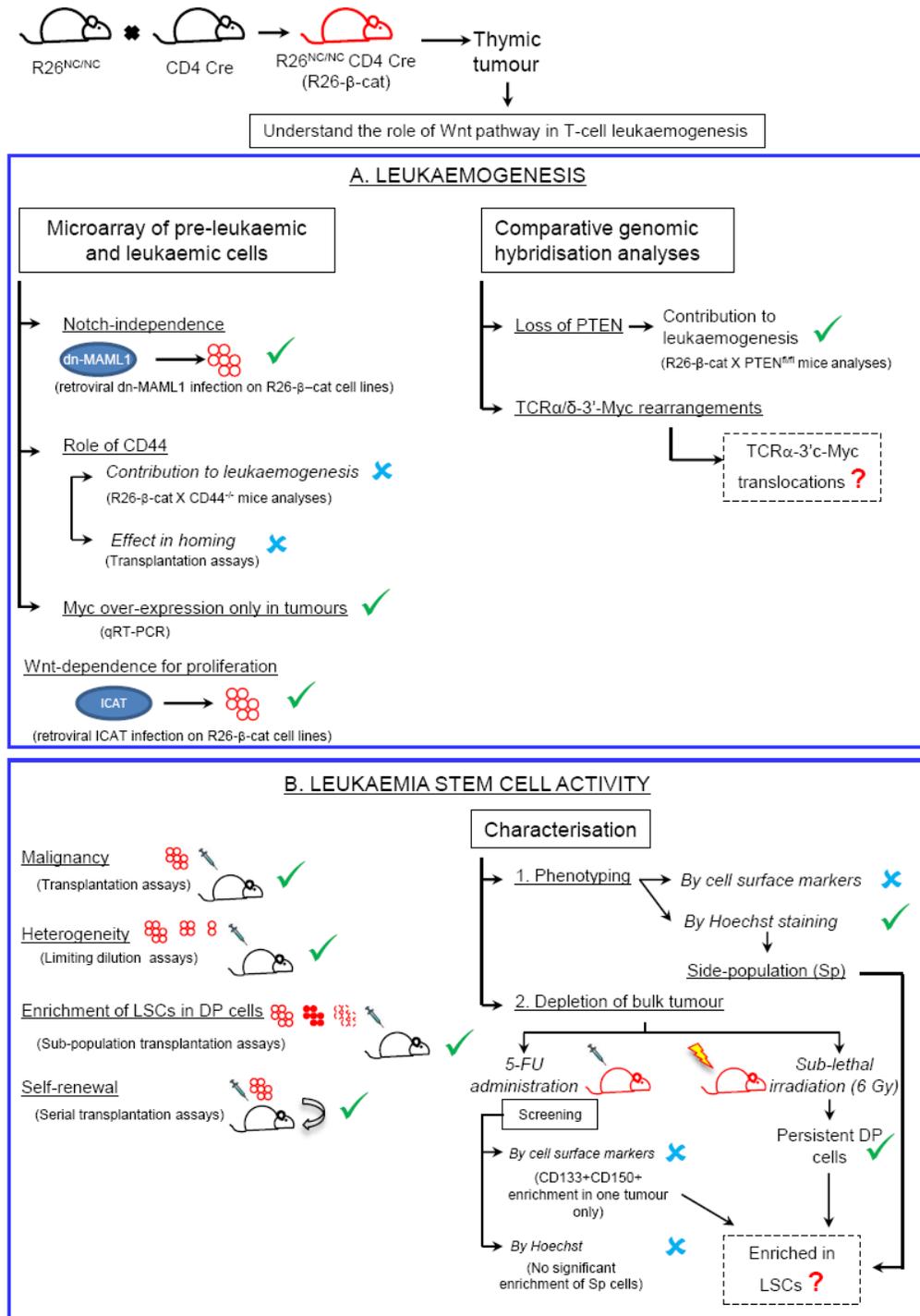


Figure 2.1. Overview of the work. R26-β-cat mice were generated by expressing stabilised β-catenin in T-cells under the control of the CD4 promoter. These mice acquired T-cell acute lymphoblastic leukaemia. We found several molecular players that co-operate with Wnt activity to induce this leukaemia. Additionally, we found that these tumours may contain leukaemia stem cells. Green ticks (✓) indicate that these players have a role in leukaemogenesis and blue crosses (✗) indicate that the respective molecular players may not have a role or the failure to reach conclusive results. Red question marks (?) indicate that these are un-answered questions.

III Results

Multi-step events in β -catenin induced T cell lymphomagenesis

Deepika Kaveri, Claus Nerlov, Philippe Kastner, Susan Chan, and Peggy Kirstetter

More than 50% of T-cell acute lymphoblastic leukaemia (T-ALL) is caused by mutations in the Notch pathway. The molecular regulators for the rest of T-ALL cases are yet to be discovered. Several studies have shown that Wnt pathway may be indispensable for T-cell development. Therefore, Wnt pathway may be one of the causative players for T-cell leukaemogenesis. To determine the role of Wnt pathway in T-cell leukaemogenesis, we have generated a mouse model R26- β -cat, which expresses stabilised form of β -catenin in T-cells under the control of CD4 promoter. In the manuscript, we have reported the identification of the molecular players that co-operate with Wnt signalling to induce this T-cell leukaemia.

Multi-step events in β -catenin induced T cell lymphomagenesis.

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ABSTRACT

Wnt signaling is important to control T cell differentiation at the early double negative DN1-DN2 stages, and is subsequently down-regulated. To assess the importance of this down-regulation, we generated a mouse line (called R26- β cat) in which high levels of active β -catenin was maintained throughout T cell differentiation. Young R26- β cat mice showed a T cell differentiation block at the CD4⁺CD8⁺ double positive (DP) stage. These blocked DP cells exhibited impaired apoptosis upon irradiation or dexamethasone treatment. All R26- β cat mice subsequently developed T-cell leukemia at the age of 5-6 months. R26- β cat leukemias were independent of Notch pathway activation and exhibited recurrent secondary genetic events leading to loss of PTEN and activation of Myc. These results establish that canonical Wnt signaling must be down-regulated in DP thymocytes for their optimal apoptotic responses and to suppress their transformation.

INTRODUCTION

T-cell acute lymphoblastic leukemia (T-ALL) refers to a group of lymphoid disorders that result from the proliferation and expansion of T lymphoid blasts in the bone marrow,

blood and other organs. ALL is the most common childhood acute leukemia (80%) but account for only 20% of adult leukemias. Advances in ALL therapy have led to long-survival rates reaching 75-80% of children. In contrast, only about 30-40% of adults achieve a long-term disease-free survival. A better understanding of the fine molecular mechanisms underlying the biology of ALL is needed to improve existing therapies for a larger number of patients. Despite the involvement of several oncogenic (Notch, Tlx3, Myc, Tal-1, JAK/STAT) and anti-oncogenic pathways (p53, Pten, ARF), an integrated view of their roles in the progression of leukemogenesis is still missing and some players remain unknown. The Wnt/ β -catenin-pathway could be one of the hidden players.

The binding of Wnt ligands to Frizzled receptors activates Wnt signaling. This interaction inhibits the activity of the destruction complex formed by the APC, Axin-1, CK1 and GSK-3 β proteins that phosphorylates β -catenin and targets this protein to the proteasome. The inhibition of β -catenin phosphorylation leads to its stabilization. As a consequence, β -catenin accumulates in the cytoplasm and translocates to the nucleus where it binds to members of the TCF/LEF family of transcription factors, thus modulating expression of a broad range of target genes. The deregulation of this pathway is the hallmark of several types of epithelial cancers (Richards *et al.*, 2012) with two common mutations: inactivation of the tumor suppressor genes APC or Axin-1, or activation of β -catenin by oncogenic mutations that render it resistant to proteolytic degradation. In the hematopoietic system, the activation of the Wnt pathway was also found in chronic (CML) and acute myeloblastic leukemia (AML), as well as in B cell chronic (CLL) and acute lymphoblastic leukemia (B-ALL) (Coluccia *et al.*, 2007; Jamieson *et al.*, 2004; Khan *et al.*, 2007; Muller-Tidow *et al.*, 2004). Epigenetic inactivation of genes encoding several Wnt pathway inhibitors have been observed in AML, B-CLL, as well as in B- and T-ALL (Chim *et al.*, 2008; Jost *et al.*, 2008; Moskalev *et al.*, 2012; Roman-Gomez *et al.*, 2007; Valencia *et al.*, 2009a). Guo *et al.* have reported that the stabilization of β -catenin induces T cells lymphomas in mice (Guo *et al.*, 2007). Moreover Pten deletion in hematopoietic stem cells (HSC) leads to β -catenin dependent T-ALL in mice (Guo *et al.*, 2008). Altogether, these results supported a role of Wnt/ β -catenin pathway activation in T lymphomagenesis.

The Wnt pathway has been implicated in various differentiation processes of hemopoietic cells as well as in T cell development. In the thymus the first lymphoid progenitor immigrants from the bone marrow (BM) are the double negative 1 (DN1) cells that phenotypically belong to CD4⁻CD8⁻CD3⁻CD25⁻CD44⁺ fraction. When these cells commit to the T cell lineage they up-regulate CD25 and become DN2 cells (CD4⁻CD8⁻CD3⁻CD25⁺CD44⁺). They then down-regulate CD44 (DN3 cells: CD4⁻CD8⁻CD3⁻CD25⁺CD44⁻) and finally CD25 (DN4 cells: CD4⁻CD8⁻CD3⁻CD25⁻CD44⁻). DN4 cells progress to the double positive stage (DP; CD4⁺CD8⁺CD3^{lo}) and rearrange the chain β of the T cell Receptor (TCR). $\alpha\beta$ TCR⁺ DP cells undergo negative and positive selections. Selected $\alpha\beta$ TCR⁺ T cells are single positive (SP) CD4⁺ or CD8⁺ cells, which then exit the thymus to circulate and colonize secondary lymphoid organs. Inhibition of Wnt signaling pathway using soluble frizzled receptor in fetal thymus organ cultures or mice deficient for either TCF-1 or β -catenin clearly demonstrated that this pathway is required for thymocytes development (Schilham *et al.*, 1998a; Staal *et al.*, 2001b; Xu *et al.*, 2003a). Surprisingly, mice deficient for γ - and β -catenin did not show T cell development defects (Jeannet *et al.*, 2008; Koch *et al.*, 2008). The lifespan of DP cells is critical for the diversity of T cell repertoire because the longer a T cell survives, more change it has to rearrange the TCR α chain and be positively selected. Experimental inhibitions or over-expressions of β -catenin in T cells have shown that β -catenin mediates enhanced survival of DP cells (Ioannidis *et al.*, 2001a; Wang *et al.*, 2011b). Thus, despite a decrease activation of the Wnt pathway during T cell maturation from the DN to the DP stage, it plays a role in the DP cells (Weerkamp *et al.*, 2006a). All these data thus suggest an important function of Wnt/ β -catenin pathway at multiple stages of T cells development.

To delimit the role of the Wnt pathway in T cell leukemogenesis, we have generated a mouse line (called R26- β cat) expressing an activated form of β -catenin protein in thymocytes. We showed that the activation of β -catenin enhanced the survival of DP thymocytes and led to a developmental block of these cells. With time, the blocked DP cells acquire secondary genetic mutations that lead to their transformation, notably Pten loss and Myc activation.

RESULTS

Regulation of the Wnt pathway is required during T cell development.

We have previously used the R26-NC allele, generated by the knock-in into the Rosa26 locus of sequences encoding a Myc-tagged stable form of β -catenin (S33Y) preceded by a floxed stop cassette (Kirstetter *et al.*, 2006). To determine the consequences of sustained β -catenin activation in T cells, we combined homozygote alleles of R26-NC with the CD4-Cre transgene which expresses the Cre recombinase under the control of the CD4 promoter (Lee *et al.*, 2001). R26^{NC/NC}CD4-Cre^{Tg/+} mice will hereafter be named R26- β cat mice and R26^{NC/NC}CD4-Cre^{+/+} control mice. The level of β -cat-S33Y in total R26- β cat thymocytes was four times higher than that of endogenous β -catenin in control thymocytes (Fig. S1A). Interestingly, the R26- β cat mice expressed the stabilized form of β -catenin in thymocytes from the DN3-DN4 cell stage onwards when endogenous β -catenin expression was down-regulated in control T cells. (Fig. S1C). Importantly the level of β -cat-S33Y in DN3, DN4 and DP thymocytes was similar to that of physiologically expressed β -catenin in DN2 cells (Fig. S1C). The R26- β cat mice therefore allow to determine the consequence of sustained levels of Wnt signaling during T cell differentiation. Flow cytometry analysis of young R26- β cat mice (6-weeks old) showed a reduced number of SP thymocytes (5-fold less CD4⁺ and 2-fold less CD8⁺ cells) (Fig. 1A, B) together with an increase of DP cells, without change in the total number of thymocytes compared with control mice (Fig. S1B). This data thus reveal a block of T-cell development at the DP cell stage. The developmental block in the thymus was reflected in peripheral organs like the spleen and lymph nodes by low number of mature T cells (8- and 2.5-fold fewer of CD4⁺ and CD8⁺ respectively) (Fig. 1A, C). Intriguingly, the analysis of the DN compartment of R26- β cat mice showed an 8-fold increase of a 'DN1'-like population and a reduction of the DN3 population when compared with control mice (Fig. 1D, E). Since the activity of the Cre recombinase starts at the DN3-DN4 cell stage, the accumulation of the 'DN1'-like cells was unlikely to result from the β -cat-S33Y expression in DN1 cells (Fig. 1F). Additionally, the CD44 gene is known to be a target of β -catenin/Tcf-4 (Wielenga *et al.*, 1999a). The CD44 expression might thus be increased in DN4 cells as a consequence of high β -catenin expression in these cells. Accordingly, the 'DN1'-like cells had completely excised the floxed cassette (Fig. 1F) and expressed high level of intracellular

β -catenin (Fig. S1C). We conclude that expression of a stable form of β -catenin in T cells from the DN3-DN4 stage onwards leads to the expansion of an abnormal CD44-positive DN4 compartment and a subsequent block of T cell development at the DP stage.

Since Wnt signaling has been implicated in the regulation of thymocyte survival, we tested the apoptotic responses to sub-lethal irradiation and dexamethasone (Dex) treatment, which are known to induce massive apoptosis of DP thymocytes. Strikingly, pre-leukemic R26- β cat mice showed abnormal persistence of DP thymocytes 48 hours after sub-lethal irradiation (6.5 Gy) when compared with control mice (Fig. 1G). In addition, no increase in the number of Annexin-V positive DP cells was seen in R26- β cat mice 6 hours after Dex treatment (Fig. 1H). Resistance to apoptosis of DP cells resulted in an increased of DP population in the thymus of R26- β cat mice 12 hours after Dex treatment when compared with control mice (Fig. 1I). Thus the constitutive expression of β -catenin in thymocytes increased survival of the DP cells.

Activation of β -catenin leads to development of T cell leukemia.

R26- β cat mice had a reduced lifespan, and died from T cell leukemia between 20 and 40 weeks of age (Fig. 2A). R26- β cat mice that became moribund around the age of 24 weeks developed only thymic lymphoma, whereas the mice that fell sick later (around 30 weeks of age) showed also transformed cells in the spleen, lymph nodes, blood and bone marrow (BM) (Fig. 2B and data not shown). Transformed cells present in the T cell leukemias exhibited mainly a DP phenotype (Fig. 2B) associated with high levels of extracellular CD3 and TCR β expressions (Fig. S2A). PCR analysis of the D β 2-J β 2 rearrangement of TCR β chain showed that the majority of the R26- β cat tumors were mono- or oligo-clonal (Fig.S2B). The R26- β cat mouse line demonstrated thus the oncogenic potential of constitutive activation of β -catenin in T cells and provides an in vivo model to study the contribution of this pathway to T-ALL.

We assessed the ability of R26- β cat tumors to re-initiate leukemias after transplantation. We transplanted 10^4 or 5×10^4 R26- β cat tumor cells (CD45.2⁺) along with 2×10^5 supporting wild type bone marrow cells (CD45.1⁺) into lethally irradiated mice (CD45.1^{+/2+}).

Transplanted mice were followed and sacrificed when clinical signs of T cell leukemia appeared. BM, thymus and spleen from moribund recipient mice were composed of 70-90% CD45.2⁺ leukemic cells with a DP phenotype similar to that of the primary tumor (Fig. S2C), showing the malignant potential of R26- β cat tumors. To determine if R26- β cat tumors were heterogeneous in their cellular constituents and if the self-renewal potential is restricted to a subset of cells within each tumor, we use serial dilution transplantation assays. We transplanted 100, 500, 2×10^3 and 10×10^3 of total tumors cells from R26- β cat lymphomas together with 2×10^5 radioprotective wild type BM cells into lethally irradiated mice. All the mice transplanted with at least 2000 of cells have developed leukemia whereas leukemia was detected in only one out of seven mice transplanted with 500 cells (Fig. 2D). Similar results were obtained with serial dilution transplantation assays using purified DP leukemic cells (data not shown). These results indicate that the tumors associated with β -catenin activation were heterogeneous and that only few cells are able to re-initiate leukemia after transplantation.

R26- β cat leukemic cells are Notch-independent and require Wnt pathway activation to proliferate.

To identify the molecular pathways involved in R26- β cat tumors, we established a global profiling of gene expression from leukemic and pre-leukemic thymocytes using Affymetrix microarrays. Six-week-old mice with a thymus of normal size and a polyclonal population of thymocytes were used to establish the pre-leukemic transcriptome. The expression profile of R26- β cat leukemic cells was compared with the profile of DP cells from non-transformed pre-leukemic and control mice. This comparison showed that the transcriptome of leukemic cells is significantly divergent from those of control cells, with more than 1800 genes deregulated (3 fold increase or decrease expression compare with control; $p \leq 0.01$, Student's t-test) in the leukemic cells (Fig. 3A). Within these genes, only few are known to be direct targets of the Wnt pathway. In contrast to the leukemias, only 142 genes were deregulated (2 fold increase or decrease; $p \leq 0.05$) in R26- β cat pre-leukemic DP cells, suggesting that the activation of the transcriptional program of leukemic cells is a late event (Fig. 3A and Table S1). We found only 11 deregulated genes shared between the pre-

leukemic and the leukemic DP cells. Altogether, these observations suggest that the expression of the activated form of β -catenin is not sufficient to activate the tumor-specific genetic program in thymocytes and that secondary genetic events are required for the T-cell transformation. To determine if the β -catenin dependent gene expression signature is related to any physiologically relevant transcriptional program, we investigated if these genes are normally regulated during T-cell differentiation, using publicly available data of various thymocytes subsets. Notably, we found that β -catenin deregulated genes contained many genes that exhibit the same expression in early positive selection DP cells ($CD4^+CD8^+CD69^+TcR\beta^{lo/int}$). These data suggest that the differentiation block induced by β -catenin activation occurs at the stage of positive selection of T cell maturation.

Interestingly, Notch1 and Notch3, as well as the target Notch genes *Dtx1*, *Nrarp* and *Il2ra* were down-regulated in R26- β cat transformed cells (Fig. 3B). Down regulation of *Il2ra* was also supported by the lack of expression of CD25 (encoded by *Il2ra* gene) on the few immature DN cells found in R26- β cat tumors (Fig. 2C). The very low mRNA expression of Notch1 and Notch3 was confirmed by RT-qPCR (Fig. 3C). To confirm that Notch signaling was not required for the proliferation of R26- β cat leukemic cells, we inhibited this pathway in a tumor cell line (149.4) derived from a R26- β cat tumor. We expressed a dominant negative form of Mastermind like 1 (a co-activator of Notch pathway), dnMAML1 (Maillard *et al.*, 2008), using retroviral transduction (Fig. 3D). dn-MAML1 expression did not affect the survival and proliferation of these cells. In contrast growth of the Notch-dependent T-ALL cell line T29 (derived from an Ikaros-deficient leukemia) (Dumortier *et al.*, 2006b) was strongly inhibited by dn-MAML1 expression. These experiments indicate that R26- β cat leukemia are independent of Notch signaling.

We next asked if the activation of the Wnt pathway remained important for the survival and proliferation of R26- β cat leukemic cells. To this aim, we expressed ICAT (catenin beta interacting protein 1), a negative regulator of Wnt signaling that inhibits the interaction between β -catenin and TCF (Tago *et al.*, 2000), in cell lines derived from R26- β cat tumors. Several cell lines derived from primary R26- β cat tumors (149.4, 149.8 (Fig. 3E, F) and data not shown) were transduced with a retroviral vector expressing an ICAT-GFP fusion protein (Mig-ICAT) or GFP alone (Mig-GFP). Two days after transduction, ICAT expressing cells

(GFP⁺) and untransduced cells (GFP⁻) were purified, mixed at a ratio of 1:1 and co-cultured. After 8 days of co-culture, the cells expressing ICAT-GFP had disappeared indicating that inhibition of the Wnt pathway in leukemic cells leads to a strong selective disadvantage (Fig. 3E, F). In contrast, ICAT expression did not affect the expansion of the Notch-dependent T29 cells (Fig. 3F). These results suggest that the activation of the Wnt pathway is not only required to initiate the transformation but remains necessary in established leukemic cells for their viability and proliferation.

CD44 is not required for the development or engraftment of R26- β cat T cell leukemias.

Among the genes induced in both pre-leukemic and leukemic DP cells, the one encoding the CD44 surface glycoprotein was particularly interesting. CD44 protein was strongly expressed on the cell surface of pre-leukemic DP cells and even at higher levels in leukemic cells when compared with control DP cells (Fig. 4A). This sequential up-regulation of CD44 was also observed in the DN compartment of pre-leukemic and leukemic cells from R26- β cat mice (Fig. 1D and 2C). CD44 is a known direct target gene of the Wnt pathway in colorectal cancer (Marhaba *et al.*, 2008a) and deletion of CD44 attenuates tumor initiation in a mouse model of colon cancer (Zeilstra *et al.*, 2008b). Interestingly CD44 was also characterized as a marker of cancer stem cells in diverse cancers (Marhaba *et al.*, 2008a). Moreover Jin *et al.*, show that the targeting of CD44 with a specific antibody eradicates cancer stem cells in acute myeloid lymphoma in human, suggesting that CD44 is a potent therapeutic target to eliminate cancer stem cell (Jin *et al.*, 2006b). In order to investigate the role of CD44 in the oncogenic process of R26- β cat lymphoma, we crossed the R26- β cat mice with mice deficient for CD44 (Protin *et al.*, 1999a). In R26- β cat/CD44^{-/-} loss of CD44 mRNA and protein expression in thymocytes was confirmed by RT-PCR (data not shown) and flow cytometry (Fig. 4C). Unexpectedly, the R26- β cat/CD44^{-/-} mice succumbed to T-cell lymphoma at a similar age as R26- β cat/CD44^{+/+} mice (Fig. 4B). The CD4 and CD8 profile of these tumors was also comparable to that of tumors from R26- β cat/CD44^{+/+} mice (Fig. 4C).

These experiments show that CD44 deletion is not affecting development of T cell leukemia dependent on Wnt pathway activation.

Because several studies suggest that homing of acute myeloid leukemia stem cells to the bone marrow (BM) and subsequent engraftment is CD44 dependent (Jin *et al.*, 2006b; Siapati *et al.*, 2011), we tested the engraftment capacity of R26- β cat/CD44^{-/-} tumor cells. Two distinct R26- β cat/CD44^{-/-} lymphomas (CD45.2 allotype) were analyzed. 10⁴ and 5x10⁴ R26- β cat/CD44^{-/-} tumor cells were transplanted into lethally irradiated CD45.1^{+/2+} recipients, along with 2x10⁵ CD45.1⁺ wild type BM cells to provide radioprotection. Mice transplanted with R26- β cat/CD44^{-/-} cells developed leukemia and died within 40 to 50 days (Fig. 4B), similar to mice transplanted with R26- β cat tumor cells. These results showed that CD44 expression is not necessary for engraftment of R26- β cat tumor cells. Analysis of BM, thymus and lymphoid organs from transplanted mice showed that transformed R26- β cat/CD44^{-/-} cells are able to engraft properly and re-initiate leukemia in host mice (97% of CD45.2⁺ in the BM and 50% in the thymus) (Fig. 4E). Leukemic cells found in different organs exclusively exhibited a DP phenotype. Collectively these data showed that establishment and progression of T cell leukemia in R26- β cat mice is independent of CD44.

Chromosomal aberrations leading to MYC over-expression in R26- β cat T-ALL.

In order to identify secondary genetic aberrations in R26- β cat tumors, we analyzed ten R26- β cat tumors by comparative genomic hybridization (CGH) arrays. We found several chromosomal regions lost or gained in more than 50% of R26- β cat tumors (Table S2). Interestingly, 50% of the R26- β cat tumors analyzed showed an amplification of the 3' end of chromosome 15 (Table S2). Moreover we noticed that six tumors showed a deletion in the 3' region of *Myc* (#794, #187, #035, #049, #031 and #296) and three other tumors an amplification (#157, #083 and #164) of the *Myc* gene on chromosome 15 (Fig. 5A). Deletion of 3' region of *Myc* may lead to the up-regulation of this oncogene by its translocation to positive regulatory regions, like the enhancer region of TCR α/δ . To test if the *Myc* downstream deletion or amplification affected its transcription, we performed RT-qPCR analysis. Strikingly *Myc* was over-expressed in β -catenin leukemic cells but that its

expression was normal in the pre-leukemic cells when compared with control thymocytes (Fig. 5B). Concurrently, these results strongly suggest that Myc over-expression in R26- β cat tumor is not due to a direct transcriptional control by the Wnt pathway (Myc is a known Wnt target) but is rather the result of a secondary chromosomal rearrangements or amplifications. Moreover, we found that all the ten R26- β cat tumors carried a deletion in the 3' part of the TCR α/δ cluster (Fig. S3). Altogether, these results showed that various chromosomal aberrations are present in R26- β cat tumors, which leads to the over-expression of Myc oncogene.

Pten deletion cooperates with β -catenin stabilization in R26- β cat leukemias.

Our CGH array analysis also revealed that half of the tumors analyzed bear partial or total (tumor #296) deletion in the Pten tumor suppressor gene (Fig. 6A). To confirm this observation, we analyzed Pten loss in these R26- β cat tumors by RT-qPCR. All the R26- β cat tumors showing genomic deletions for Pten, as well as two tumors with normal Pten gene copy numbers (#157 and #188), expressed reduced level of Pten when compared with control thymocytes (Fig. 6B). Moreover, PTEN protein levels in these tumors were also diminished when compared with control cells (Fig. 6C). Only #049 and #083 tumors cells expressed PTEN levels comparable to control thymocytes, suggesting that Pten deletion is not the only mechanism leading to this down-regulation. Furthermore we observed that cell lines established from R26- β cat tumors, which initially expressed PTEN, had selectively lost PTEN expression (Fig. 6D) suggesting that Pten loss provides a selective advantage. These data suggest that Pten deletion frequently occurs in R26- β cat leukemias and could cooperate with the oncogenic process of transformation.

To investigate the synergy between the loss of Pten and β -catenin activation, we crossed floxed alleles of Pten (Suzuki *et al.*, 2001) into the R26- β -catenin background to allow the deletion of Pten in thymocytes by the CD4-Cre transgene, concomitantly with the activation of β -catenin. Pten^{fl/fl} R26- β cat mice developed T cell lymphoma and died within 12 weeks (data not shown) as observed previously for Pten^{fl/fl} CD4Cre^{Tg/+} mice (Hagenbeek and Spits, 2008). Interestingly Pten^{fl/+}R26- β cat mice deleted for only one allele of Pten showed

accelerated T-cell leukemogenesis (Fig. S6) when compared with $Pten^{+/+}$ R26- β cat mice and died within 24 weeks (Fig. 6E). These data demonstrate that $Pten$ deletion cooperates with the expression of stable β -catenin in T-cell leukemogenesis. Expression of PTEN was also analyzed in $Pten^{fl/+}$ R26- β cat mice by western blot. Young $Pten^{fl/+}$ R26- β cat mice (6-weeks old) without sign of leukemogenesis showed reduced level of PTEN in total thymocytes compared with $Pten^{+/+}$ control thymocytes. Strikingly, all the $Pten^{fl/+}$ R26- β cat tumors analyzed expressed very low or no PTEN protein indicating that the second allele of $Pten$ was lost or silenced in most of the tumors cells. Overall these results demonstrate that $Pten$ deletion synergizes with β -catenin activation during the oncogenic process of R26- β cat T cell leukemia.

DISCUSSION

In this report, we provide clear evidence that constitutive activation of β -catenin from DN3-DN4 stage onwards leads to a developmental block of thymocytes at the DP stage and to T-ALL in older mice. Interestingly R26- β cat tumors are independent of Notch pathway activation. In agreement with our results Guo *et al.* obtained similar data using the CD4Cre-Ctnnb1^{A_{ex3}} mice, which express another stable form of β -catenin in thymocytes, reinforcing the fact that β -catenin activation causes T cell transformation. Our studies showed that R26- β cat tumors cell were found not only in the thymus but also in peripheral lymphoid organs arguing that β -catenin activation caused T cell leukemia in mice. Human T-ALL is a highly heterogeneous group of diseases, which comprises a large number of different subtypes, with Notch activation occurring in >50% of cases. Our results and those from other groups (Guo *et al.*, 2007) suggest that Wnt pathway activation may characterize a new subset of T-ALL, independent of Notch activation.

We observed an additional developmental defect at the DN4 cell stage in the R26- β cat mice compared with CD4Cre-Ctnnb1^{A_{ex3}} mice. This difference could be in part explained by the fact that we started to detect high level of β -catenin earlier. In R26- β cat mice high level of β -catenin were observed in DN3 cells concomitantly with recombined R26 allele. At the DN4

cell stage most of the R26- β cat cells bear a fully excised R26 locus leading to a significant higher expression of intracellular β -catenin in these cells whereas CD4Cre-Ctnnb1 ^{Δ ex3} mice showed higher level of β -catenin only in DP cells. The exact stage of this early defect was difficult to determine since CD44 expression was up-regulated in DN3 and DN4 cells. As a consequence, expression of CD44 in DN4 R26- β cat cells rendered these cells phenotypically similar to DN1 cells. Reminiscent with our data for DN3-DN4 cells, intermediate Wnt activation resulted in reduced percentage of DN3 and a burst of DN4 thymocytes suggesting that Wnt activity enhances immature stages of thymocytes (Luis *et al.*, 2011b).

The CD44 cell surface protein, a known therapeutic target in several cancers and leukemia, was highly expressed in normal and leukemic R26- β cat T cells. Mutations inactivating the tumor suppressor Apc (APC^{Min/+}) or activating β -catenin cause constitutive activation of Wnt pathway and initiate colorectal cancer (CRC). The expression of CD44 is dramatically increased in aberrant crypt foci in both humans and tumor-susceptible APC^{Min/+} mice, suggesting a role for CD44 in intestinal tumorigenesis (Zeilstra *et al.*, 2008b). Deletion of CD44 in APC^{Min/+} mice attenuates intestinal tumorigenesis. These results showed that CD44 does not affect proliferation of intestinal stem cell but increase apoptosis. Surprisingly, deletion of CD44 in R26- β cat mice did not reduce T cell lymphoma development. However, we did not investigate apoptosis of CD44^{-/-}R26- β cat T cells. CD44 cell surface glycoprotein regulates growth, survival, migration and differentiation and thereby is prone to be involved in tumor migration and metastasis. Indeed, homing of AML-LSC to their niche and subsequent engraftment is CD44-dependent (Jin *et al.*, 2006b), demonstrating that CD44 signaling plays a pivotal role in AML. Even if CD44 expression has been reported for several subgroups of T-ALL (Kindler *et al.*, 2008; Vaskova *et al.*, 2005), its role remained controversial in this type of leukemia. Our results showed that CD44 deletion did not impair tumor development as well as homing and engraftment of R26- β cat tumor cells. Hence, the high level of CD44 expression in these tumors cells might occur as a neutral consequence of Wnt pathway activation, without a major role in tumor cell biology.

In this report we showed that β -catenin activation enhances the survival of DP thymocytes after sub-lethal irradiation or dexamethasone treatment. These data are in agreement with previous findings showing that Wnt signaling regulates thymocyte

apoptosis. For instance, deletion of Tcf1 leads to massive apoptosis of DP thymocytes (Ioannidis *et al.*, 2001a) indicating a physiological role for Wnt signaling in the regulation of thymocytes survival. Wang *et al.* have also shown that β -catenin/Tcf1 pathway regulates the expression of Bcl-x_L, a key regulator of DP cell survival (Chao and Korsmeyer, 1997; Ma *et al.*, 1995; Wang *et al.*, 2011b). Thus, Wnt pathway needs to be tightly regulated in DP cells because ablation of Wnt signaling leads to massive death of these cells whereas over-activation of this pathway increases their survival. However, the mechanisms regulating such fine-tuning remain elusive. Enhanced lifespan of DP thymocytes may also generate an auspicious context for the acquisition of additional oncogenic mutations.

Interestingly, β -catenin activation was described as a secondary event in murine T cell leukemia induced by the deletion of the tumor suppressor Pten (Guo *et al.*, 2008). Conversely, secondary inactivation of Pten or more generally inactivation of PI3K/AKT/mTOR pathway could cooperate with primary activation of the Wnt pathway in oncogenic processes. This hypothesis was sustained by the reduced expression of the PTEN protein and the Pten deletion observed in 50% of the R26- β cat tumors analyzed. Moreover genetic ablation of one allele of Pten synergized with β -catenin activation in R26- β cat T cell leukemia in which a loss of Pten heterozygosity was often observed. Recently, cooperation of β -catenin activation and Pten loss have been described in several other cancers like colorectal and bladder cancer (Ahmad *et al.*, 2011; Richards *et al.*, 2012) as well as in follicular cell tumors (Byun *et al.*, 2011). Moreover, the Notch-1 pathway was not altered in the T-ALL which developed in Pten-deficient mice (Guo *et al.*, 2008). Reciprocally, recent studies of a group of pediatric T-ALL patients have shown that Notch-1 activating mutations seemed to have a lower incidence of Pten/Akt aberrations (Zuurbier *et al.*, 2012) suggesting that Notch-1 activating mutations and Pten/Akt mutations are associated with different T-ALL subgroups. Altogether these observations suggest that Pten loss and β -catenin activation may contribute to leukemogenesis in a T-ALL subgroup defined by lack of Notch-1 mutations and Pten deletions.

Our work also showed that recurring genetic aberrations in the 3' region of the Myc locus lead to increased level of MYC in R26- β cat T-ALL. These findings suggest that Myc is not induced as a result of Wnt pathway activation, even though it is known to be a Wnt

target gene in CRC (van de Wetering *et al.*, 2002b). Rather, Myc activation occurs through secondary genomic rearrangements. Interestingly ablation of Myc in CD4Cre-Ctnnb1^{Δex3} mice suppressed lymphomagenesis (Guo *et al.*, 2007). Additionally T-ALL from Pten-deficient mice, which exhibited activated β-catenin, frequently carried translocation bringing together the enhancer region of the TCRα and the 3' region of the Myc locus (Guo *et al.*, 2008). Interestingly in R26-βcat tumors we also observed chromosomal deletions in the 3' part of the TCRα/δ cluster suggesting that chromosomal breakpoint occurred frequently near the TCRα enhancer. We show that Pten loss and Myc activation are also frequent secondary events in tumors where the initiating event is β-catenin activation. The convergence of these three genetic events during T-ALL progression suggests that they elicit biologically complementary effects. Thus, β-catenin activation may primarily regulate survival, while Pten loss and Myc activation may regulate cell growth and metabolism.

We also show that inhibition of the Wnt pathway activation in R26-βcat leukemic cell lines using ICAT, a small molecule that specifically disrupts β-catenin and TCF interaction, inhibited the growth of these cells and led to their disappearance. Consistently with this result, the expression of ICAT under the lck promoter (ICAT-Tg) enhanced thymocyte apoptosis (Hossain *et al.*, 2008a). Hence, inhibition of Wnt pathway alone in R26-βcat tumor cells is sufficient to suppress the proliferation of transformed cells even after they had acquired secondary mutations leading to Pten deletion or Myc over-expression. We hypothesized that Wnt pathway is required for the onset and maintenance of leukemic initiating cells whereas secondary events are necessary for the propagation and expansion of leukemic cells. Interestingly, targeting Wnt pathway with molecules inhibiting β-catenin and TCF interaction could constitute therapeutic tools for T-ALL dependent on Wnt activation.

Our transplantation assays of leukemic R26-βcat cells have shown that R26-βcat tumors are heterogeneous and that only a few cells were able to re-initiate the leukemia in host mice. This result strongly supports the existence of rare leukemic stem cells (LSC) in this T-ALL. Guo *et al.* also described the existence of LSCs in T-ALL from Pten deficient mice (Guo *et al.*, 2008). Moreover β-catenin activation is associated with LSC formation and T-ALL development in this mice model. This suggest that β-catenin activity is not only required for the propagation of leukemic cells in T-ALL but is also required for the formation and the

self-renewal of these LSCs. The fact that most anticancer therapies are directed against the bulk of the tumor, and possibly spare the LSCs, may lie at the heart of treatment failures with conventional modalities or relapses. Hence, characterization of LSCs is essential to define efficient targeted therapies. The R26- β cat mice constitute therefore an excellent model to identify and characterize such LSCs.

Finally, the implication of Wnt/ β -catenin pathway in several mouse models of T-ALL suggests that this pathway may also contribute to human T-cell leukemogenesis. Our study suggest that T-ALL lacking Notch1 activation and exhibiting Pten loss as well as Myc over-expression may be candidates to analyze for Wnt pathway activation.

MATERIALS AND METHODS

Mice.

R26^{NC/NC} (Kirstetter *et al.*, 2006), CD4-Cre^{Tg/+} (Lee *et al.*, 2001), CD44 deficient mice CD44^{tm1Hbg/J} (hereafter called CD44^{-/-} mice)(Protin *et al.*, 1999a) and floxed Pten (Suzuki *et al.*, 2001) mice have been described previously. Mice were killed at different time points after birth to monitor T-cell development and to monitor the onset of T-cell leukemia. Clinical signs of T-cell leukemia included lethargy, ruffling of fur, hunched posture, shallow breathing and social isolation. Mice with the R26-NC and R26-LC allele were analyzed as previously described (Kirstetter *et al.*, 2006). CD44^{tm1Hbg/J} mice were genotyped by PCR with primers as follows forward 5'-ACCCAGAGGCATACCAGCTG-3', reverse 5'-TGCCCAGCAAACCTTTCTTCT-3' for the WT allele and 5'-TGCTGCAAGGCGATTAAGTT-3' for the null allele and mice with floxed Pten with primers forward 5'-TGGATTCGACTTAGACTTGACCT-3' and reverse 5'- GCGGTGTCATAATGTCTCTCAG-3'.

Cell lines and culture.

Cell lines derived from primary tumors were grown in RPMI 1640, 25mM Hepes, 10% FCS, 1mM sodium pyruvate, 1% antibiotics and 50 μ g/ml gentamicin. Cells were frozen at an early

passage (usually after 1 or 2 weeks of amplification), and were thawed and used for a 1-month period. For these experiments, the cells were plated at 10^6 cells/ml and the medium was changed every day. To generate ICAT-expressing 149 cells, we first cloned the Icat cDNA obtained by RT-PCR from wild type thymus DNA (using 5'-GAAGATCTGCCACCATGAACCGCGAGGGAGCA-3' and 5'-GGAATTCGAAGCTTGCTGCCTCCGGTCTTCCGT-3' primers) into the MigR1 retroviral vector (provided by W. Pear). High titer of ICAT and dn-MAML1 (Mig-dnMAML1 provided by W. Pear) retroviral supernatants were produced after transfection in the Eco-Phoenix packaging cell line (provided by G. Nolan). To generate 149.4-dn-MAML1, 149.4 and 149.8-ICAT cells, cell lines were transduced according to standard protocols. After 24 hours, the dn-MAML1-GFP⁺ cells were analyzed on BD FACSCalibur™ everyday and the ICAT-GFP⁺ and -GFP⁻ cells were sorted on BD FACSAria™II and co-cultured in a ratio of 1:1 for 8 days. During the co-culture, cells were counted and analyzed by flow cytometry daily.

Flow cytometry and cell sorting.

Single cell suspension of thymuses, spleen and bone marrow from mice were prepared and stained with fluorochrome- or biotin-conjugated antibodies. Antibodies and reagents were purchased as follows: BD Pharmingen, anti-CD3 (145-2C11, Bio), anti-CD4 (RM4-5, Bio or PE), anti-CD8 (53-6.7, Bio or FITC or PerCP-Cy5.5), anti-CD25 (PC61, PE or APC), anti-CD44 (IM7.8.1, Bio or FITC or PECy7), anti-TcR β (H57-597, Bio), anti-CD45.1 (A20, PECy7) and Streptavidin-PECy7; Biolegend, anti-CD45.2 (104.2, AlexaFluor-700); eBioscience, Streptavidin-PECy5.5; Jackson ImmunoResearch, Streptavidin-Cy5. For intracellular staining of β -catenin, thymocytes were first stained for surface markers and then immediately fixed in 1% paraformaldehyde at RT and permeabilized in PBS containing 0.1% saponin, 2% FCS, 0.2% NaN₃. Cells were stained for 30 min with an anti- β -catenin-FITC antibody (clone 14, BD Transduction Laboratories) or a mouse IgG1-FITC isotype control (MOPC-31C, BD Pharmingen), washed, and analyzed by flow cytometry. Apoptosis detection by flow cytometry was performed using the AnnexinV-FITC apoptosis detection kit (BD Biosciences) and TO-PRO®-3 iodide (Molecular Probes) according manufacturer's recommendations. Cells were analyzed on a FACSCalibur™ (BD BioSciences) or a LSR™II (BD BioSciences) and

sorted on a FACSAriaTMII special order system (BD Biosciences). Sort purity was >98%. Results were analyzed using the FlowJo software (TreeStar).

Western blot and Immunoprecipitation.

Immunoprecipitation and western blot analysis of β -catenin were performed as noted before (Kirstetter *et al.*, 2006). Homogenized cell suspensions from spleen, thymocytes and cell lines were lysed in 60mM Tris pH-6.8, 2% SDS, 0.1% glycerol, 0.0025% bromophenol blue and 2.5% β -mercaptoethanol. The proteins of 10^6 cells were fractionated on a 12% SDS-acrylamide gel and transferred to Immobilon®-P transfer membrane (Millipore). Membranes were blocked in PBS-0.1%Tween (PBS-T) with 5% non-fat dry milk for 1 hour and then incubated with an anti-Pten (#9552, Cell Signaling) or an anti- β -actin (clone AC-15, Sigma) antibody. Membranes were washed three times in PBS-T and probed with an anti-rabbit-HRP (SC-2004, Santa Cruz) conjugated antibody in PBS-T for 1 hour. After three washes with PBS-T, the proteins were detected by enhanced chemiluminescence (ImmobilonTM Western, Millipore).

RT-qPCR and PCR.

Total RNA was isolated using the RNeasy Midi Kit (Qiagen) according the manufacturer's instructions (including on-column DNase digestion). Reverse transcription was performed on 500ng of total RNA using oligo(dT)₁₈ and Superscript II (Invitrogen) in a total volume of 20 μ l. qPCR were performed using 1x SYBR Green (Sigma) in a LightCyclerTM480 (Roche). qPCR conditions were: 95°C for 5min, followed by 45 cycles of 95°C for 15s, 60°C for 20s and 72°C for 25s. mRNA extension primers are as follows: Pten forward 5'-GCAGCTTCTGCCATCTCTCT-3', Pten reverse 5'-TCTGCAGGAAATCCCATAGC-3', myc forward 5'-GGAATTTTTGTCTATTTGGGGACAG-3' and myc reverse 5'-TAGTCGAGGTCATAGTTCCTGTTGG-3'. The mRNA levels were normalized using Hprt or Ubiquitin primers as described previously (Kirstetter *et al.*, 2006) and (Kurata *et al.*, 2002). PCR of D \otimes 2-J \otimes 2 rearrangements were performed as previously described (Dumortier *et al.*, 2006b).

Microarray analysis.

DP thymocytes from pre-leukemic R26- β cat and control mice were purified by flow cytometry (purity>98%). RNA from control and pre-leukemic R26- β cat DP thymocytes as well as R26- β cat total tumors cells were extracted with the RNeasy kit (Qiagen), and used for transcriptome analysis with Affymetrix 430 2.0 arrays, according to standard procedures.

Transplantation assay.

Thymocytes transplantation assay were performed by tail vein injection. CD45.2⁺ tumors cells were mixed with 2×10^5 radio-protective bone marrow cells (CD45.1⁺) and transplanted into lethally irradiated congenic mice (CD45.1^{+/2+}). CD45.2⁺ leukemic cells were measured by flow cytometry of peripheral blood leukocytes after transplantation.

CGH array analysis.

Genomic DNA from R26- β cat tumors cells and control thymocytes was extracted using NucleoSpin®Tissue (Macherey-Nagel) and processed for hybridization by the Microarray and Sequencing platform at the IGBMC. The DNA was hybridized to 244K Whole Mouse Genome Chip (Agilent Technologies) according to the manufacturer's instructions. A mix of five control thymus from male mice (R26^{NC/NC}CD4Cre^{+/+}) DNA were used as reference for five tumors from male R26- β cat mice (R26^{NC/NC}CD4Cre^{Tg/+}) and five female control thymus for five females tumors. Data were assembled and analyzed using the Gain and Loss Analysis of DNA (GLAD) algorithm (Hupe *et al.*, 2004) or PISSCO algorithm based on a Taut String method (Dembélé D. *et al.*, manuscript in preparation).

ACKNOWLEDGMENTS

We thank T. Mak (Campbell Family Institute for Breast Cancer Research, Toronto) and A.

Suzuki (Akita University School of Medicine, Akita, Japan) for floxed PTEN mice; W. Pear for the MigR1 vector system; G. Nolan for the Eco-Phoenix cells; P. Marchal for technical assistance; D. Dembélé for help in the microarray and CGH array analyses and the staff of the transgenic and animal facilities for excellent technical assistance.

This work was supported by grants from the Association pour la Recherche sur le Cancer, the Fondation de France, La Ligue Contre le Cancer (équipe labellisée La Ligue 2007, 2010) and Institut National du Cancer and institute funding from Inserm, CNRS, and l'Université de Strasbourg. D.K. received a predoctoral fellowship from the IGBMC International PhD program and from the Fondation pour la Recherche Médicale. P.K. received a postdoctoral fellowship from Association pour la Recherche sur le Cancer.

AUTHORSHIP CONTRIBUTION

DK, PK, SC and P. Kirstetter designed and performed experiments, analyzed data and wrote the paper; NC contributed to scientific discussions.

DISCLOSURE OF CONFLICT OF INTEREST

The authors have no conflicting financial interest.

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Fig. 1. Constitutive activation of β -catenin affects T cell development. **A)** Flow cytometry of T cells from R26- β cat and control mice stained with anti-CD4 and anti-CD8 antibodies. **B)** Frequency of DN (CD4⁻CD8⁻), DP (CD4⁺CD8⁺), CD4⁺ and CD8⁺ T cell sub-populations in the thymus and **C)** in the spleen (data show the mean \pm s.d. of 6 mice per genotype). **, $p \leq 0.001$. **(D, E)** Phenotype analysis **(D)** and frequency **(E)** of CD4⁻CD8⁻CD3⁻ DN1 (CD44⁺CD25⁻), DN2 (CD44⁺CD25⁺), DN3 (CD44⁻CD25⁺) and DN4 (CD44⁻CD25⁻) thymic cells from R26- β cat and control mice (mean \pm s.d from 4 mice per genotype). **F)** PCR analysis of thymic DN and tail cells from R26- β cat and control mice reveals a band of 660bp for the R26 floxed allele and 330bp for the excised allele. Note that most of the 'DN1' like cells from the R26- β cat mice carry the excised R26- β cat allele. **G)** Phenotypic analysis of thymic cells from control and R26- β cat mice 48h after sub-lethal irradiation (6.5 Gy). **H)** Frequency of apoptotic thymic cells (AnnexinV⁺) 6h after intraperitoneal injection of 25mg/kg of dexamethasone. (mean \pm s.d. of 4 mice per genotype). *, $p \leq 0.05$. **I)** Flow cytometry analysis of thymic cells representative of control or R26- β cat mice 12h after dexamethasone treatment. All the data were obtained with 6-week-old mice and are representative of at least three independent experiments.

Fig. 2. Activation of β -catenin in T cells leads to T cell leukemia. **A)** Kaplan-Meier plot showing the survival curve of R26- β cat vs. control mice. **B)** Phenotype of T cells from the thymus and spleen from control (R26^{NC/NC}CD4Cre^{+/+}) and leukemic R26- β cat mice. **C)** Staining of CD4⁻CD8⁻CD3⁻ cells from thymus of control (R26^{NC/NC}CD4Cre^{+/+}) and leukemic R26- β cat mice for the expression of CD44 and CD25. Data are representative of 6 mice per genotype. **D)** Kaplan-Meier plot illustrating the survival curves of mice transplanted with different numbers of total leukemic R26- β cat cells.

Fig. 3. β -catenin lymphomas are Notch-independent and Wnt pathway activation is required for tumor cell proliferation. **A)** Transcriptome profiling of control (R26^{NC/NC}CD4Cre^{+/+}) (n=2), pre-leukemic (n=3) and leukemic (n=4) DP cells. Genes under- or over-expressed were selected with a fold change of expression > 3 ($p \leq 0.01$) compared with

pre-leukemic (PL) or control (Ctl) cells. Expression changes are color-coded: red indicating up-regulation and green down-regulation. **B)** Expression of Notch family members or target genes in R26- β cat DP leukemic cells (L). **C)** RT-qPCR analysis of Notch1 and Notch3 in R26- β cat DP leukemic cells. Values are shown relative to that of ubiquitin mRNA and represent the average of 3 controls and 6 leukemic R26- β cat mice (error bar, s.d). $p \leq 0.01$. **D)** The 149.4 cell line derived from a R26- β cat tumor as well as in T29 cell line derived from an $Ik^{L/L}$ T cell tumor (Notch dependent)(Dumortier *et al.*, 2006b) were transduced with the Mig or Mig-dnMAML1 retroviral vectors at day 0 and cultured for 7 days. Transduced cells expressed GFP and are distinguishable from non-transduced ones. The percentage of GFP⁺ cells is shown over 7 days of culture. **E)** The 149.4 and 149.8 cell lines were transduced with the Mig or Mig-Icat retroviral vectors. Two days later (day 0) transduced (GFP⁺) and non-transduced (GFP⁻) cells were sorted and co-cultured for 8 days. Flow cytometry analysis of GFP-positive and -negative cells in this co-culture at day 1 and 8 are shown. Data are representative of two independent experiments. **F)** Cumulative numbers of GFP⁺ cells in the experiment described in E) are shown over 8 days of co-culture.

Fig. 4. CD44 is not required for R26- β cat leukemia development or engraftment. **A)** CD44 expression on CD4⁺CD8⁺ cells from R26- β cat pre-leukemic (6-week-old) and leukemic mice compared with control (R26^{NC/NC}CD4Cre^{+/+}) mice (solid grey). **B)** Kaplan-Meier plot showing the survival curves of R26- β cat (R26^{NC/NC}CD4Cre^{Tg/+}CD44^{+/+}), CD44-deficient R26- β cat (R26^{NC/NC}CD4Cre^{Tg/+}CD44^{-/-}), or CD44-deficient control (R26^{NC/NC}CD4Cre^{+/+}CD44^{-/-}) mice. **C)** Phenotype of a CD44-deficient R26- β cat (R26^{NC/NC}CD4Cre^{Tg/+}CD44^{-/-}) tumor compared with control thymuses from CD44 deficient (R26^{NC/NC}CD4Cre^{+/+}CD44^{-/-}) or heterozygote (R26^{NC/NC}CD4Cre^{+/+}CD44^{+/+}) mice. Left panels show the CD25 and CD44 expression of immature CD4⁻CD8⁻CD3⁻ thymocytes. Right panels show the CD4 and CD8 expression in total thymocytes. **D)** Kaplan-Meier plot showing survival of mice transplanted with 10⁴ or 5x10⁴ cells from two distinct CD44-deficient R26- β cat tumors. **E)** Bone marrow and thymus of mice transplanted with CD44-deficient R26- β cat tumor cells were evaluated for CD45.1 and CD45.2 to identify CD45.2⁺ tumor cells, and with CD4 and CD8 to analyze the tumor phenotype (gated on CD45.2⁺ cells).

Fig. 5. Myc Over-expression. **A)** CGH array profile of chromosome 15 showing ten R26- β cat tumors. The y-axis represents the \log_2 of the ratios of the combined dye swap experiments of leukemic/control DNA normalized data (black stars) and the smooth segmentation based on a Taut String method (red line). The position of the CGH probe is indicated on the x-axis. The arrow indicates the location and orientation of genes. **B)** RT-qPCR analysis of Myc mRNA levels in thymocytes from 6-week-old R26- β cat mice (PL), R26- β cat leukemic cells (L) and control (R26^{NC/NC}CD4Cre^{+/+}) thymocytes. Values are shown relative to that of ubiquitin mRNA and represent the average of 4 mice per genotype performed in triplicate (error bar, s.d).

Figure 6. Pten deletion cooperates with β -catenin activation in T cell lymphomagenesis.

A) CGH array analysis of a region of chromosome 19 from ten R26- β cat tumors. The y-axis represents the \log_2 of the ratios of the combined dye swap experiments of tumor/control DNA raw normalized data (black circles). The breakpoint detection and status assignment (gain, loss or normal) was performed using the PISSCO algorithm based on a Taut String smoothing method (red line) (Dembélé D. *et al.* manuscript in preparation). The position of the CGH probe is indicated on the x-axis. The arrow indicates the location and orientation of the Pten gene. **B)** RT-qPCR analysis of Pten mRNA levels from seven distinct R26- β cat tumors compared with control (R26^{NC/NC}CD4Cre^{+/+}) thymocytes. Values are shown relative to Hprt mRNA and represent the average of two independent experiments performed in triplicate (error bar, s.d). **C)** Western blot for PTEN in total cell extracts from eight R26- β cat tumors and two control thymuses. **D)** Western blot for PTEN from three primary R26- β cat tumors (T) and their derived cell lines (CL) compared with control (R26^{NC/NC}CD4Cre^{+/+}) thymocytes. **E)** Kaplan-Meier plot showing the survival curves of control mice (R26^{NC/NC}CD4Cre^{+/+}Pten^{F/+}) and R26- β cat mice with wild type (R26^{NC/NC}CD4Cre^{Tg/+}Pten^{+/+}) or heterozygote (R26^{NC/NC}CD4Cre^{Tg/+}Pten^{F/+}) Pten allele. **F)** Western blot for PTEN expression in four distinct heterozygote Pten R26- β cat tumors (1 to 4) or splenocytes (Spl), in pre-leukemic thymocytes (PL) from 6-week-old (R26^{NC/NC}CD4Cre^{Tg/+}Pten^{F/+}) mice and in control (R26^{NC/NC}CD4Cre^{+/+}Pten^{F/+}) thymocytes.

Fig. 1

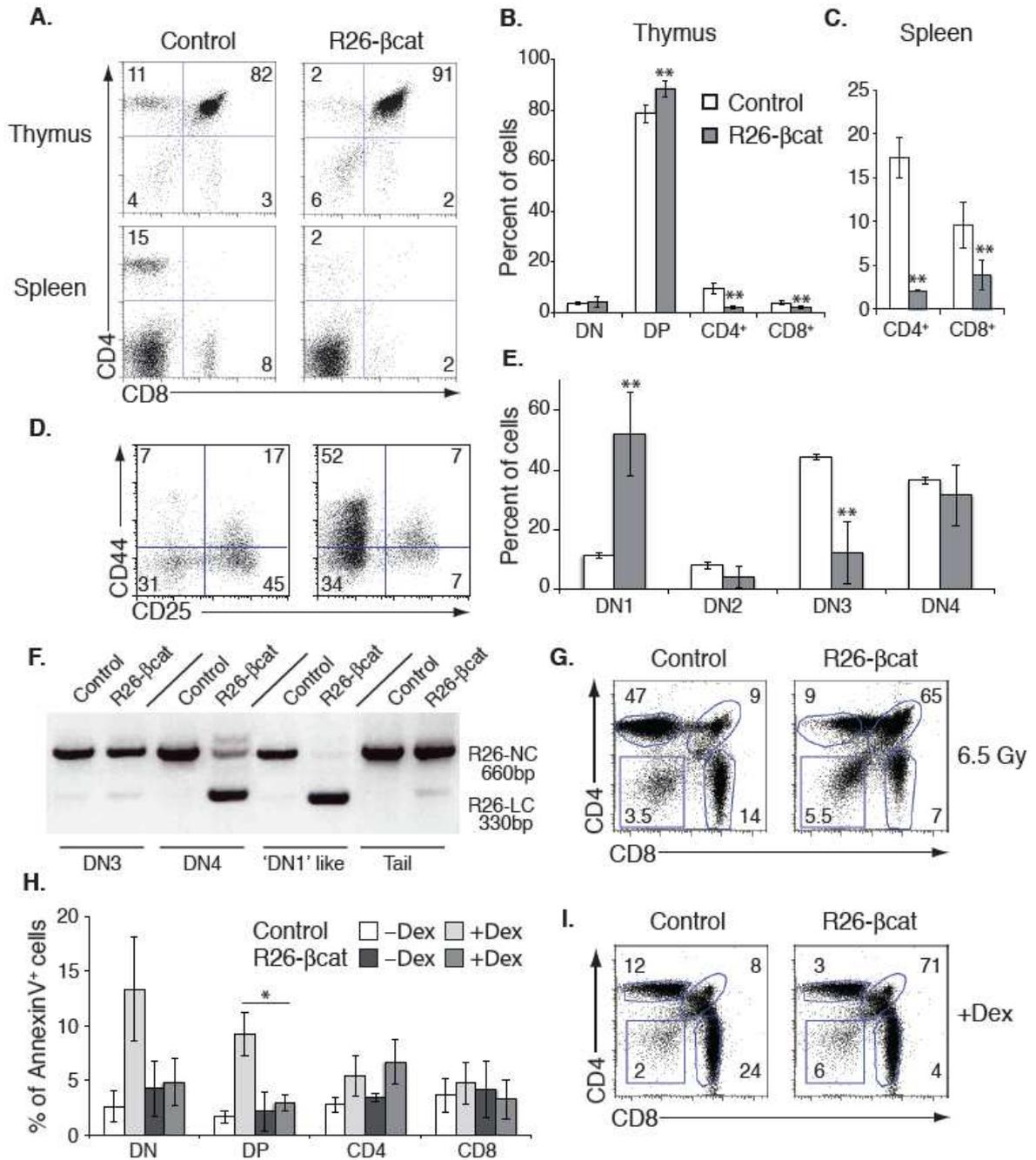


Fig. 2

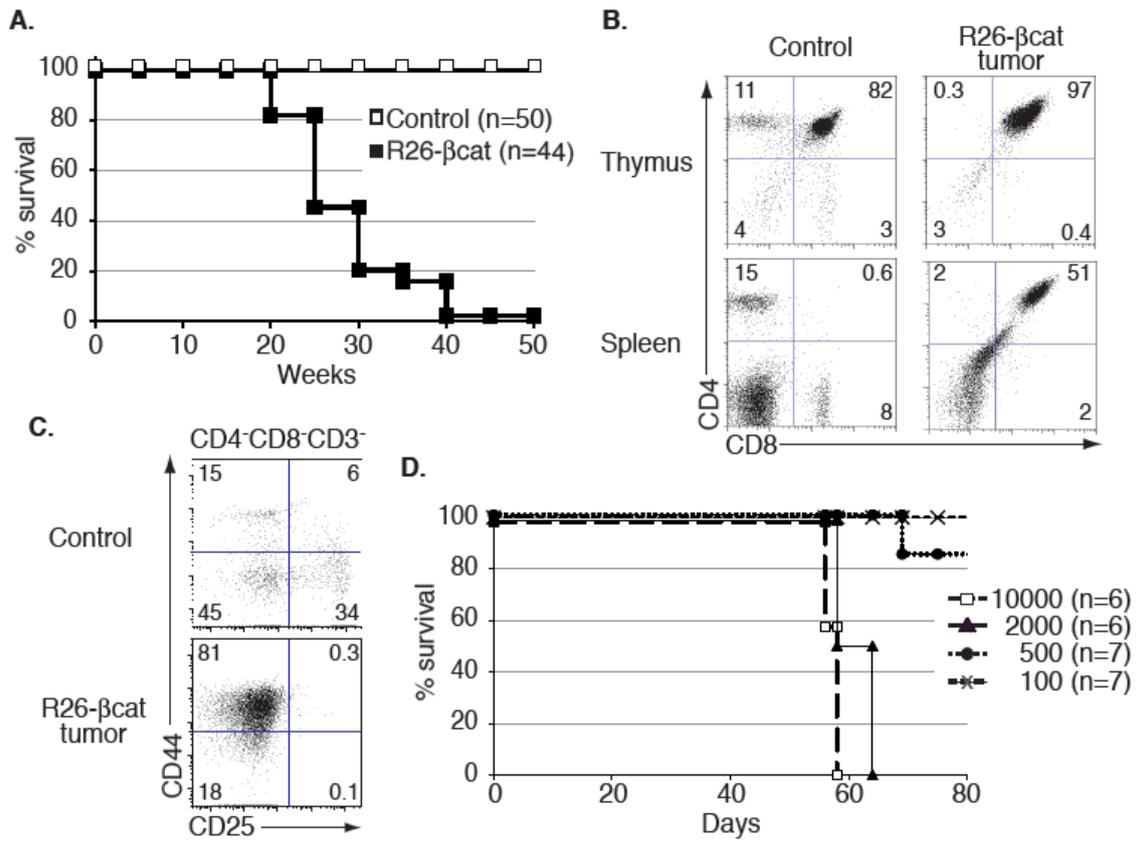


Fig. 3

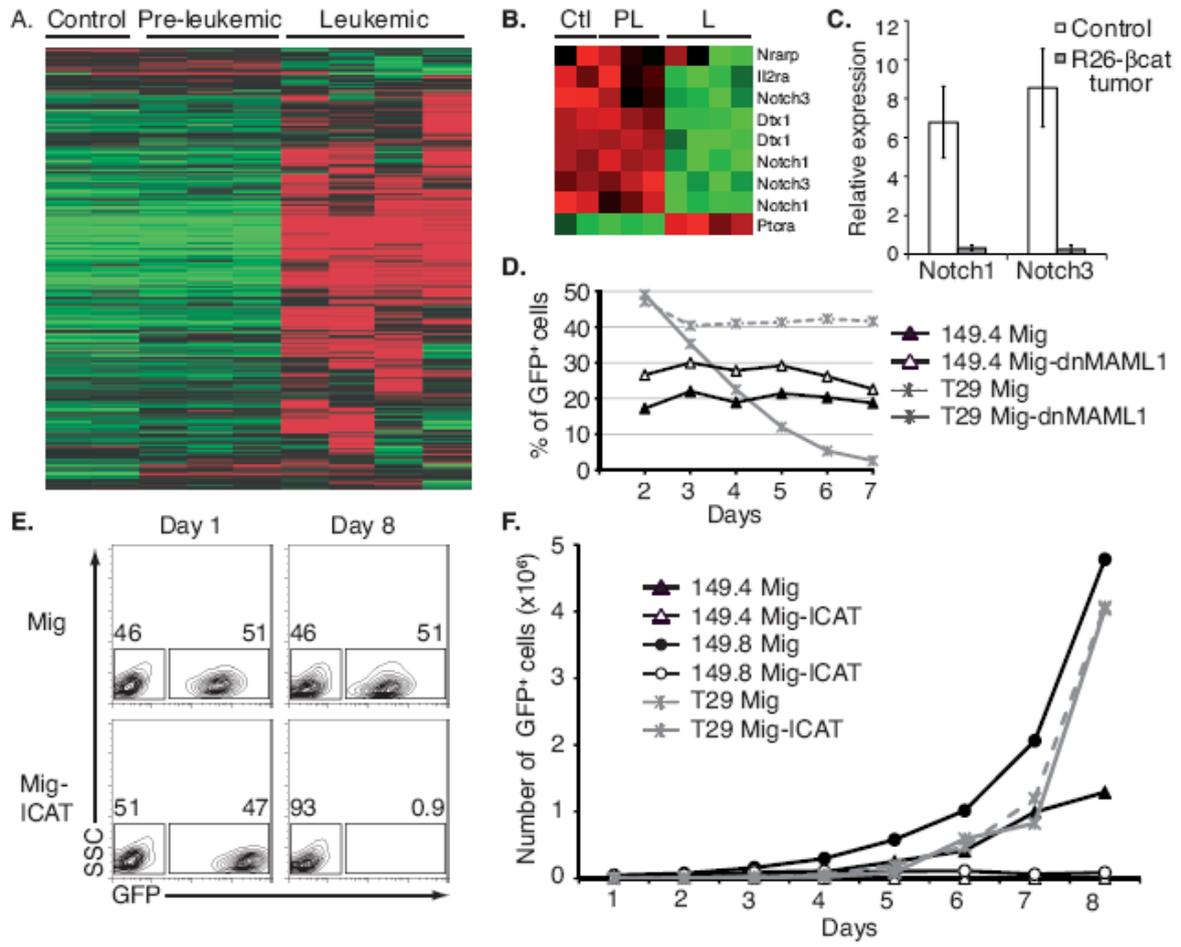


Fig. 4

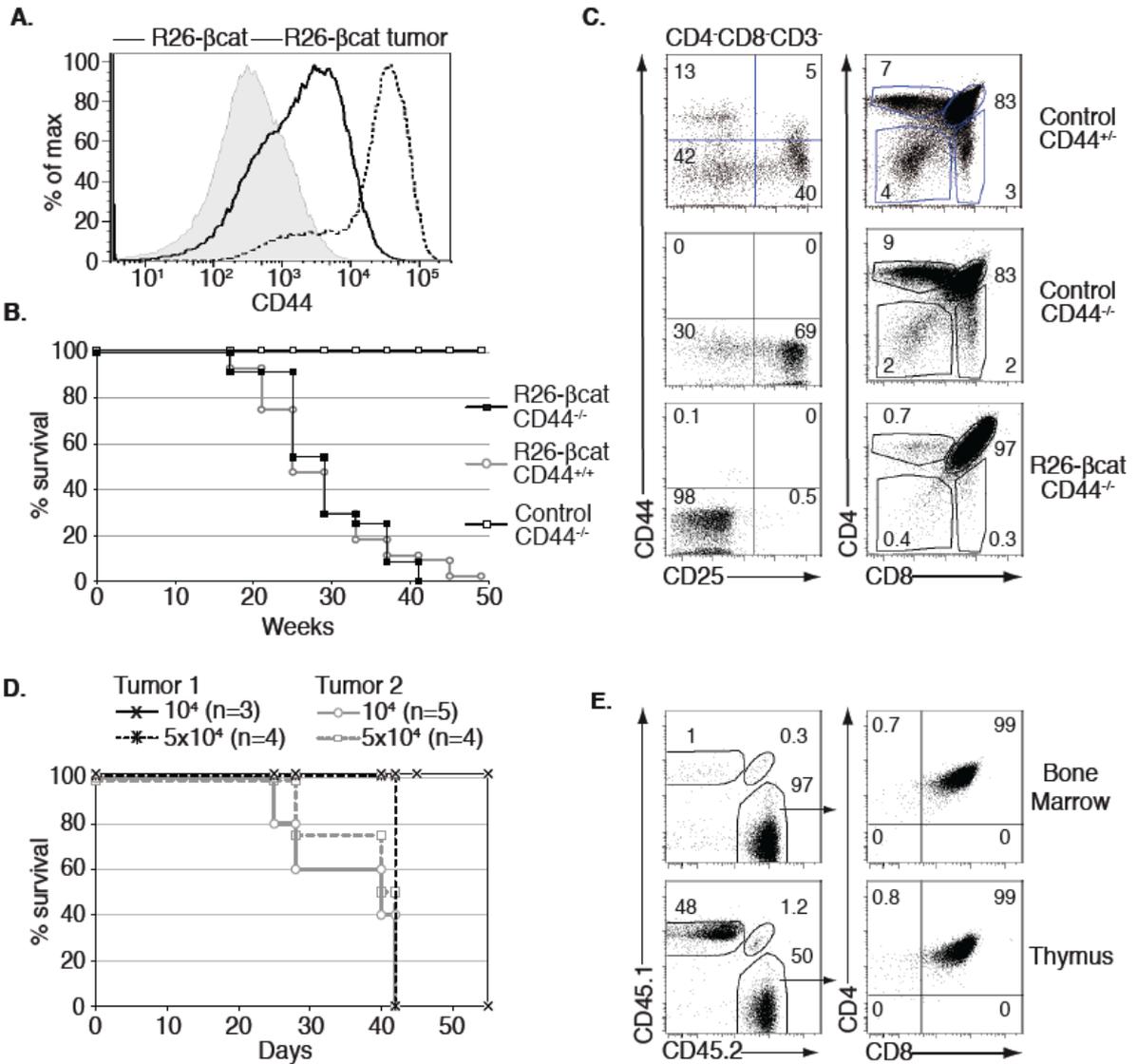
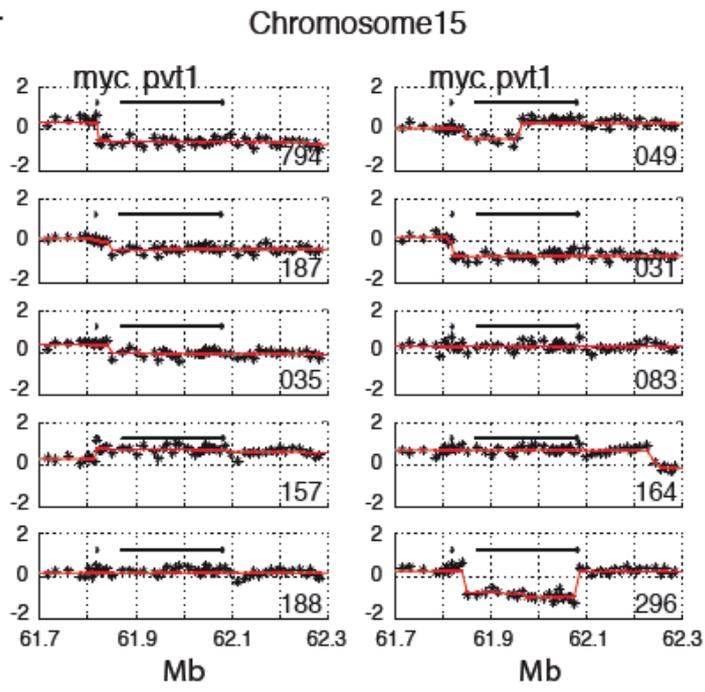


Fig. 5

A.



B.

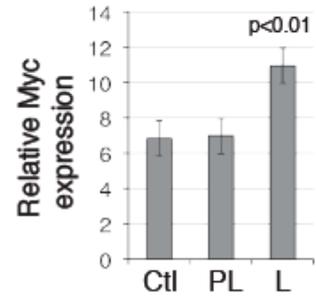


Fig. 6

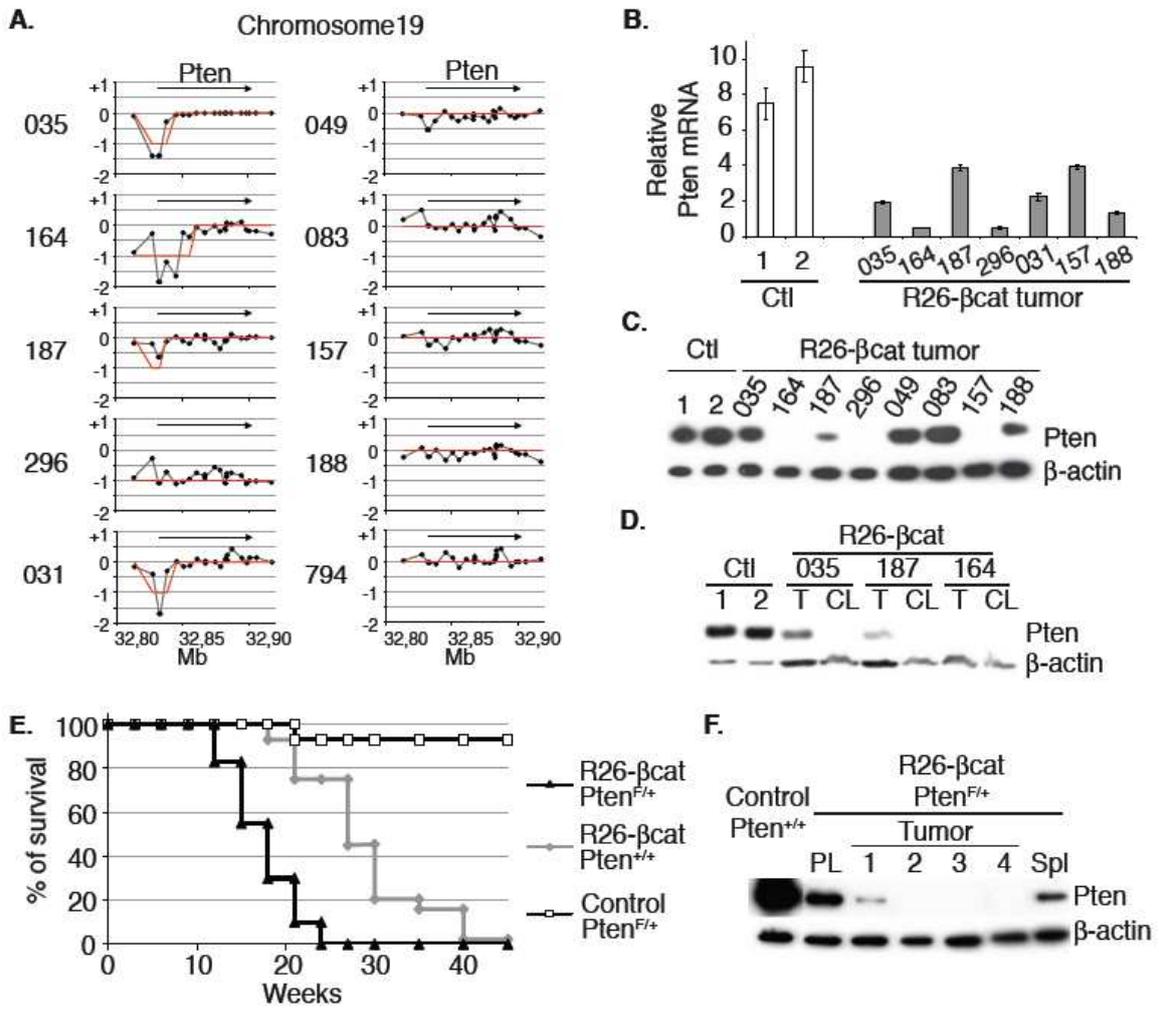


Table S1 – Genes which show transcriptional alterations in pre-leukemic R26-βcat mice.

Up-regulated genes Probe Set ID	Gene Name	Gene Symbol	Fold Change
1457039_at	cat eye syndrome chromosome region, candidate 2 homolog (human)	Cecr2	103,1
1459202_at	16 days embryo head cDNA, RIKEN full-length enriched library, clone:C130034D14 product	---	11,2
1431014_at	cat eye syndrome chromosome region, candidate 2 homolog (human)	Cecr2	11,1
1433683_at	RNA binding motif protein 35b	Rbm35b	7,7
1423760_at	CD44 antigen	Cd44	7,3
1434376_at	CD44 antigen	Cd44	7,2
1454867_at	meningioma 1	Mn1	6,3
1446140_at	3 days neonate thymus cDNA, RIKEN full-length enriched library, clone:A630047A13 product	---	5,8
1426263_at	cell adhesion molecule 4	Cadm4	5,5
1442434_at	DNA segment, Chr 8, ERATO Doi 82, expressed	D8Ert82e	5,4
1417960_at	cytoplasmic polyadenylation element binding protein 1	Cpeb1	5,1
1435710_at	expressed sequence AI661384	AI661384	4,7
1424008_a_at	RNA binding protein with multiple splicing 2	Rbpms2	4,7
1451588_at	RIKEN cDNA 181002C23 gene	181002C23Rik	4,4
1428964_at	solute carrier family 25 (mitochondrial carrier), member 18	Slc25a18	4,0
1455359_at	protein tyrosine phosphatase, non-receptor type 14	Ptpn14	3,9
1436442_at	protein tyrosine phosphatase, non-receptor type 14	Ptpn14	3,9
1442467_at	neuron navigator 2	Nav2	3,9
1421499_a_at	protein tyrosine phosphatase, non-receptor type 14	Ptpn14	3,8
1417937_at	dapper homolog 1, antagonist of beta-catenin (xenopus)	Dact1	3,6
1449470_at	distal-less homeobox 1	Dlx1	3,5
1422046_at	Integrin alpha M	Itgam	3,5
1444706_at	Neuron navigator 2	Nav2	3,5
1429071_at	malic enzyme 3, NADP(+)-dependent, mitochondrial	Me3	3,5
1458524_at	fibronectin type III domain containing 3a	Fndc3a	3,4
1431175_at	RIKEN cDNA 1810019D21 gene	1810019D21Rik	3,4
1452483_a_at	CD44 antigen	Cd44	3,4
1418943_at	RIKEN cDNA B230120H23 gene	B230120H23Rik	3,3
1427035_at	solute carrier family 39 (zinc transporter), member 14	Slc39a14	3,3
1435209_at	cDNA sequence BC057079	BC057079	3,2
1455622_at	podocalyxin-like 2	Podxl2	3,0
1444780_at	neuron navigator 2	Nav2	3,0
1446653_at	---	---	3,0
1427714_at	SET and MYND domain containing 1	Smyd1	2,9
1447559_at	expressed sequence AI834762	AI834762	2,9
1435981_at	neuron navigator 2	Nav2	2,9
1441667_s_at	SET and MYND domain containing 1	Smyd1	2,8
1443669_at	Transcribed locus	---	2,8
1440103_at	Transcribed locus	---	2,7
1417663_a_at	N-myc downstream regulated gene 3	Ndrq3	2,6
1421594_a_at	synaptotagmin-like 2	Sytl2	2,6
1418301_at	interferon regulatory factor 6	Irf6	2,6
1451474_a_at	poly (ADP-ribose) polymerase family, member 8	Parp8	2,5
1432085_at	RIKEN cDNA 4930425O10 gene	4930425O10Rik	2,5
1444080_at	RIKEN cDNA E430014L09 gene	E430014L09Rik	2,5
1448788_at	Cd200 antigen	Cd200	2,5
1423549_at	solute carrier family 1 (glutamate/neutral amino acid transporter), member 4	Slc1a4	2,5
1438528_at	3 days neonate thymus cDNA, RIKEN full-length enriched library, clone:A630047A13 product	---	2,5
1420705_at	forkhead box B1	Foxb1	2,4
1445695_at	0 day neonate lung cDNA, RIKEN full-length enriched library, clone:E030049G11 product	---	2,4
1439686_at	3 days neonate thymus cDNA, RIKEN full-length enriched library, clone:A630056D15 product	---	2,4
1433133_at	EDAR (ectodysplasin-A receptor)-associated death domain	Edaradd	2,4
1439343_at	Transcribed locus	---	2,4
1434326_x_at	coronin, actin binding protein, 2B	Coro2b	2,4
1452160_at	TCDD-inducible poly(ADP-ribose) polymerase	Tiparp	2,4
1417894_at	G protein-coupled receptor 97	Gpr97	2,3
1449712_s_at	VATPase, H+ transporting, lysosomal V1 subunit E1	Atp6v1e1	2,3
1450203_at	SET and MYND domain containing 1	Smyd1	2,3
1440721_at	RIKEN cDNA 5930433N17 gene	5930433N17Rik	2,3
1449593_at	---	---	2,3
1439930_at	16 days neonate thymus cDNA, RIKEN full-length enriched library, clone:A130046D02 product	---	2,2
1417849_at	zinc finger protein 704	Zfp704	2,2
1423572_at	Bcl2-like 2	Bcl2l2	2,2
1426721_s_at	TCDD-inducible poly(ADP-ribose) polymerase	Tiparp	2,2
1417664_a_at	N-myc downstream regulated gene 3	Ndrq3	2,2
1441467_at	3 days neonate thymus cDNA, RIKEN full-length enriched library, clone:A630052I23 product	---	2,2
1457605_at	0 day neonate lung cDNA, RIKEN full-length enriched library, clone:E030049G11 product	---	2,2
1442966_at	---	---	2,2
1434510_at	3'-phosphoadenosine 5'-phosphosulfate synthase 2	Papss2	2,2
1435927_at	DENN/MADD domain containing 3	Dennd3	2,2
1444538_at	---	---	2,2
1445813_at	WD repeat domain 27	Wdr27	2,2
1417848_at	zinc finger protein 704	Zfp704	2,2
1451644_a_at	RIKEN cDNA 0610037M15 gene	0610037M15Rik	2,2
1432650_at	RIKEN cDNA 2510019K15 gene	2510019K15Rik	2,2
1452161_at	TCDD-inducible poly(ADP-ribose) polymerase	Tiparp	2,1
1424567_at	tetraspanin 2	Tspan2	2,1
1440372_at	Transcribed locus	---	2,1
1445285_at	Transcribed locus, moderately similar to XP_872120.2 similar to histamine H3 receptor [Bos taurus]	---	2,1
1425673_at	LIM domain containing preferred translocation partner in lipoma	Lpp	2,1
1457189_at	---	---	2,1
1423885_at	laminin, gamma 1	Lamc1	2,1
1446627_at	---	---	2,1

1456046_at	CD93 antigen	Cd93	2,1
1435444_at	activating transcription factor 6	Atf6	2,1
1448908_at	phosphatidic acid phosphatase type 2B	Ppap2b	2,1
1434451_at	predicted gene, ENSMUSG00000074917	ENSMUSG00000074917	2,1
1436666_at	0 day neonate lung cDNA, RIKEN full-length enriched library, clone:E030042E08 product	---	2,1
1423277_at	protein tyrosine phosphatase, receptor type, K	Ptpkr	2,1
1451998_at	taspase, threonine aspartase 1	Tasp1	2,0
1442157_at	RIKEN cDNA A930041H05 gene	A930041H05Rik	2,0
1444667_at	bromodomain, testis-specific	Brdt	2,0
1457835_at	predicted gene, EG218444	EG218444	2,0
1437602_at	predicted gene, OTTMUSG00000005491	OTTMUSG00000005491	2,0
1438294_at	ataxin 1	Atxn1	2,0
1434755_at	coronin, actin binding protein, 2B	Coro2b	2,0
1440892_at	---	---	2,0

Down-regulated genes

Probe Set ID	Gene Name	Gene Symbol	Fold Change
1453328_at	RIKEN cDNA 2700008G24 gene	2700008G24Rik	11,4
1437181_at	pellino 2	Peli2	9,7
1435137_s_at	RIKEN cDNA 1200015M12 gene	1200015M12Rik	6,7
1452418_at	RIKEN cDNA 1200016E24 gene	1200016E24Rik	5,5
1428595_at	solute carrier family 6 (neurotransmitter transporter), member 19	Slc6a19	5,1
1427932_s_at	RIKEN cDNA 1200003I10 gene	1200003I10Rik	4,5
1455442_at	solute carrier family 6 (neurotransmitter transporter), member 19	Slc6a19	4,3
1437605_at	nephrosis 2 homolog, podocin (human)	Nphs2	3,9
1447360_at	TSC22 domain family, member 1	Tsc22d1	3,7
1429172_a_at	on-SMC condensin I complex, subunit G	Ncapg	3,6
1434881_s_at	potassium channel tetramerisation domain containing 12	Kctd12	3,5
1424127_at	eyes absent 2 homolog (Drosophila)	Eya2	3,4
1436994_a_at	histone cluster 1, H1c	Hist1h1c	3,0
1454880_s_at	Bcl2 modifying factor	Bmf	3,0
1448943_at	neuropilin 1	Nrp1	2,9
1429658_a_at	structural maintenance of chromosomes 2	Smc2	2,9
1448617_at	CD53 antigen	Cd53	2,7
1460118_at	Transcribed locus	---	2,7
1419497_at	cyclin-dependent kinase inhibitor 1B	Cdkn1b	2,7
1443602_at	Transcribed locus	---	2,7
1422398_at	histone cluster 1, H1e	Hist1h1e	2,7
1438307_at	high mobility group box 2	Hmgb2	2,7
1417270_at	WD repeat domain 12	Wdr12	2,7
1418084_at	neuropilin 1	Nrp1	2,6
1453238_s_at	RIKEN cDNA 3930401B19 gene	3930401B19Rik	2,6
1427655_a_at	RIKEN cDNA A630038E17 gene	A630038E17Rik	2,6
1448944_at	neuropilin 1	Nrp1	2,6
1416101_a_at	histone cluster 1, H1c	Hist1h1c	2,5
1417094_at	acyl-CoA thioesterase 7	Acot7	2,5
1417654_at	syndecan 4	Sdc4	2,5
1429159_at	inter-alpha (globulin) inhibitor H5	Itih5	2,5
1435800_a_at	cold shock domain protein A	Csda	2,5
1431106_a_at	RIKEN cDNA 9530053H05 gene	9530053H05Rik	2,5
1452459_at	asp (abnormal spindle)-like, microcephaly associated (Drosophila)	Aspm	2,5
1439040_at	centromere protein E	Cenpe	2,4
1425398_at	H2b histone family member	LOC665622	2,4
1450156_a_at	hyaluronan mediated motility receptor (RHAMM)	Hmnmr	2,4
1438833_at	cancer susceptibility candidate 5	Casc5	2,4
1447474_at	receptor (calcitonin) activity modifying protein 1	Ramp1	2,4
1422760_at	peptidyl arginine deiminase, type IV	Padi4	2,3
1448793_a_at	syndecan 4	Sdc4	2,3
1435171_at	similar to splicing coactivator subunit SRm300	LOC100046169	2,3
1448752_at	carbonic anhydrase 2	Car2	2,3
1428304_at	establishment of cohesion 1 homolog 2 (S. cerevisiae)	Esc02	2,3
1421214_at	cytidine monophospho-N-acetylneuraminic acid hydroxylase	Cmah	2,3
1451012_a_at	cold shock domain protein A	Csda	2,3
1434734_at	RIKEN cDNA E130016E03 gene	E130016E03Rik	2,3
1421037_at	neuronal PAS domain protein 2	Npas2	2,2
1449171_at	Ttk protein kinase	Ttk	2,2
1459804_at	CREB binding protein	Crebbp	2,2
1418497_at	fibroblast growth factor 13	Fgf13	2,2
1450521_a_at	T-cell receptor gamma, variable 6	Tcrg-V6	2,2
1417623_at	solute carrier family 12, member 2	Slc12a2	2,2
1428043_a_at	cytidine monophospho-N-acetylneuraminic acid hydroxylase	Cmah	2,2
1417445_at	NDC80 homolog, kinetochore complex component (S. cerevisiae)	Ndc80	2,2
1452115_a_at	polo-like kinase 4 (Drosophila)	Plk4	2,2
1416529_at	epithelial membrane protein 1	Emp1	2,2
1419152_at	RIKEN cDNA 2810417H13 gene	2810417H13Rik	2,2
1429660_s_at	structural maintenance of chromosomes 2	Smc2	2,1
1456652_at	denticleless homolog (Drosophila)	Dtl	2,1
1433681_x_at	calpain 3	Capn3	2,1
1435640_x_at	RIKEN cDNA A130040M12 gene	A130040M12Rik	2,1
1421340_at	mitogen activated protein kinase kinase 5	Map3k5	2,1
1426782_at	G protein-coupled receptor 125	Gpr125	2,1
1456449_at	---	---	2,1
1454171_x_at	RIKEN cDNA 9530053H05 gene	9530053H05Rik	2,1
1429171_a_at	on-SMC condensin I complex, subunit G	Ncapg	2,1
1417821_at	DNA segment, Chr 17, human D6S56E 5	D17H6S56E-5	2,1
1459400_at	RIKEN cDNA A630038E17 gene	A630038E17Rik	2,1
1424046_at	budding uninhibited by benzimidazoles 1 homolog (S. cerevisiae)	Bub1	2,1

1458374_at	expressed sequence C79407	C79407	2,1
1435740_at	predicted gene, ENSMUSG00000072684	ENSMUSG00000072684	2,1
1423602_at	Tnf receptor-associated factor 1	Traf1	2,1
1435306_a_at	kinesin family member 11	Kif11	2,1
1418281_at	RAD51 homolog (S. cerevisiae)	Rad51	2,0
1417707_at	RIKEN cDNA B230342M21 gene	B230342M21Rik	2,0
1416418_at	gamma-aminobutyric acid (GABA(A)) receptor-associated protein-like 1	Gabarapl1	2,0
1458126_at	RIKEN cDNA D030041H20 gene	D030041H20Rik	2,0
1453323_at	RIKEN cDNA 2900079G21 gene	2900079G21Rik	2,0
1438774_s_at	phosphoglucomutase 2-like 1	Pgm2l1	2,0
1452166_a_at	keratin 10	Krt10	2,0

Table S2. Chromosomal regions lost or gained in R26- β cat tumors analyzed by CGH array. Regions were selected when 3 consecutive probes are considered as lost or gained in at least 50% of the tumors analyzed.

REGIONS LOST			
Chromosome	Start	End	RefSeq
2	157,861,749	157,884,459	Rprd1b
4	111,726,014	113,309,541	Skint4; Skint3; Skint9; Skint2; Skint10; Skint6; Skint5
4	145,018,403	147,179,003	Smarca5-ps; Gm13212; Gm13242; Gm13238; 1700095A21Rik; Gm13247; Gm13251; Rex2; Gm13034; Gm13051; Zfp600; Rex2; 4933438K21Rik; Gm13139; 1700029I01Rik; Gm13252; Gm13254; 2610305D13Rik; Zfp534; Gm13157
7	7,312,489	9,046,999	Vmn2r31; Vmn2r32; Vmn2r33; Vmn2r34; Vmn2r35; Vmn2r36; Vmn2r47; Vmn2r46; Vmn2r36; Vmn2r35; Vmn2r37
7	21,117,504	21,555,512	Vmn1r104; Vmn1r152; Gm5726; Gm8677; Gm4201; Gm4141; Gm4541; Vmn1r155; Vmn1r107; Vmn1r130; Vmn1r137; Vmn1r165; Gm4172; Vmn1r111; Gm4220; Gm4179; Gm4567
8	22,622,959	22,642,669	Gm15308
11	3,026,911	3,094,185	Pisd-ps1; Pisd-ps3, Sfi1
11	116,609,271	116,615,925	BC018473
12	114,930,317	114,960,452	
12	115,662,583	116,170,763	
12	116,746,379	117,071,450	
14	26,701,644	26,833,029	Anxa11; Plac9; D14Ert449e; Cphx; Duxb1; Gm10391; Gm10394
14	53,430,721	54,810,474	A430107P09Rik
14	69,871,627	70,082,968	Slc25a37; Synb; Gm16677; Entpd44; Lox12
18	9,819,613	10,016,625	Colec12; Thoc1; Usp14

REGIONS GAINED			
Chromosome	Start	End	RefSeq
3	60,148,602	60,537,324	Mbnl1
6	41,100,250	41,480,704	Try4; Try5; Try10; Prss3; Gm5771; Gm5409; GM10334; Prss1; Prss2
15	66,079,838	66,992,818	Kcnq3; Lrrc6; Tmem71; Phf2011; Tg; Sla; Wisp1; Ndrgr1; St3gal1
15	71,875,152	103,485,071	

Fig. S1. Constitutive expression of S-33- β -catenin in T cells. **A)** Immunoprecipitation with an anti- β -catenin antibody of whole cell extracts from thymocytes analyzed by western blot with antibodies recognizing the c-myc tag or β -catenin. **B)** Absolute numbers of cells in the thymus and spleen of 6-week-old mice. Mean \pm s.d from 10 mice per genotype. **C)** Intracellular β -catenin levels in 'DN1'-like, DN2, DN3, DN4, DP (CD4⁺CD8⁺), CD4⁺ and CD8⁺ thymocytes from 6-week-old R26- β cat and control mice. The data are representative of two independent experiments.

Fig. S2. R26- β cat tumors are mono- or oligo-clonal and malignant. **A)** Flow cytometry of TCR β and CD3 levels of thymic DP and CD4⁺ cells from 6-week-old control (R26^{NC/NC}CD4Cre^{+/+}) and R26- β cat mice and R26- β cat tumor. **B)** PCR analysis of D β 2-J β 2 rearrangement from DNA of control thymocytes and R26- β cat tumor cells. **C)** CD4 and CD8 expression of cells from a primary R26- β cat tumor (left) and from the bone marrow, thymus and spleen of mice transplanted with the same R26- β cat tumor (right).

Fig. S3. Chromosomal aberrations in the TCR α /TcR δ cluster

CGH array profiles of chromosome 14 for ten R26- β cat tumors. The y-axis represents the log₂ of the ratios of the combined dye swap experiments of tumor/control DNA normalized data (black stars) and the smooth segmentation based on a Taut String method (red line). The position of the CGH probe is indicated on the x-axis. The arrow indicates the location and orientation of genes. The arrows indicate the location and the orientation of the genes encoding for the constant chain (trdc) and the variable region 5 (trdv5) of the T cell receptor delta, the constant region of the T cell receptor alpha (trac) and the defender against cell death 1 protein (dad1).

Fig. S4. Phenotype of Pten mono-allelic R26- β cat tumors. CD4 and CD8 expression of a Pten mono-allelic R26- β cat (R26^{NC/NC}CD4Cre^{Tg/+}Pten^{F/+}) tumor and control (R26^{NC/NC}CD4Cre^{+/+}Pten^{F/+}) thymocytes.

Fig. S1

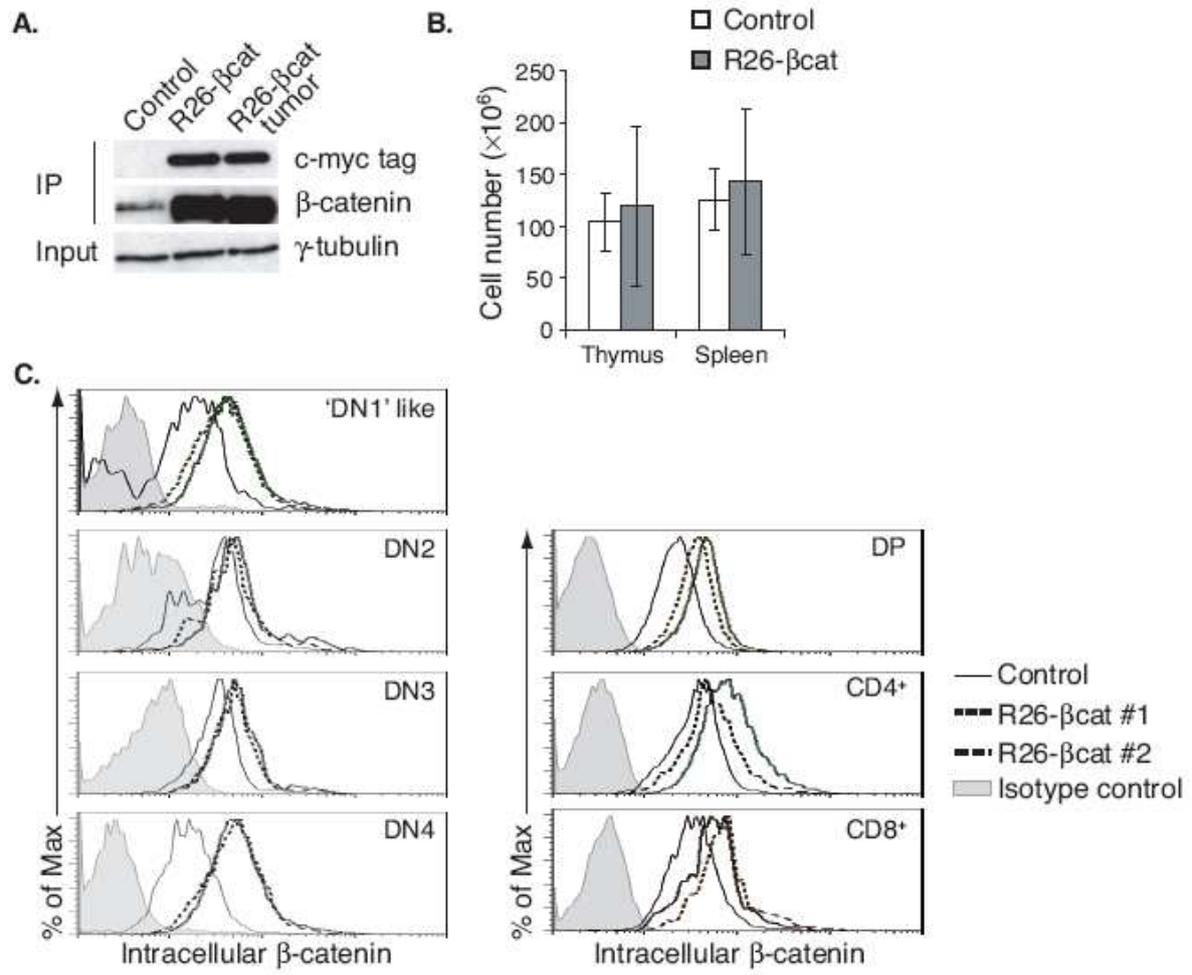


Fig. S2

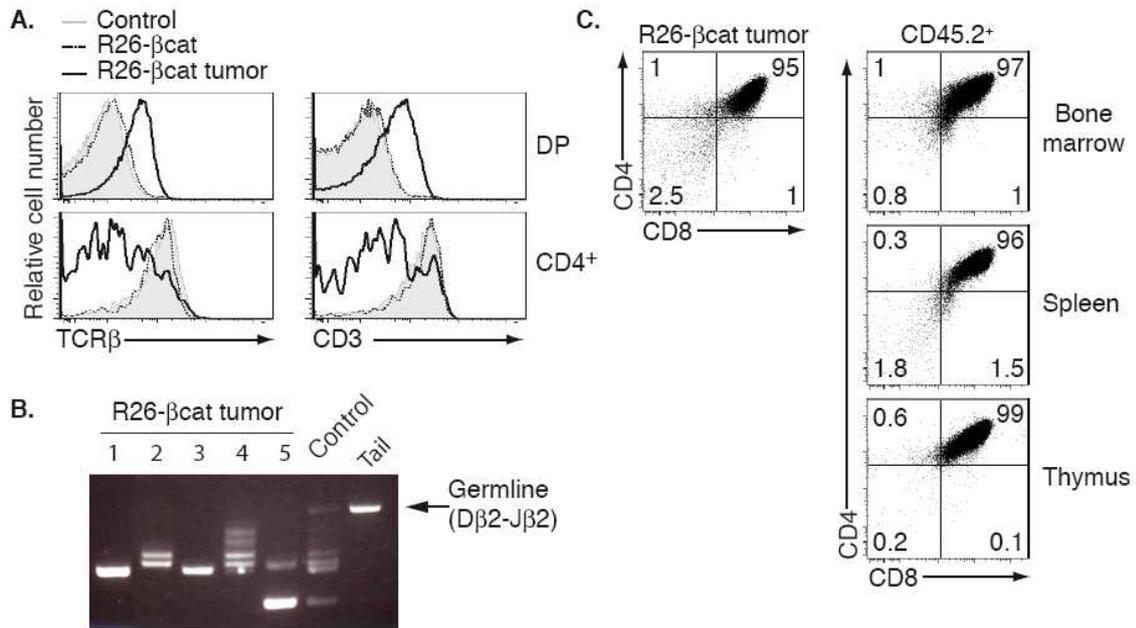


Fig. S3

Chromosome14

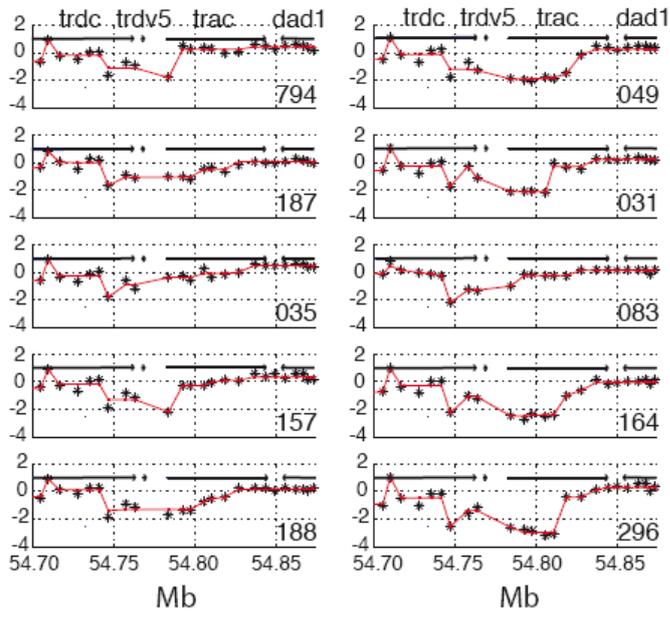
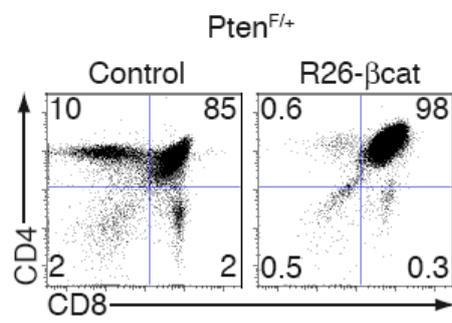


Fig. S4



III. Complementary results

III.I R26- β -cat tumours exhibit leukaemia stem cell activity

III.I.1 β -catenin is efficiently excised in DP cells of R26- β -cat thymic tumour

Since the R26- β -cat mice begin to show T-cell developmental defects at 4-6 weeks and acquire T-ALL only later during their life, we wanted to check if there are any differences in the neo-cassette excision in the sub-populations of pre-leukaemic and leukaemic stages. To determine this, we checked for the presence of the neo-cassette by PCR (refer manuscript Fig 1F). The primers (marked by red arrows in the R26- β -cat construct shown in Fig 1.14) amplify both the floxed (660bp) and excised (330bp) forms of R26 allele. In the pre-leukaemic mice, we detected the presence of both excised and floxed alleles in the DN cells of two randomly chosen tumours. Considering that cre-recombinase expression in R26- β -cat mice begins at DN3 stage (refer manuscript Fig S1 D), this is what we expected to occur in DN subset. In the DP cells, most cells appear to have the excised fragment. But, it is clear that all DP cells have not undergone excision. However, the majority of mature CD4⁺ and CD8⁺ cells in the thymus appear excised. Interestingly, in the splenic mature cells, most cells seem to have escaped the excision (Fig 3.1 A). Furthermore, in DN subsets of three independent leukaemias we were able to detect both excised and floxed alleles, similar to previous results. However, this differed among the three tumours analysed showing that some or most cells in the leukaemic DN subset have escaped excision. The DP cells had clearly undergone efficient excision in all tumours analysed. CD4⁺ cells in leukaemic stages however show only partial excision (Fig 3.1 B).

This result shows that excision begins relatively late during development in the thymocytes as soon as cre expression is initiated. Only CD4⁺ and CD8⁺ cells that escape excision at the pre-leukaemic stage seem to be able to circulate into the peripheral lymphoid organs like spleen resulting in accumulation of cells that have undergone excision in the thymus.

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Additionally, complete excision of DP cells was achieved only at the leukaemic stage. This data also supports the fact that pre-leukaemic mice show T-cell developmental block at DN4-DP stages and that because Wnt pathway is activated in the DP cells, they do not mature to SP cells creating a block.

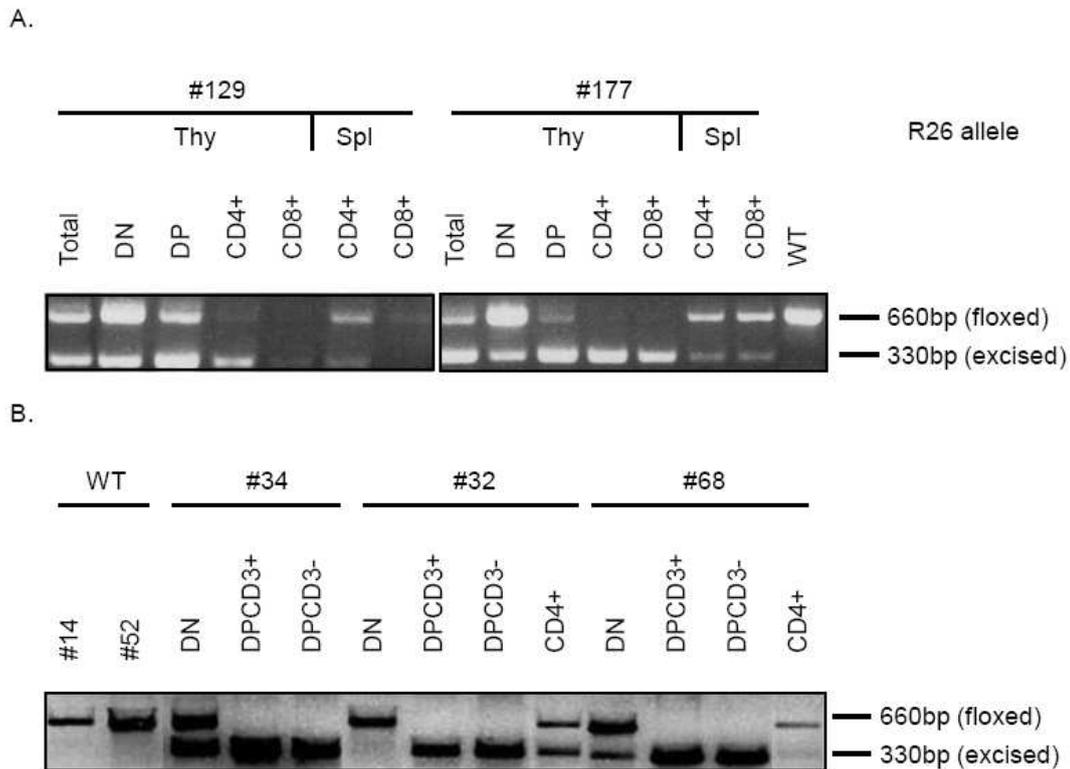


Figure 3.1. β -catenin excision in pre-leukaemic and leukaemic sub-populations. PCR analysis of DNA from pre-leukaemic (A) and leukaemic (B) sorted thymus sub-populations showing the R26 floxed allele (660bp) and R26- β -cat excised allele (330bp). The cells were sorted based on CD4, CD8 and CD3 cell surface markers. Two independent tumours have been used for pre-leukaemic and three for leukaemic analysis.

[III.1.2 R26- \$\beta\$ -cat tumours are malignant](#)

To address if the R26- β -cat tumours are malignant, we employed transplantation assays in our preliminary experiments. We transplanted groups of mice (3-5 per group) with 50000 or 10000 total tumour cells (CD45.2⁺) into lethally irradiated recipient mice (CD45.1⁺) by intravenous injection in the tail vein. We injected 200000 wild type BM (WT BM) cells

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(CD45.1/.2⁺) along with the tumour cells for radioprotection (Fig 3.2 A), allowing to us to follow WT BM cells and tumour cells in the same micro-environment which can be deduced simply by checking the percentages of cells expressing different leukocyte common antigen, CD45. Additionally, we can determine the irradiation efficiency by checking the percentage of CD45.1⁺ cells remaining in the recipient and the transplantation efficiency by percentage of CD45.1/.2⁺ cells. We consider a transplantation assay 'successful' if i) the host CD45.1⁺ cells are rather few, implying that the irradiation was efficient and ii) we are able to detect supporting WT BM cells in recipient mice, verifying our injections. Mice were bled 3 weeks after transplantation to check the expansion of leukaemia. Mice that showed high percentages of tumour-derived cells were observed daily and were sacrificed when sick. From the results obtained, we observed that all mice, irrespective of the number of tumour cells injected, developed leukaemia 30-50 days post-transplantation (Fig 3.2 B). The transplanted mice have small thymus, splenomegaly, paler liver with infiltration of leukaemic cells, 'whiter' bones and occasional larger inguinal or mesenteric lymph nodes. Interestingly, there was no difference in the tumour incidence between the groups of recipient mice injected with 50000 or 10000 tumour cells. Bones, spleen and thymi of all recipients were analysed. Bones and the spleen show the highest percentage (30-60%) of tumour-derived cells while thymi show the presence of 0-50% CD45.2⁺ cells. All CD45.2⁺ cells found in the recipient mice (from the BM, spleen and thymus) were DP cells like the primary tumour (Fig 3.2 C). This result shows that R26- β -cat tumours are malignant. For all our further assays, we have used a group of mice transplanted with 10000 total tumour cells as 'control group' since these results clearly indicated that 10000 cells were sufficient for reinitiating leukaemia.

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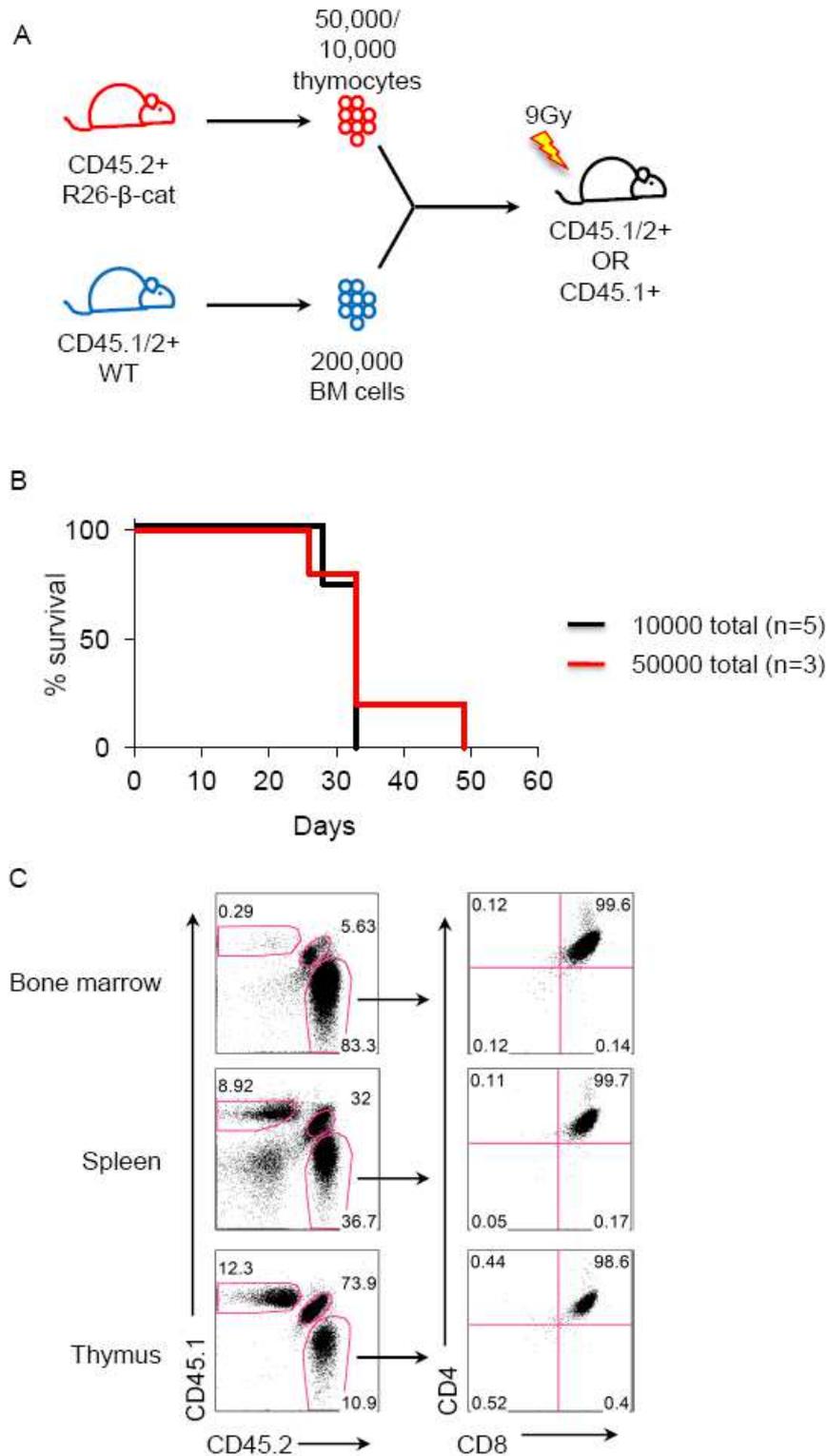


Figure 3.2. Ten thousand R26-β-cat thymocytes are sufficient to re-initiate tumours. A) Schematic representation of the experimental strategy, B) Kaplan-Meier survival curves showing tumour incidence among mice transplanted (CD45.1⁺) with tumour cells (CD45.2⁺) and supporting WT BM cells (CD45.1/2⁺) and C) Representative flowcytometry analysis of bone marrow, spleen and thymus of a recipient mouse transplanted with 10000 R26-β-cat tumour cells (CD45.2⁺) (left panel) and phenotype of CD45.2⁺ cells showing DP cells (right panel).

III.1.3 DP cells alone can regenerate leukaemia

As mentioned previously, the cancer stem cell hypothesis suggests that a tumour can be either homogeneous, in which case every single tumour cell has the capacity of regenerating tumours; or heterogeneous, in which case only a subset or subsets of cells possess such competence of producing tumours. These cells are called cancer stem cells (CSC) or leukaemia-initiating cells (LSCs) (Fig 1.8). We were intrigued to recognize if the R26- β -cat model was heterogeneous.

To check this, we planned to determine the capacity of different T-cell populations to re-initiate leukaemia in recipient mice.

To achieve the same, we stained tumour cell suspensions (CD45.2⁺) with antibodies CD4, CD8, CD44 and CD3. To be sure that we have chosen the cells that express activated β -catenin, we always sort cells that are high for cell surface marker CD44, since it is one of the target genes of Wnt pathway. We chose to sort the CD3^{-/lo} DP cells since WT DP cells express low or no CD3. In this manner, we sorted the following T-cell populations: DNCD44^{hi}CD3^{lo}, DP44^{hi}CD3^{lo} and CD4⁺CD44^{hi}CD3⁺ (Fig 3.3 A). Since we already know that 10000 of bulk tumour cells regenerates leukaemia, we used this as control. We then transplanted 10000 of each of these sorted populations along with 200000 WT BM cells (CD45.1/2⁺ or CD45.1⁺ depending upon the recipient mice genotype) into lethally irradiated recipient mice (CD45.1/2⁺ or CD45.1⁺). The mice were bled after 3 weeks and were sacrificed when sick. Interestingly, mice that were injected with DP cells alone developed leukaemia. Analysing these mice further, we noted that all CD45.2⁺ in the bones, spleen and thymi were DP cells. The mice that were transplanted with 10000 DP cells diseased at around the same time as the mice transplanted with 10000 bulk tumours (control group) (Fig 3.3 B).

These results show that the R26- β -cat tumours are heterogeneous and that the LSCs are contained in the DP population of the tumour.

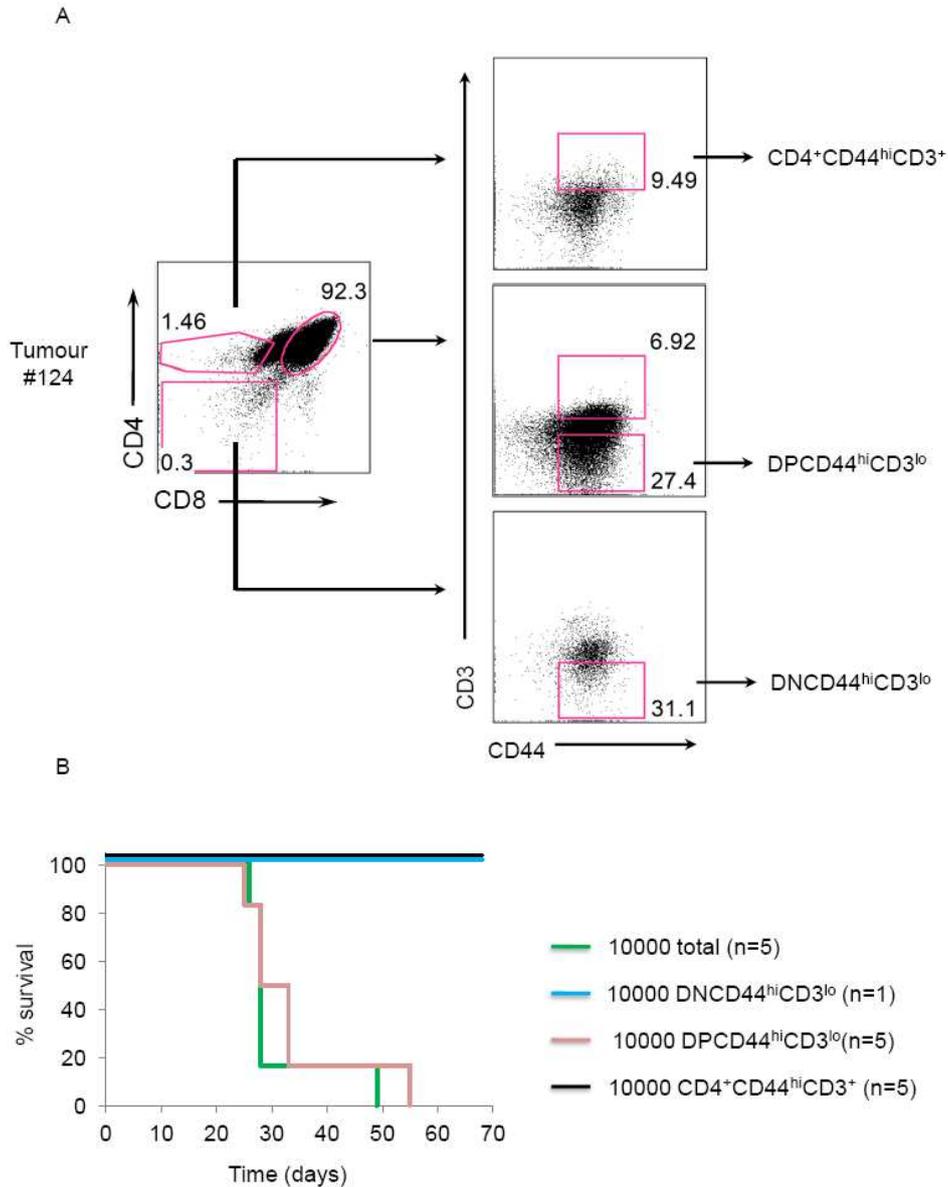


Figure 3.3. R26- β -cat DP cells alone can re-initiate leukaemia. A) Flow cytometry dot plots representing gates used to sort different R26- β -cat T-cell populations using cell surface markers CD4, CD8, CD44 and CD3 and B) Kaplan-Meier curve of mice transplanted with 10000 of different T-cell sub-populations. This is representative of 5 independent experiments.

III.1.4 DPCD3⁺ and DPCD3^{lo} cells possess equal potentials to regenerate leukaemia

Unlike the WT, most R26- β -cat DP cells express intermediate levels of CD3 (Fig 3.4 A). CD3 plays an important role in surface expression and signal transduction of the pre-TCR and

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TCR complexes (Dave, 2000). We speculated that CD3 marker might be able to help us identify the LSC-enriched population since the expression of CD3 has been reported on T-ALL LSCs earlier (Guo et al., 2008).

To determine if DP cells expressing CD3 had different leukaemic potentials than DPCD3^{lo} cells, we sorted DPCD44^{hi}CD3⁺ and DPCD44^{hi}CD3^{lo} from a single tumour (Fig 3.4 B). We transplanted 10000 of each of these two sub populations (CD45.2⁺) into lethally irradiated recipient mice (CD45.1/.2⁺) with 200000 WT BM competitor cells (CD45.1⁺) by intravenous injection through tail vein. Surprisingly, we did not notice any difference in their leukaemia re-initiating potential (Fig 3.4 C). They not only develop tumours at the same time, but also the phenotypes of tumour derived cells from both the groups of mice are similar (Fig 3.4 D).

This result excludes the fact that CD3 could have any role in leukaemia re-initiating potential of DP cells in R26- β -cat cells.

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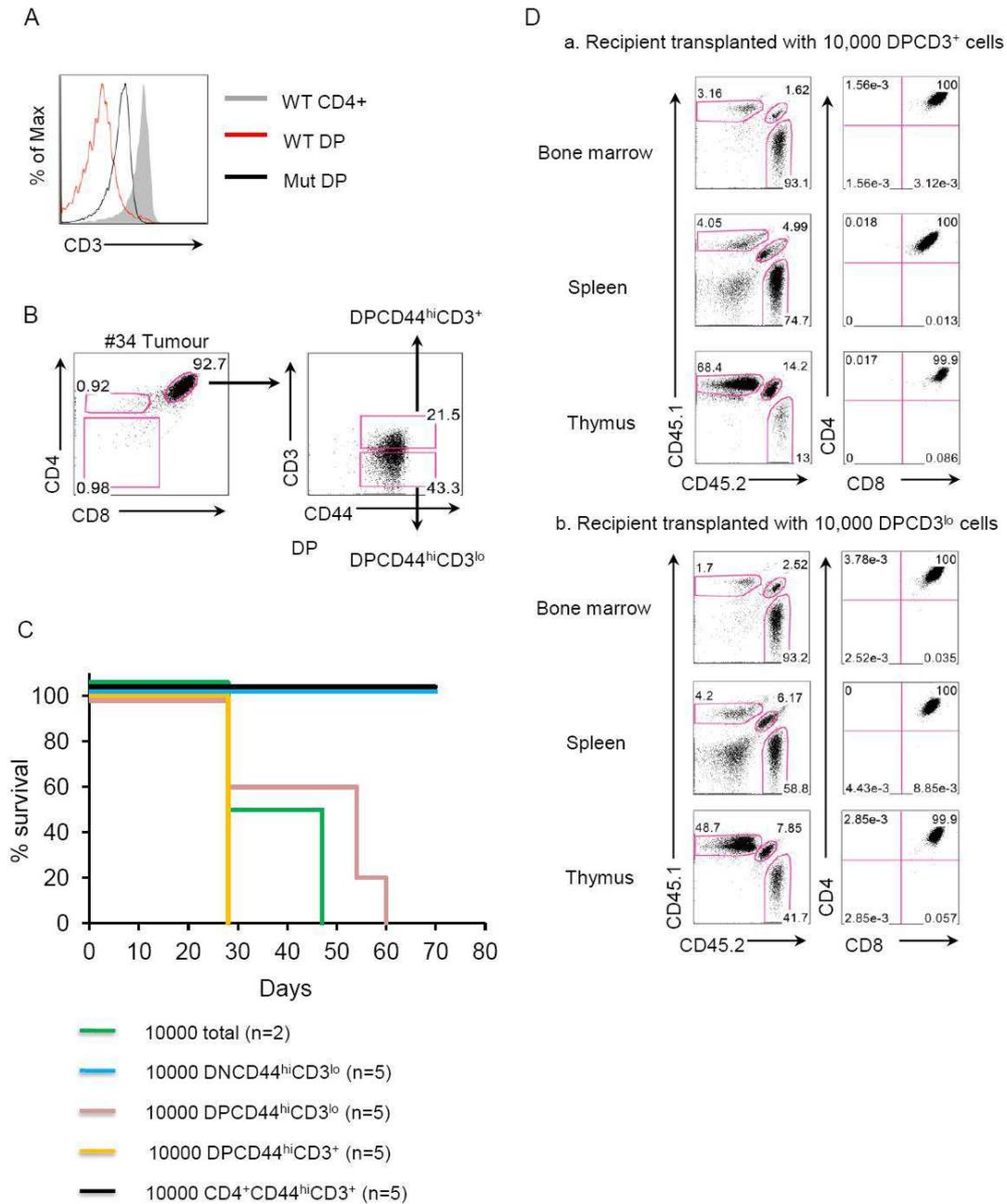


Figure 3.4. DPCD3⁺ and DPCD3^{lo} cells have equal tumour-reinitiating potential. A) Histograms showing CD3 expression in WT and R26- β -cat thymocytes, WT CD4⁺ cells (in grey) is used as positive control for CD3 expression, B) Flow cytometry plots showing sorters gates for tumour #34 based on CD4, CD8, CD44 and CD3 cell surface expression, C) Kaplan-Meier plots showing tumour latency in mice transplanted (CD45.1/2⁺) with 10000 DPCD3⁺ or DPCD3^{lo} of R26- β -cat tumour (CD45.2⁺) along with supporting WT BM cells (CD45.1⁺) and D) Representative flow cytometry analysis of mice transplanted with DPCD3⁺ and DPCD3^{lo} cells showing donor contribution (left panel), tumour profile CD4,CD8 (right panel). This is representative of 4 independent experiments.

[III.1.5 An atypical R26-β-cat tumour](#)

We have shown that DP cells alone are able to re-initiate leukaemia in lethally irradiated hosts suggesting that LSCs of R26-β-cat tumours are enriched in these cells. While this was true for 5 independent tumours analysed, we encountered a tumour which showed different results. In this case, we sorted DNCD44^{hi}CD3^{lo}, DPCD44^{hi}CD3^{lo}, DPCD44^{hi}CD3⁺ and CD4⁺CD44^{hi}CD3⁺ populations as in our previous experiments (Fig 3.5 A). We transplanted 10000 of these sorted cells (CD45.2⁺) into lethally irradiated recipient mice (CD45.1/2⁺) along with 200000 WT BM cells (CD45.1⁺) to support reconstitution by intravenous injection into the tail vein. As control, we transplanted 10000 total tumour cells in a group of recipient mice. We monitored these recipient mice and sacrificed them when their health deteriorated. Unlike our earlier experiments, all transplanted mice developed leukaemia within 80 days post-transplantation with a similar latency (Fig 3.5 B). Firstly, mice transplanted with total, DPCD3^{lo} and DPCD3⁺ cells developed leukaemia. Unexpectedly, mice transplanted with CD4⁺ and DN cells also developed leukaemia. Secondly, while the leukaemic CD45.2⁺ cells from mice transplanted with total and DP populations remained DP cells, the cells from mice transplanted with DN were DP or CD8⁺CD4^{lo}, and the ones transplanted with CD4⁺ cells remained CD4⁺ with few DP cells in all organs (Fig 3.5 C). However, all mice transplanted with DN cells did not develop leukaemia. These collective data showed that in this particular R26-β-cat tumour analysed, all sub-populations of the thymus appeared to have similar tumour re-initiating potentials.

This particular tumour did not represent the bulk R26-β-cat tumours generally observed. Since there was a single tumour with such altered heterogeneity, the results from this experiment did not contribute to our findings.

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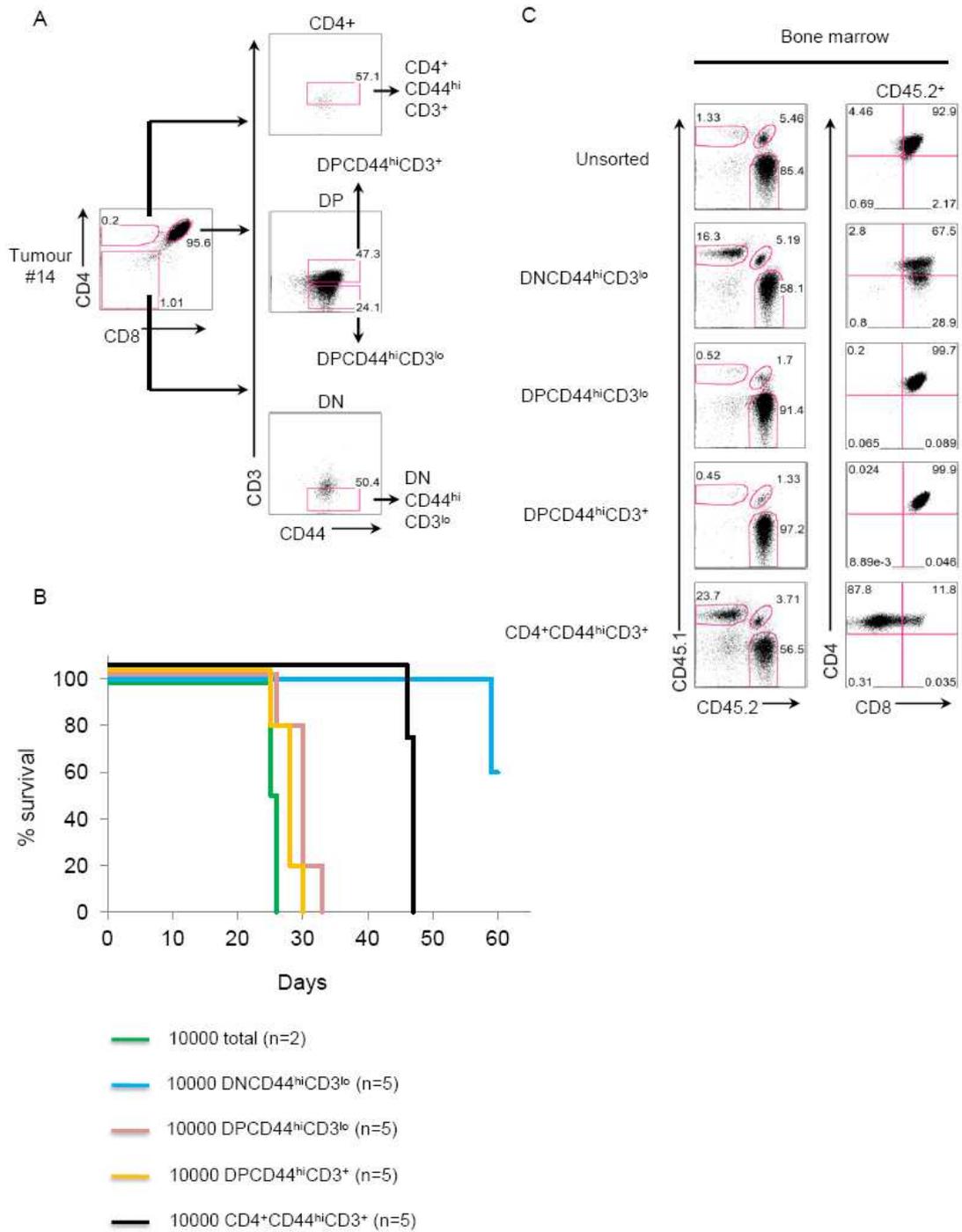


Figure 3.5. An atypical R26- β -cat tumour. A) Flow cytometry plots showing gates used to sort tumour cells based on CD4, CD8, CD44 and CD3 cell surface markers, B) Kaplan-Meier survival curve of mice transplanted (CD45.1/.2⁺) with 10000 tumour cells (CD45.2⁺) along with supporting WT BM cells (CD45.1⁺) and C) Flow cytometry analysis of representative bone marrows of mice from different groups transplanted with different sub-populations. Left panel shows the donor contribution (CD45.2⁺) and right panel shows the tumour profile of CD45.2⁺ cells.

III.1.6 LSCs are present at similar frequencies in R26-β-cat tumours

We established with limiting dilution assay that R26-β-cat tumours are heterogeneous and the frequency of LSCs is between 1/2000 to 1/500 cells in total tumours (refer manuscript Fig 2D). To confirm if LSCs are indeed present in the same range in all tumours, we performed yet another limiting dilution assay using DP cells instead of total tumour cells. We sorted DPCD44^{hi}CD3^{lo} cells from a R26-β-cat thymic tumour. We transplanted 10000, 5000, 2000 and 500 of sorted cells (CD45.2⁺) along with supporting WT BM cells (CD45.1/2⁺) into lethally irradiated recipients (CD45.1⁺) by intravenous injections. We monitored the health of mice and sacrificed recipients as they got sick. We noted that all recipients that received 10000, 5000 and 2000 DPCD44^{hi}CD3^{lo} cells developed leukaemia within 100 days post-transplantation. Additionally, only 50% of the mice transplanted with 500 cells developed leukaemia (Fig 3.6). This result suggests that 1/580 (frequency calculation by L-Calc software) cells are able to re-initiate leukaemia (range defined by ± SE = 1 in 969 to 1 in 348). Interestingly, the frequency obtained with this assay is similar to the frequency obtained with limiting dilution assay using total tumour cells (1/1187; range defined by ± SE = 1 in 1737 to 1 in 811). Collectively, these results demonstrate and confirm that LSCs of R26-β-cat leukaemia are present in a range of 1/580 to 1/1187 cells.

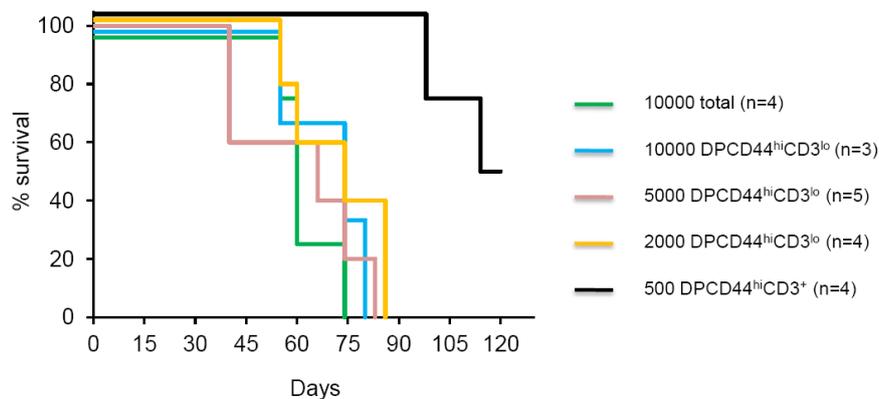


Figure 3.6. R26-β-cat LSCs are present at similar frequencies. Kaplan-Meier survival curve of limiting dilution assay in which lethally irradiated recipient mice were injected with reducing number of DPCD44^{hi}CD3^{lo} cells (10000, 5000, 2000 and 500).

III.1.7 Self-renewal of R26- β -cat LICs appears to be exhaustive

LSCs, like normal stem cells, possess properties of self-renewal and quiescence. Wnt signalling has been shown to induce proliferation and is believed to have a role in stem cell self-renewal (Attar and Scadden, 2004, Staal, 2004 #62). We planned to determine if the LSCs of this tumour has the capacity of self-renewal by serial dilution assays.

To be homogenous and do different analyses on few tumours, we often 'amplify' primary R26- β -cat tumours. To amplify a tumour, we inject large number of cells (5-10 million – CD45.2⁺) into lethally irradiated recipients along with supporting WT BM cells to mimic the primary tumour. These mice develop tumours as early as within 20 days. We then sort the CD45.2⁺ tumour cells from BM and use these cells as duplicates primary tumour cells to perform serial dilution assay.

To perform serial dilution assays, we used the amplified tumour cells from three different tumours (#123, #92, and #164). We transplanted 10000 of amplified tumour cells (CD45.2⁺) into a group of 3-5 mice making them the primary recipients for these assays. When the primary recipient mice developed tumours, we sorted the donor-derived tumour cells and transplanted 10000 cells into secondary recipients and so on (Fig 3.7 A).

We observed that through serial transplantations, the tumour latency increased (Fig 3.7 B). Although the time taken to re-initiate tumours in new varied among tumours, the trend was similar. The delay often ended by cells not being able to self-renew. Most tumours (3 out of 5 serial transplantations) could not self-renew beyond tertiary transplantations.

The model of clonal evolution of cancer stem cells led us to believe that there exists a possibility of certain 'superior clones' to be selected through serial dilution assays. It is likely that these clones are present in fewer numbers and are diluted through transplantation assays. It was also evident from these experiments that amplification of tumours does not

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actually mimic the primary tumour. From these results, we conclude that the R26- β -cat tumours can self-renew. However, their capacity to self-renew appears to be exhaustive.

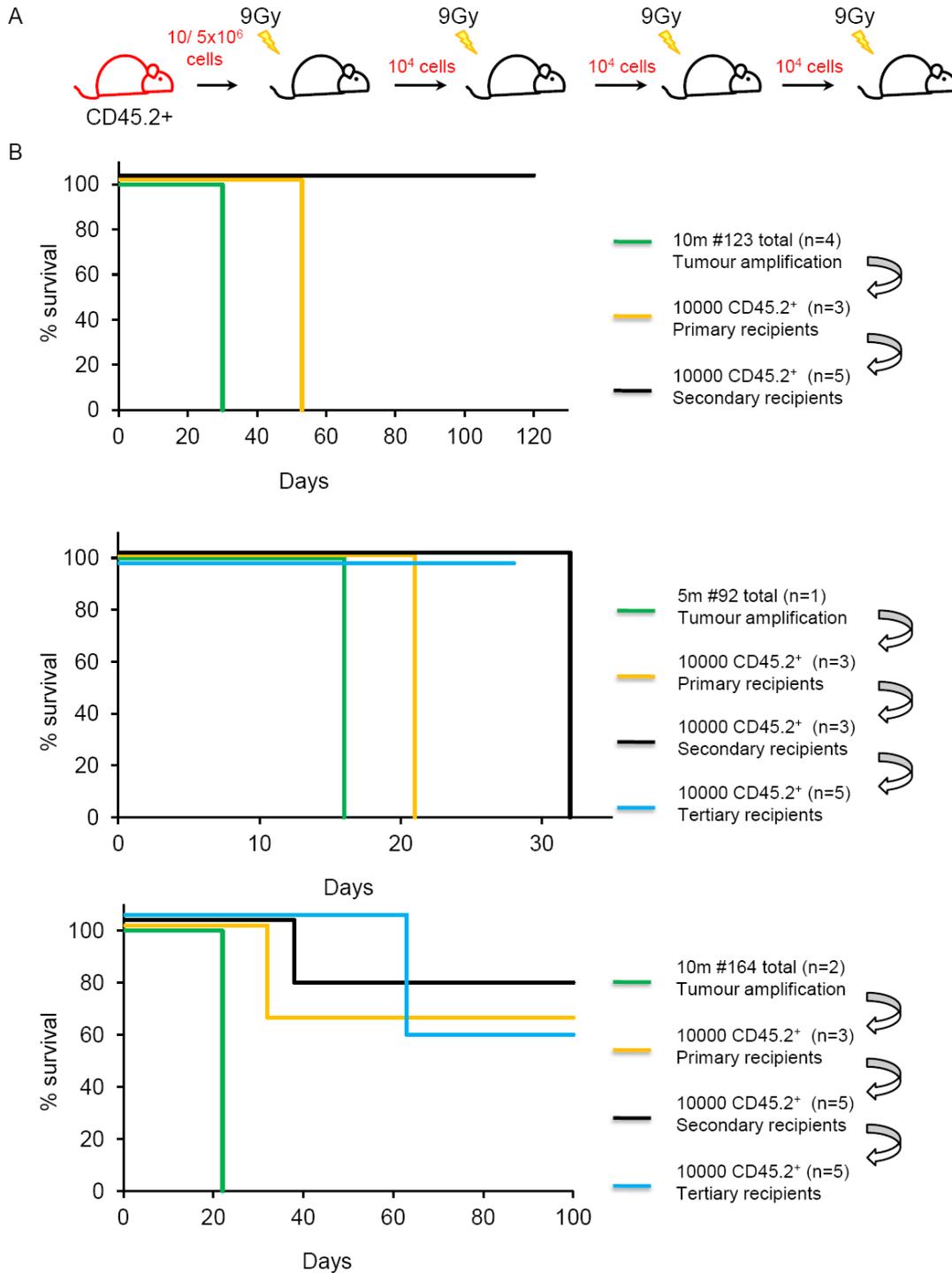


Figure 3.7. R26- β -cat LSCs appear to have exhaustive self-renewal. A) Schematic representation of serial dilution assays and B) Kaplan-Meier survival curves of mice in serial dilution assays of 3 independent amplified tumours. All transplantation assays were done by sorting CD45.2+

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tumour cells from the previous recipients' bones and by injecting these into lethally irradiated recipients.

From these results, we conclude that:

- i. DP cells undergo efficient neo-cassette excision,
- ii. R26- β -cat tumours are malignant,
- iii. DP cells alone can re-initiate tumours,
- iv. DPCD3⁺ and DPCD3^{lo} cells possess equal self-renewal potentials,
- v. LSCs of R26- β -cat tumours are present at a frequency approximately 1/2000 to 1/500 (1/580 – 1/1187) and
- vi. LSCs of R26- β -cat tumours appear to lose their capacity to self-renew through serial transplantation.

III.II Characterisation of leukaemia stem cells of R26- β -cat tumours

Our results show that R26- β -cat mice display LSC activity. To better understand LSCs, our next step was to try to characterize them. To achieve this, we adopted several methods to enable us to mark and isolate LSCs.

III.II.1 R26- β -cat thymocytes have an altered phenotype

To try to be able to identify a unique population that could be enriched in LSCs in this tumour, we planned to screen the tumours with a wide array of markers including activation markers, stem cell and cancer stem cell markers. Among them were Sca-1, CD200, CD138, CD71, CD2, CD5, CD44, CD69, CD3, CD133 and CD150.

Stem cell antigen-1 (Sca-1) is one of the most important markers used to identify HSCs in mouse. Sca-1 encodes a glycosylphosphatidylinositol (GPI)-linked cell surface protein, present in the plasma membrane of HSCs and thought to influence signalling by altering protein-protein interactions (Holmes and Stanford, 2007). Although Sca-1 is highly expressed in HSCs, it is reported to be not essential for HSC homeostasis as Sca-1 deficient mice show no obvious developmental defects, suggesting the involvement of Sca-1 in self-renewal of stem cells (Ito et al., 2003).

CD200 is a transmembrane glycoprotein expressed on B-, T- and dendritic cells. It is constantly shown to be over-expressed in CLL (chronic lymphoblastic leukaemia) (McWhirter et al., 2006). We found that CD200 was upregulated in the transcriptome of R26- β -cat leukaemia. Therefore, CD200 may be a useful marker to identify LSCs.

Syndecan-1/CD138 is an important transmembrane heparan sulphate proteoglycan and is expressed in distinct stages of differentiation of many cell types. CD138 expression has

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defined the CSCs of several multiple myeloma models and some B-cell leukaemias (Ghosh and Matsui, 2009; Huff and Matsui, 2008).

CD71 or transferrin receptor is an important carrier protein in most cells. Increased expression of CD71 is known to mark enhanced cellular activity indicating that CD71⁺ cells are highly proliferative. Recently, anti-CD71 antibody treatments have been experimented on adult T-ALL as potential therapy. Cells that do not express CD71 are thought to be quiescent and possess self-renewing abilities (Callens et al., 2008). Therefore, CD71⁻ cells may be an interesting population and may mark the LSCs.

CD2 is a surface glycoprotein found on T-cells and NK cells. Over-expression of CD2 is common feature of chronic lymphoid leukaemias (CLL). Novel drugs designed to target LSCs have aimed to use anti-CD2 antibodies (Matutes, 2008).

CD5 is a surface glycoprotein found on both B- and T-cells. The absence of CD5 on leukaemic cells has been reported to be a 'sign' of malignancy in CLL (Cortelazzo *et al.*, 2011).

CD69 is lectin protein expressed on stimulated and activated T-cells, amongst others. CD69 expression is known to play a role in lymphoid proliferation and signal transduction (Zola, 2000). CD69 expression may perhaps enable us to determine T-cell functionality statuses.

CD44 is a transmembrane glycoprotein that is a marker for immature T-cells. Hyaluronic acid (HA) and its derivatives are the most common ligands for CD44; however, there are many more ligands known to bind to CD44 (Hertweck *et al.*, 2011). It has been recently discovered to be over expressed in several types of cancer including haematological malignancies. There are accumulating evidence that CD44 plays a role in promoting leukaemias (Zoller, 2011). CD44 is a marker for breast cancer LSCs (Honeth et al., 2008). Recently, it was shown that human AML progression was delayed or almost abolished by using monoclonal antibodies against CD44 in xenografts (Jin *et al.*, 2006a).

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CD133 or Prominin-1 is a surface glycoprotein found on HSCs and early haematopoietic progenitors. Several recent studies described CD133 as a marker for CSCs of brain and colorectal cancers (Luo and Han, 2006). Therefore, this marker serves as an interesting candidate for our studies.

CD150 is a signalling lymphocyte activation molecule (SLAM) that regulates several leukocyte functions. It is selectively expressed among primitive mouse progenitors and HSCs (Sintes et al., 2008). SLAM family of markers have been recently linked with LSCs of haematological malignancies by serial xenotransplantation experiments (Wang et al.).

For the above mentioned reasons, we screened R26- β -cat tumours for these markers. By scanning all the markers, we observed that Sca-1, CD200, CD138, CD71, CD2, CD5, CD69, CD44, TCR β and CD3 markers were expressed at higher levels in R26- β -cat tumours (Fig 3.8 A, B). CD133 and CD150 expression however varied between mutants; while a small population of CD133⁺ population (~3-10%) was noted in some tumours, some others did not show any expression of CD133 (Fig 3.8 C, D).

Unexpectedly, we were not able to distinguish populations that are unique in the R26- β -cat tumours with all the above mentioned markers. However, it was possible for us to ascertain that phenotype of R26- β -cat tumour was altered and that these tumours are heterogeneous with respect to these cell surface marker expression.

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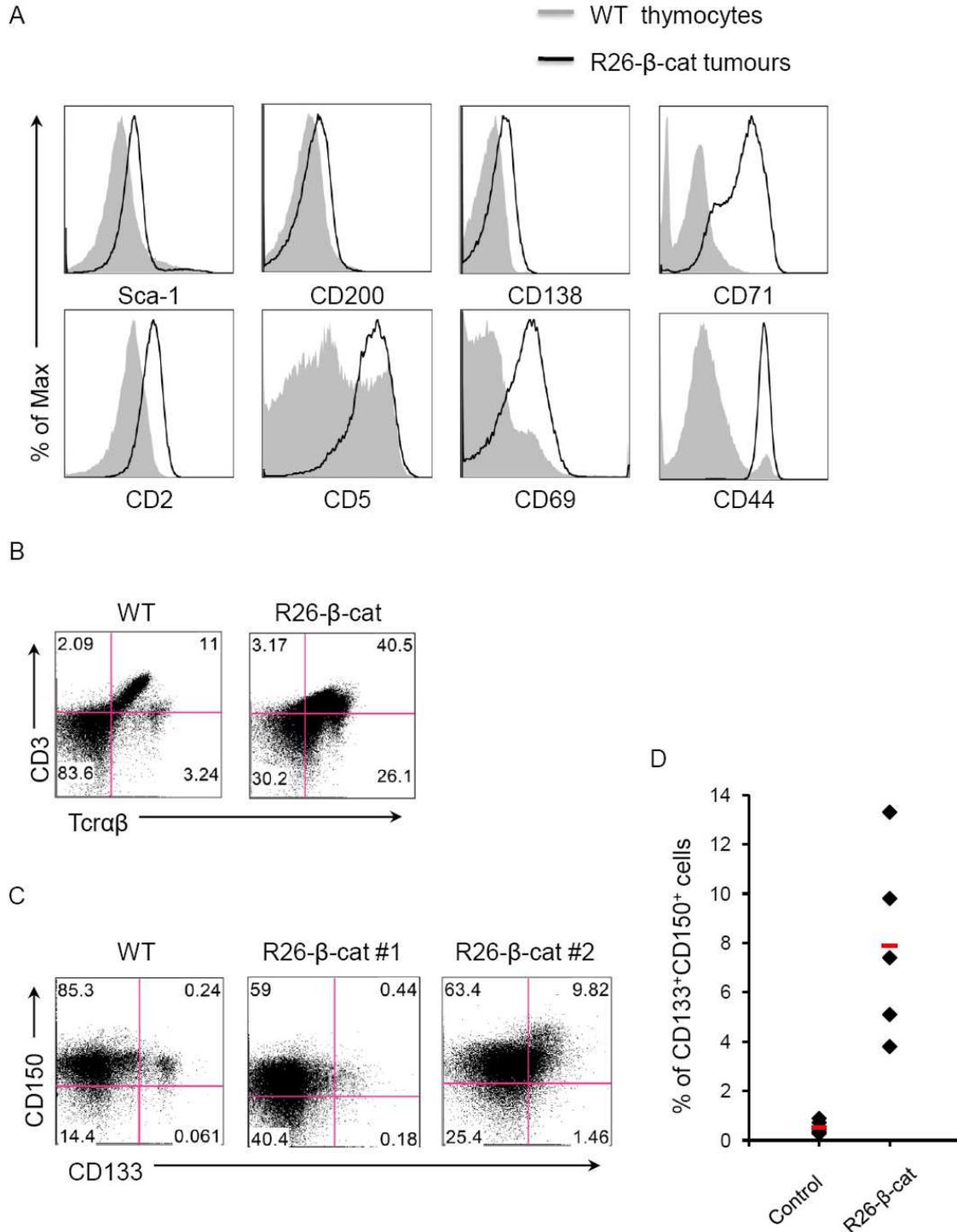


Figure 3.8. R26- β -cat tumours have altered phenotype. A) Histograms of WT and R26- β -cat thymocytes showing difference in expression of Sca-1, CD200, CD138, CD71, CD2, CD5, CD69, CD44, (B) Dot plots showing the expression analysis of CD3, TCR β , (C) CD133, CD150 expression in WT thymocytes and R26- β -cat thymocytes by flow cytometry. This data is representative of 12 independent tumours and (D) Percentage of CD133⁺CD150⁺ cells from WT and R26- β -cat thymocytes of 5 independent samples each. Red lines represent mean percentage in WT thymocytes and R26- β -cat tumours.

III.II.2 Side-population in R26- β -cat tumours

Since most of the markers screened were heterogeneously expressed, these results did not help us identify a unique population that could be regarded as LSCs in this tumour.

Therefore, we next sought to try to identify LSCs of this tumour using properties of CSCs or LSCs that have been well-described. A well-studied population that has been shown to display drug resistance and is quiescent is the side-population (Sp) cells. These cells have been shown to express ATP-binding cassette (ABC) transporter which helps in extrusion of some dyes including Hoechst 33342. Sp cells are shown to be composed of most immature cells mainly in the BM. Some Sp cells are also present in liver, kidney, lung, skin, brain and heart. Recently, Sp cells have been detected in many cancers including haematological malignancies. In few cancers, Sp cells have been shown to have clonogenic and tumorigenic properties which also are the key features of CSCs (Moserle *et al.*, 2009). Treatment with Verapamil, a calcium channel blocker is used to inhibit the ABC transporter and constitutes as negative control for Hoechst staining. All our Sp gates are based on respective Verapamil controls.

Our analysis revealed that R26- β -cat thymic tumours had a population of Sp that was present in greater percentages than in the WT thymi. The percentage of the mutant Sp however varied and was not consistent among tumours (Fig 3.9 B). This result suggests that Sp cells could serve as good candidates as LSCs of R26- β -cat tumours.

Furthermore, to identify the LSCs, we made use of the drug 5-fluorouracil (5-FU). 5-FU is a chemotherapy agent that is used to eradicate tumour mass in patients till date. This drug is an inhibitor of the enzyme thymidylate synthase which is the rate-limiting enzyme for thymidine synthesis. 5-FU has been described as one of the most potent inhibitors of this enzyme. With the aim of deleting the majority of the cells that are dividing and leave behind a quiescent cell population, we administered 5-FU to mutant mice. LSCs of many cells lines including those of human cancers have been previously characterized using this method

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(Zhang *et al.*, 2010). Therefore, if Sp cells were LSCs, they would be enriched after 5-FU treatment.

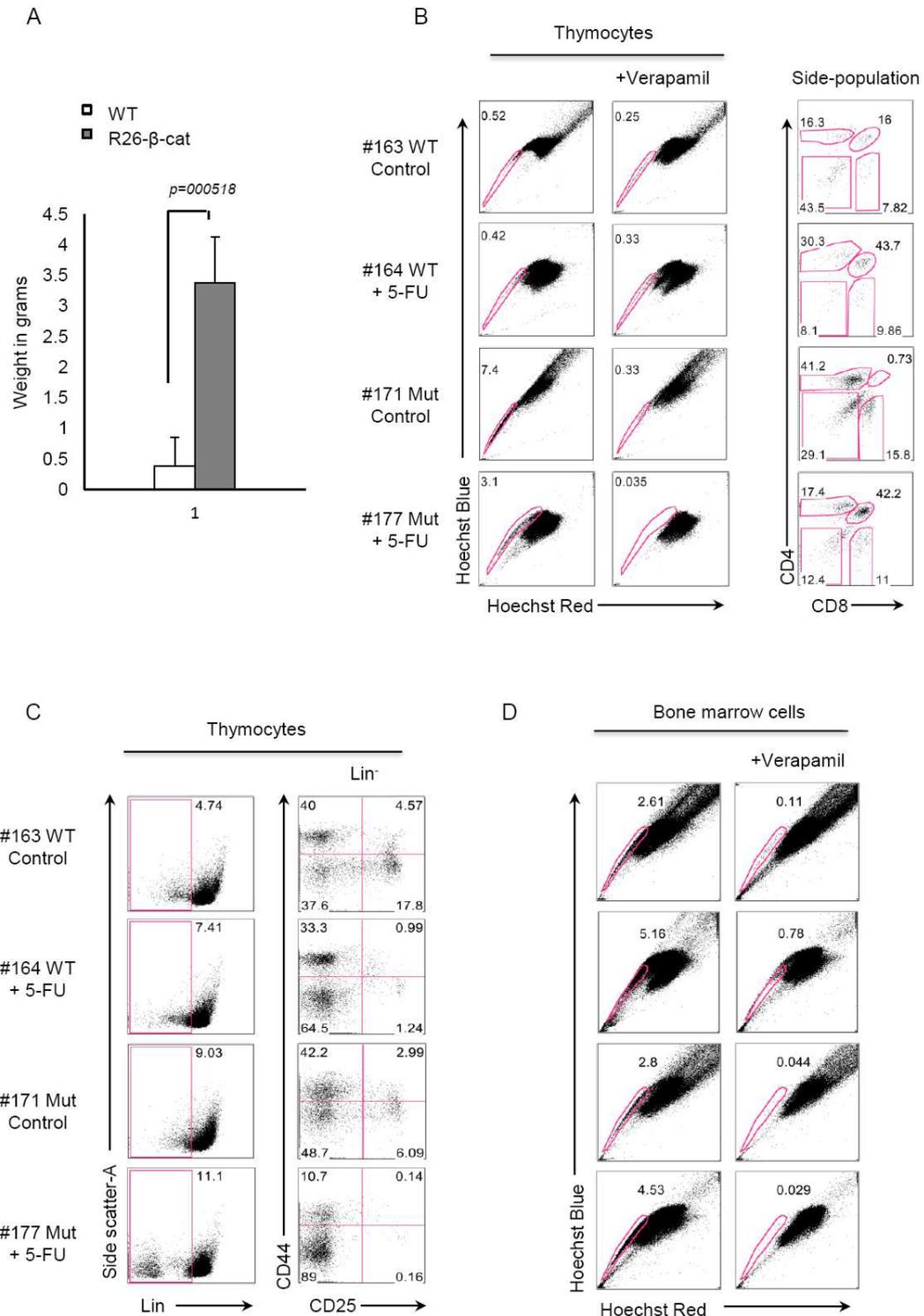


Figure 3.9. Side-population analyses of R26-β-cat tumours. A) Estimation of difference in weight loss of control and mice treated with 5-FU, B) Flow cytometry analysis of Hoechst 33342 staining of 5-FU treated WT and R26-β-cat tumours showing side-population (Sp) with (middle panel)

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or without (left panel) Verapamil (control) and the phenotype of Sp (right panel), C) analysis of 5-FU administered WT thymocytes and R26- β -cat tumours showing DN sub-populations. (CD4⁻CD8⁻CD3⁻) Lin⁻ cells are gated (left panel) and represented with CD44 and CD25 (right panel) and D) analysis of side-population staining of 5-FU administered WT and R26- β -cat bone marrow cells with (left panel) or without (right panel) Verapamil. This data is representative of two independent experiments (WT control (n=2), WT+5-FU (n=3), R26- β -cat control (n=2) and R26- β -cat +5-FU (n=3)). All plots are gated on TOPRO3⁻ cells.

In this regard, we administered the drug 5-FU by intraperitoneal injection in WT and R26- β -cat leukaemic mice at 150mg/kg. 48 hours later, we analysed mice injected with 5-FU or not (control). We weighed the mice before and after injections and noticed that while the WT mice lost 1.3% of their total mass, the mutant mice lost an average of 13.8% (Fig 3.9 A). This loss of mass in R26- β -cat mice was significantly higher than the mass lost by WT mice. The overall cellularity of the organs (BM, Spleen and Thymus) analysed was reduced (~3 fold). Mutant mice that were sick prior to injection had become slightly more active after injection reflecting the regression of the tumour. The effect of 5-FU on WT and R26- β -cat remained similar in the DN sub-populations. Both showed an enrichment of DN4 subset and loss of DN3 cells (Fig 3.9 C).

Additionally, we also stained BM cells (which are known to contain Sp cells) of WT and R26- β -cat mice, treated with 5-FU or not, with Hoechst dye. As expected, the Sp from BM of both WT and mutant were enriched with 5-FU treatment (Fig 3.9 D). This served as a control that showed that the 5-FU administration and staining was correct.

[III.II.3 5-FU treatment enriches a rare CD133⁺CD150⁺ population in some R26- \$\beta\$ -cat tumours](#)

Because Sp analysis on R26- β -cat was not very clear in terms of identifying a drug-resistant population, we employed alternate methods. We tried to characterise 5-FU treated R26- β -cat and WT mice with markers that we used for immuno-phenotyping with the aim of identifying

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a population expressing certain markers that could be enriched after the treatment. To do so, we used amplified tumours.

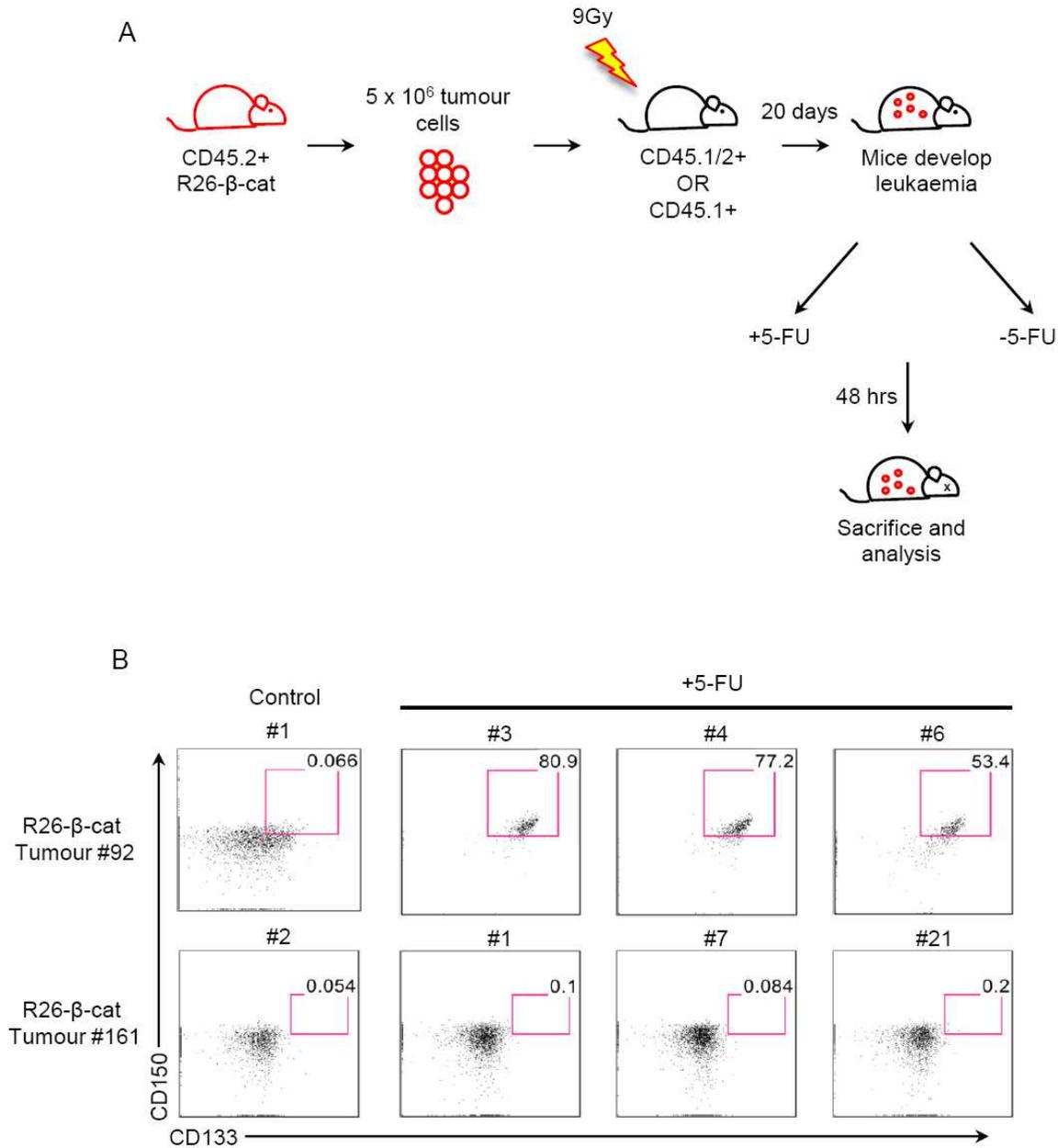


Figure 3.10. CD133⁺CD150⁺, a drug-resistant population enriched in some R26-β-cat tumours. A) Schematic representation of 5-FU administration strategy in this experiment and B) analysis of markers CD133 and CD150 expression in two independent amplified tumours after 5-FU administration in different recipient mice. The graphs represent TOPRO3⁺CD45.2⁺DP cells for alive, tumour, DP cells respectively. Tumours that show enrichment, n=1; tumours that did not show enrichment, n=2.

As these mice developed leukaemia, we administered 5-FU in the mice by intraperitoneal injections. 48 hours later, we analysed mice for different markers mentioned earlier (Fig 3.10 A). We observed that for all recipients that received tumour #92, a population of CD133⁺CD150⁺ was enriched (Fig 3.10 B). This enrichment was specific to the CD45.2⁺ BM cells of the 5-FU treated mice. The spleen and thymi showed no such enrichment. However, 2 other tumours that were analysed showed no such enrichment in the organs analysed. Moreover, since CD133 expression was not consistent among all tumours, CD133⁺CD150⁺ population enrichment after 5-FU treatment also seemed reflecting the variability among primary tumour's expression of these markers.

[III.II.4 Leukaemic cells expressing CD133, CD150, Sca-1 and CD200 have higher tumour re-initiating potential in some R26-β-cat lymphomas](#)

By screening a wide array of stem cell and CSC markers, we determined that R26-β-cat tumours have an altered phenotype compared to WT thymocytes (Fig 3.8). We observed that Sca-1 was over-expressed in R26-β-cat tumours and Sca-1 is an important marker for HSCs that may have a role in self-renewal. We noticed that CD200 was upregulated in the transcriptome of R26-β-cat tumours. Furthermore, we noted that some R26-β-cat mice treated with 5-FU showed an enrichment of CD133⁺CD150⁺ leukaemic cells (Fig 3.10 B). Additionally, we wanted to address the issue of 'importance' of these markers. To determine if cells expressing CD133, CD150, Sca-1 and CD200 have higher tumorigenic potential, we first amplified a R26-β-cat tumour (CD45.2⁺). We sacrificed these mice as they got sick and stained harvested BM cells with CD133, CD150, Sca-1 and CD200 cell surface markers (Fig 3.11 A). From the donor-derived BM cells, we sorted populations that were positive and negative for these markers. We transplanted 10000 of total (CD45.2⁺), positive (CD133⁺CD150⁺Sca-1⁺CD200⁺) and (CD133⁻CD150⁻Sca-1⁻CD200⁻) into lethally irradiated recipients (CD45.1/.2⁺) along with 200000 WT BM cells for radioprotection, by intravenous

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injections (Fig 3.11 B). Mice transplanted with total tumour cells and population expressing all the cell surface markers developed tumours within 60 days post-transplantation (Fig 3.11 C). Donor-derived cells harvested from BM, spleen and thymi of these mice were all DP cells, like the original primary tumour. The DP cells appeared to not maintain the CD133 and CD150 expression (Fig 3.11 D). However, mice transplanted with population not expressing these markers failed to re-initiate tumours in lethally irradiated hosts.

From these results, we conclude that LSCs of R26- β -cat tumours may be enriched in the stem and cancer stem cell marker expressing population within the DP cells. We speculate that there is a possibility that R26- β -cat tumour LSCs may be contained in the stem cell-like populations in the DP cells. However, since the cell surface marker expression lack homogeneity among tumours, LSCs of individual tumours may express different markers. Although these results could not mark CD133⁺CD150⁺Sca-1⁺CD200⁺ cells as LSCs of R26- β -cat tumours, we could theorize that these markers may characterize LSCs in some R26- β -cat tumours.

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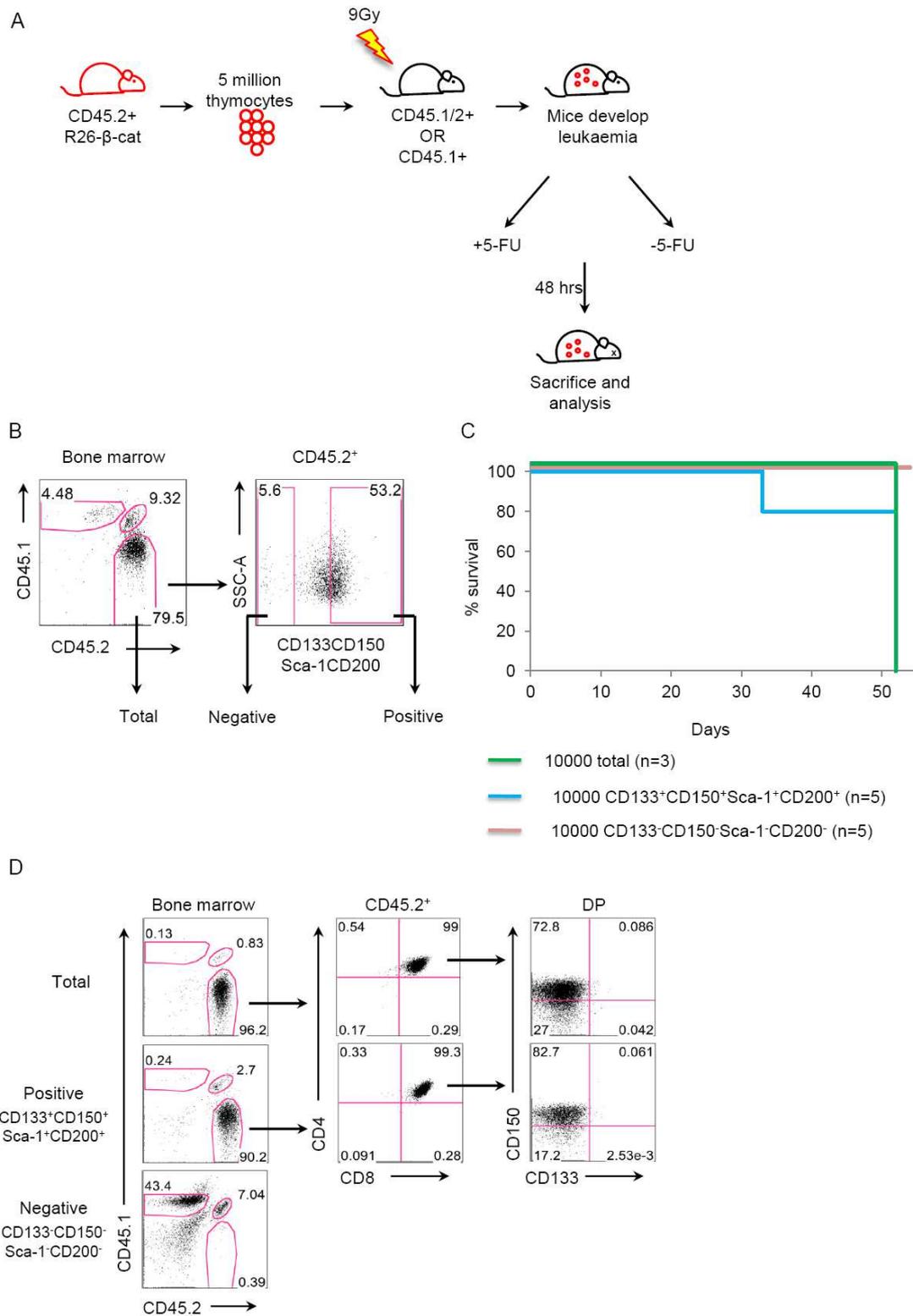


Figure 3.11. Tumour re-initiation potential of CD133⁺CD150⁺Sca-1⁺CD200⁺ cells. A) Schematic representation of experimental strategy, B) Flow cytometry plots of gates used to sort CD45.2⁺ (donor-derived tumour cells from bone marrow of mice transplanted to amplify tumour) for total, positive (CD133⁺CD150⁺Sca-1⁺CD200⁺) and negative (CD133⁻CD150⁻Sca-1⁻CD200⁻) cells for transplantation, C) Kaplan-Meier survival curves of mice transplanted with population (10000 cells) expressing or not expressing CD133, CD150, CD200 or Sca-1 markers, and total tumour cells

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(CD45.2⁺) and D) Flow cytometry analysis of bone marrow of mice representative of each group showing donor contribution (CD45.2⁺) (left panel), their phenotype for CD4, CD8 (middle panel) and CD133, CD150 (right panel).

III.II.5 A subset of DP cells appear to be radio-resistant

With earlier analyses, we observed that R26- β -cat thymocytes consisted of potential LSC populations like Sp and drug-resistant CD133⁺CD150⁺ cell surface marker expressing DP cells (Fig 3.9 and 3.10). The results from these experiments were not consistent among different tumours. Therefore, we attempted to try to identify radio-resistant DP cells. Radio-resistance is property of CSCs to survive through radiation therapies in patients, resulting in relapse. CSCs and stem cells are known to have efficient DNA repair mechanisms allowing them to persist through the treatment (Rich, 2007). Radiation, in this case X-rays, creates several DNA breaks, both single and double stranded breaks, and results in cell death. Most dividing cells are killed leaving behind the quiescent, radio-resistant cells. These cells could be potential LSCs. There is accumulating evidence that CSCs are inherently resistant to radiation.

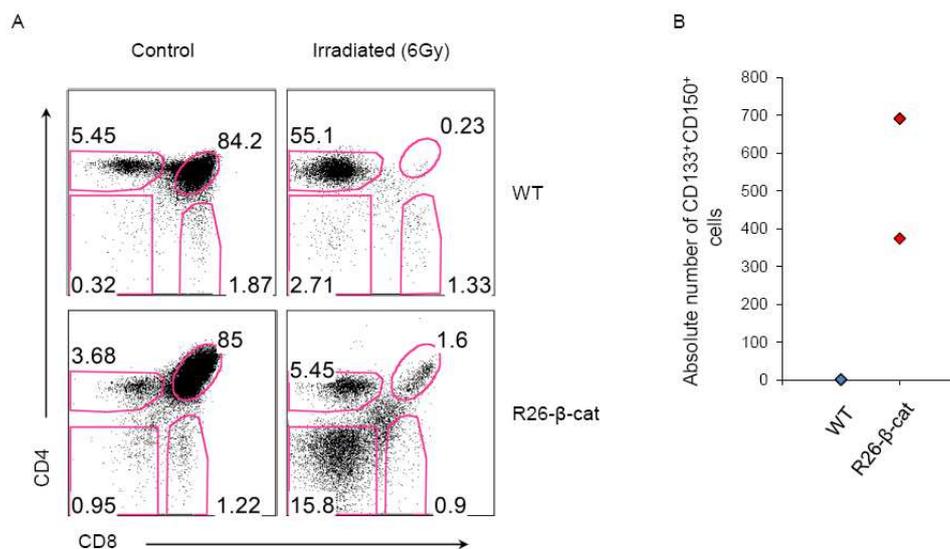


Figure 3.12. A sub-population of DP cells of R26- β -cat tumours appear to be radio-resistant. A) Flow cytometry analysis of TOPRO3⁻ cells for CD4 and CD8 markers in control (non-

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irradiated) and sub-lethally irradiated WT (n=1) and mutant leukaemic R26- β -cat mice (n=2) and B) Graphic representation of number of DP cells after irradiation in WT and R26- β -cat thymocytes.

To determine if R26- β -cat tumour cells contained some radiation-resistant, we irradiated WT and sick R26- β -cat mice using an X-ray source with a sub-lethal dose of 6Gy. 48 hours later, we analysed irradiated mice and used non-irradiated mice as control. We observed that in WT mice, DP cells did not survive the sub-lethal dose and were killed by irradiation. We also noted an enrichment of CD4⁺ cells in WT (~55%) along with lesser enrichment (~5%) in R26- β -cat tumours (Fig 3.12 A). There was a large enrichment of DN cells (~15%) in the mutant thymus. Additionally, in the mutants there was a residual DP population that survived the irradiation (Fig 3.12 B). Since we have previously found that LSCs are contained in the DP cells, we speculate that this population might therefore be enriched LSCs of R26- β -cat tumours.

These results show that some DP cells appear to be radio-resistant within R26- β -cat tumours.

Taken together, our attempts to characterise LSCs of R26- β -cat showed that;

- i) Sp cells are present in the R26- β -cat thymic lymphomas,
- ii) Sp cells are not enriched by 5-FU treatment,
- iii) a CD133⁺CD150⁺ population appear to be enriched in some tumours but not in others after 5-FU treatment,
- iv) CD133, CD150, Sca-1 and CD200 expressing R26- β -cat thymocytes possess self-renewal potential in some tumours and
- v) some DP cells are radio-resistant.

III.III Molecular mechanisms regulating R26- β -cat leukaemogenesis

Our preliminary data on the transcriptome profiles by Affymetrix microarray on pre-leukaemic mutants (6 weeks) showed 142 deregulated genes. Interestingly, WT and leukaemic mice showed that 1800 genes were deregulated (refer manuscript Fig 3A, Table S1). This suggests that secondary events that follow activated β -catenin accumulation contribute to leukaemogenesis. This could also explain why these tumours take 6-8 months to advance.

III.III.1 R26- β -cat cell lines mimic R26- β -cat tumours

For our molecular studies, we made use of cell lines that we have generated from R26- β -cat tumours. The cell lines were generated by culturing them at very high numbers (10-20 million) for 4-7 days. After about a week of growth, some tumours give rise to robustly proliferating cells, which can be maintained in culture. We have generated >10 cell lines from R26- β -cat tumours and have labelled them in '149' series (149.1, 149.2 ...etc).

To demonstrate that these cell lines mimic the R26- β -cat tumours, we analysed them by flow cytometry for intracellular β -catenin expression. All cell lines maintained the expression of intracellular β -catenin (Fig 3.13 A, B). To assess their malignant potential, we transplanted 10000 of these cells along with WT BM supporting cells into lethally irradiated recipients by intravenous injection into tail vein. We observed that these mice developed leukaemia like the primary tumours (Fig 3.13 A). The donor-derived tumour cells from the transplanted mice resembled those of the primary tumours. Therefore, our *in vitro* studies on these cell lines are likely to be relevant to the biology of R26- β -cat tumours *in vivo*.

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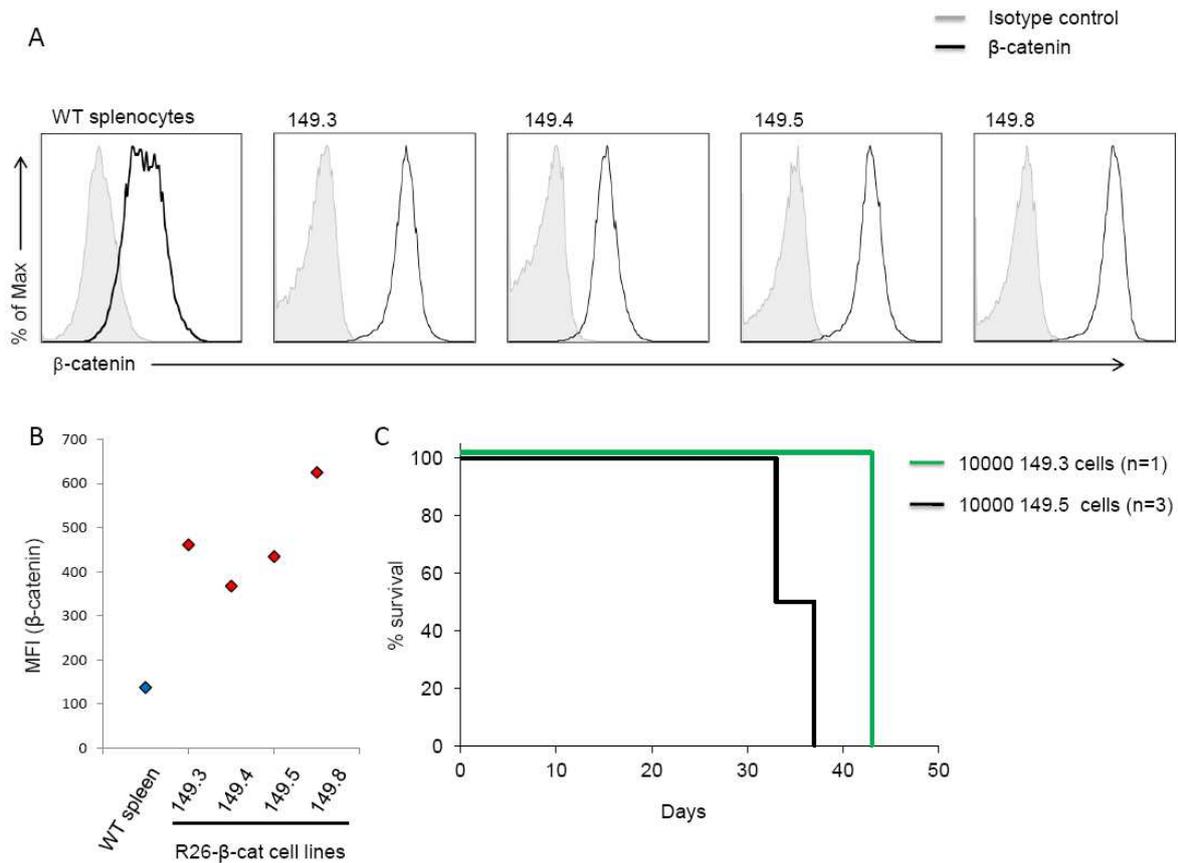


Figure 3.13. R26-β-cat cell lines mimic primary tumours. A) Flow cytometry analysis of intracellular β-catenin expression in R26-β-cat cell lines with respective isotype controls, B) Mean fluorescence intensity of β-catenin in different cell lines and WT spleen and C) Kaplan-Meier survival curve of mice transplanted with 10000 cells from R26-β-cat cell lines along with 200000 WT BM supporting cells.

III.III.2 R26-β-cat cell lines express very low level of Notch1

The microarray results showed down-regulation of Notch1 in leukaemic DP cells of R26-β-cat tumours (refer manuscript Fig 3B, C, D). In addition to these results, we also verified for the intracellular expression of Notch1 in R26-β-cat cell lines by flow cytometry. As expected, we observed that the expression level of Notch1 was very low in all the R26-β-cat cell lines analysed compared to a Notch-dependent cell line T-29 (derived from Ik^{L/L} T-ALL, (Dumortier *et al.*, 2006a)) (Fig 3.14 A, B).

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This data supports the microarray results and confirms that R26- β -cat cell lines not express Notch1, the major receptor mediating Notch signalling in T-ALL

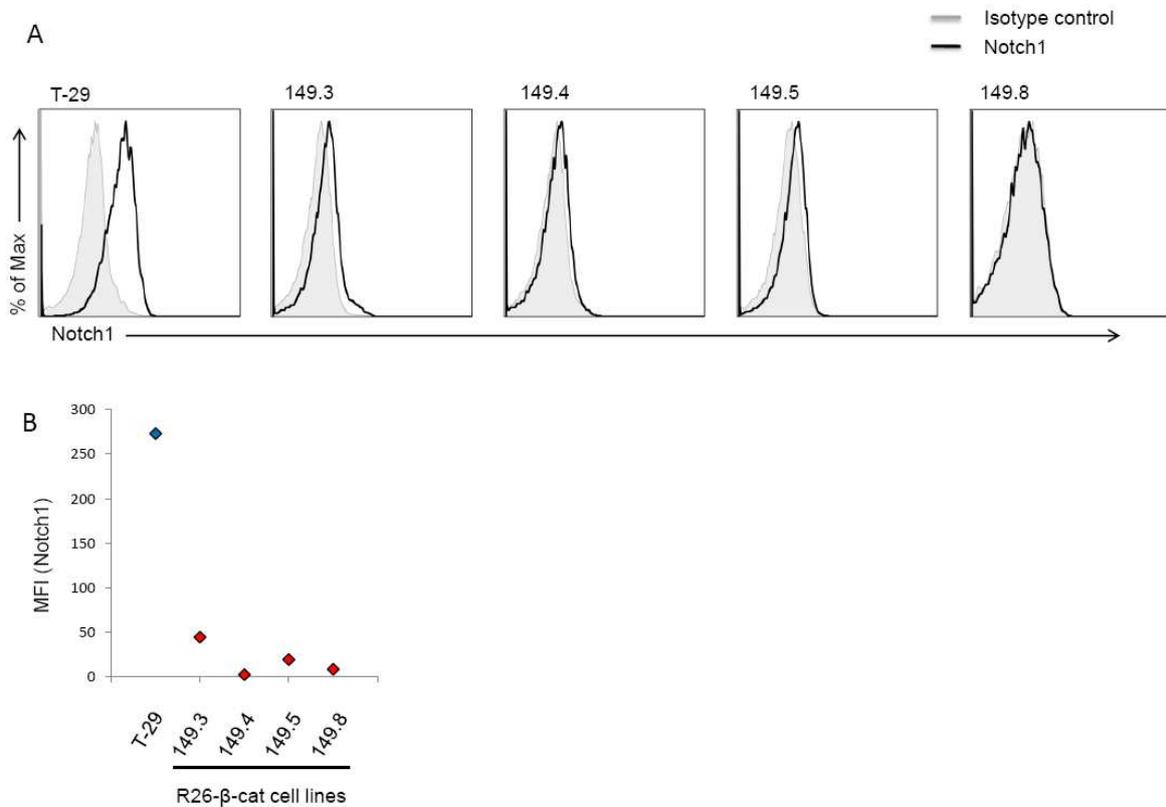


Figure 3.14. R26- β -cat cell lines express low or no Notch1. A) Flow cytometry analysis of intracellular Notch1 expression in R26- β -cat cell lines with respective isotype controls, B) Mean fluorescence intensity of Notch1 in different R26- β -cat cell lines and T-29 (cell line derived from Notch-dependent $Ik^{L/L}$ tumour)

III.III.3 Inhibition of the Wnt pathway in R26- β -cat cell lines

We have determined that inhibition of Wnt pathway by retroviral (MiG $Icat$ -GFP) transduction of $Icat$ (catenin- β interacting protein) in R26- β -cat cell lines suppresses their proliferation (refer manuscript Fig 3E, F). Prior to using this, we tried three other approaches to achieve efficient inhibition of Wnt signalling.

- Inhibition using dn-Tcf4 – We cloned cDNA of dominant negative form of Tcf4, dn-Tcf4 (Kolligs et al., 1999) into a lentiviral vector (pTRIP MND-GFP (Gerby et al.) such

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that it would generate a fusion protein dn-Tcf4-GFP driven under the T-cell specific MND promoter. Puromycin resistance gene was also cloned for selection. The dn-Tcf4 protein binds to Wnt target sequences but cannot interact with β -catenin, thereby inhibiting the expression of Wnt target genes. After infection of R26- β -cat cell lines with pTRIP MND dn-Tcf4-GFP lentiviruses, we did not notice any difference in growth of these cells compared to those infected with the empty vector (pTRIP MND-GFP). We were not able to find a suitable antibody to detect dn-Tcf4 protein in the infected cell lines to prove that Tcf4 was in fact expressed in the infected cells. We were not able to conclude from these results if the fusion protein is not functional or if inhibition of Wnt pathway has no effect on these cell lines. Therefore, these results were not conclusive.

- b) Inhibition using Quercetin – Quercetin is a flavonol, a subclass of flavonoids. Flavonoids are components of normal human diet. Studies have shown that inhibition of Wnt pathway by Quercetin can block the initiation of colorectal cancers in mice bearing crypt foci (Matsukawa et al., 1997). Furthermore, Quercetin has been shown to efficiently inhibit Wnt pathway in human colon cancer cell lines (Matsukawa et al., 1997). When we treated the R26- β -cat cell lines, they did not proliferate well in culture after Quercetin treatment. This was true not only for R26- β -cat cell lines, but also for T-29 and HEK293 cells. Since 10 μ g/mL of Quercetin appeared toxic to cells, we tried to titer the concentration on all cell lines. As low a concentration as 10ng/mL was also toxic to all cell lines (R26- β -cat, T-29 and HEK293). Therefore, we abandoned this method and switched to the next strategy.
- c) Inhibition using Icat in lentiviruses – Since the last two methods were not successful, we made use of Icat to achieve efficient inhibition of Wnt pathway. We cloned cDNA of mouse Icat into pTRIP MND-GFP retroviral vector where Icat expression would be driven by MND promoter and this would produce a fusion protein, Icat-GFP. We also cloned puromycin resistance for selection. We infected R26- β -cat cell lines with

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vector containing Icat or empty vector and analysed 48 hours post-infection. We did not detect any GFP expression until 5 days post-infection. However, the GFP expression was very low and was not maintained in spite of selection with puromycin.

Thus, we did not use these results to contribute to our data.

III.III.4 CD44 expression is reduced by inhibition of Wnt pathway

We demonstrated that R26- β -cat cell lines did not proliferate well when Wnt pathway was inhibited using Icat (refer manuscript Fig 3E, F). CD44 is a direct Wnt target gene (Hertweck *et al.*, 2011; Zoller, 2011). We also investigated the expression of CD44 in Icat-infected cell lines (149.4 and 149.5). We detected a two-fold reduction in CD44 mean fluorescence intensity (MFI) in MiG Icat-GFP-infected R26- β -cat cell lines when compared to those infected with empty vector (MiG GFP) (Fig 3.15 A, B). CD44 expression in MiG-ICAT-GFP cells suggests that the Wnt pathway was inhibited.

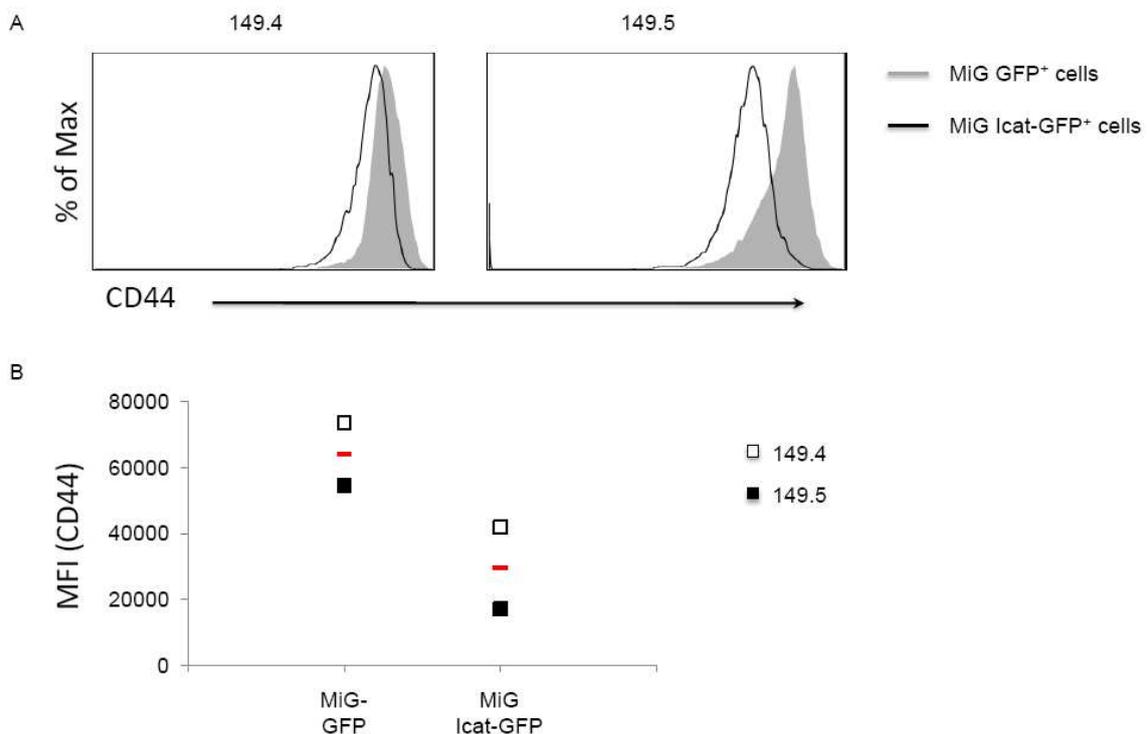


Figure 3.15. CD44 expression is reduced by ICAT-mediated inhibition of the Wnt pathway in R26- β -cat cell lines. A) Histograms of CD44 expression in two different cell lines

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infected with empty vector (MiG GFP) and Icat containing vector (MiG Icat-GFP) and B) MFI of CD44 expression in both cell lines.

Taken together, we conclude that;

- i. R26- β -cat cell lines express high level of β -catenin and are able to re-initiate tumours in lethally irradiated hosts similar to R26- β -cat tumours,
- ii. R26- β -cat cell lines do not express Notch1 and
- iii. Icat infected R26- β -cat cell lines have reduced CD44 MFI indicating that Wnt pathway is inhibited in these cell lines by Icat.

IV. Discussion and perspectives

In this section, I have tried not to repeat the discussions that are in the manuscript. However, this is an extension of the discussion in the manuscript and complementary results.

Oncogenesis is a complex multistep process that often involves mutations that favour transformation. We report a murine model, R26- β -cat, that expresses stabilised form of β -catenin in T-cells. Young mice show developmental blocks at DN4 and DP stages. DP cells of pre-leukaemic mice exhibit resistance to apoptosis. Eventually, these mice acquire T-ALL. The thymic tumour consists mostly of DP cells. Further, we show that R26- β -cat tumour proliferation is Notch-independent and Wnt-dependent. Additionally, CD44 did not appear to have a role in leukemogenesis and homing of R26- β -cat tumour cells. We also demonstrated that loss of PTEN and over-expression of Myc was favoured and may constitute secondary events that contribute to this leukaemogenesis (Fig 4.1 and 4.2).

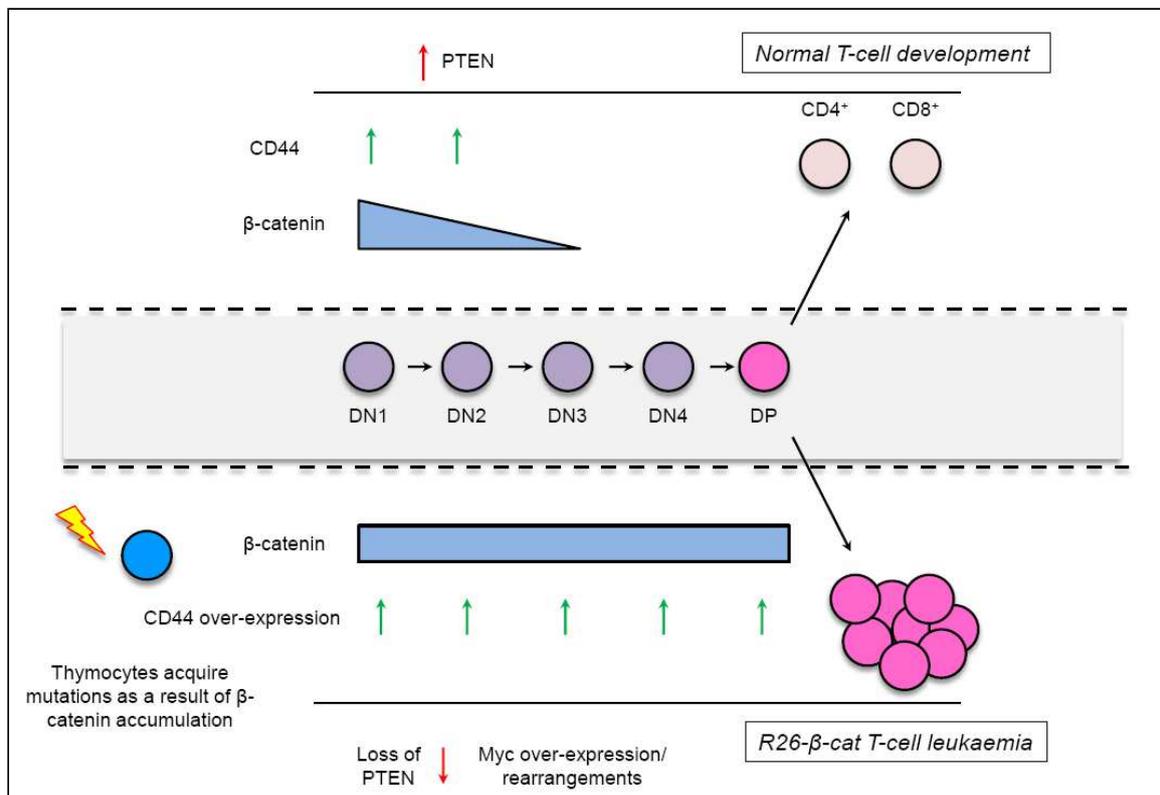


Figure 4.1. Schematic representation of R26- β -cat leukaemogenesis. Over-expression of stabilised β -catenin during T-cell development results in acquisition of secondary genetic events and eventually T-ALL. CD44 over-expression and loss of PTEN follow β -catenin accumulation accompanied Myc genomic rearrangements. These events together contribute to R26- β -cat leukaemogenesis.

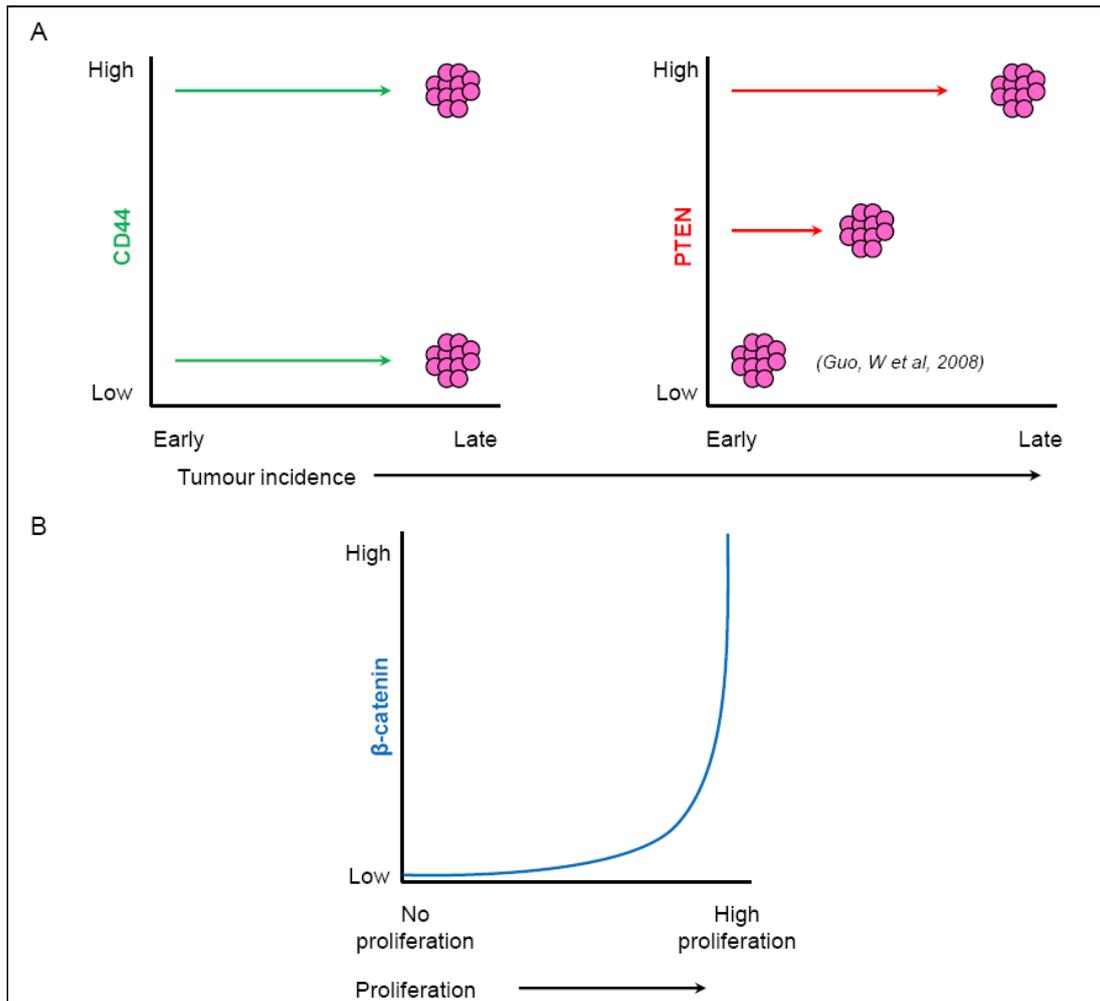


Figure 4.2. Molecular dependence of R26- β -cat tumours. A) CD44 signalling does not play a role in Wnt-dependent R26- β -cat leukaemogenesis. R26- β -cat mice acquire tumour with or without CD44 at the same latency. Loss of PTEN in R26- β -cat mice can significantly accelerate T-ALL development. Here ‘early’ refers to ~15 weeks and ‘late’ refers to ~30 weeks. B) Inhibition of Wnt pathway can attenuate growth of R26- β -cat tumour cell lines.

Our project provides significant insight into the mechanisms that may be involved in Notch-independent T-ALL. Recently, the study on Lck/CD4-Cre Ctnnb1 ^{Δ ex3} mice (which lack the third exon of β -catenin containing the phosphorylation domains), showed that β -catenin dependence of this leukaemia, accompanied by c-Myc upregulation, did not require Notch activation (Guo et al., 2007).

IV.1 How is the R26-β-cat model different from the Ctnnb^{Δex3} model?

The role of Wnt pathway in T-cell leukaemogenesis has recently gained importance. The work of Guo *et al* (Guo *et al.*, 2007), showed that over-expressing β-catenin in T-cell can lead to leukaemia using the Ctnnb^{Δex3} (Lck/ CD4-Cre). Although they have studied the consequences of activating β-cat through T-cell development; following are some differences between the R26-β-cat and Ctnnb^{Δex3} models (Table 4.1).

Table 4.1 Comparison of Ctnnb^{Δex3} Lck/CD4Cre and R26-β-cat T-cell lymphomas.
(Blue – Pre-leukaemia, Red – Leukaemia)

		LckCre-Ctnnb ^{Δex3} LckCre-Ctnnb ^{Δex3} lymphoma	CD4Cre-Ctnnb ^{Δex3} CD4Cre-Ctnnb ^{Δex3} lymphoma	R26-β-cat R26-β-cat tumour	WT
β-catenin expression in T-cell development	DP	intermediate high	intermediate high	intermediate high	low
	CD4+	intermediate high	intermediate high	intermediate high	low
T-cell developmental block		DP	DP	DN4, DP	-
TCRβ expression	DP	low high	intermediate high	low intermediate	high
	CD4+	low-high intermediate-high	intermediate intermediate	normal intermediate	
Clonality		oligoclonal	oligoclonal	mono/ oligoclonal	polyclonal
Leukaemia acquisition		~16 weeks	~14 weeks	24-40 weeks	-

- a) Primarily, studying human protein gives a ‘real picture’ of the behaviour of the protein. Such studies may have more appropriate relevance in drug designing allowing efficacy of testing drugs on the relevant human protein. The Ctnnb^{Δex3} line was generated by inserting loxp sites in exon3 of mouse *Ctnnb* gene encoding β-catenin. Exon3 contains the β-catenin phosphorylation domain and crossing these

mice with Lck-Cre or CD4-Cre allowed excision of exon3, rendering the β -catenin protein active. We have generated R26- β -cat by inserting an activated form of human β -catenin protein in ubiquitously expressed rosa26 locus preceded by neomycin resistance cassette flanked by loxP sites. Crossing these mice with CD4-Cre mice allowed efficient expression of activated human β -catenin protein. Although the strategies are the similar, the R26- β -cat model allows study the behaviour of the human β -catenin protein. This may be comparable and have more applicability to human leukaemia.

- b) Expression levels of activated β -catenin from ubiquitous rosa26 locus and *Ctnnb1* (β -catenin encoding gene) might be different although driven with the same CD4-cre recombinase.
- c) Additionally, cre-mediated excision in *Ctnnb1* ^{Δ ex3} mice results in complete loss of exon3 of β -catenin encoding gene. Exon3 contains 4 different phosphorylation sites (Serine 33, 37, 45 and Threonine 41) of β -catenin protein and removal of the complete exon may affect regulation of some other signalling pathways. On the other hand, R26- β -cat mice were generated by a point mutation at Serine 33 (S33). This is one of the common Wnt pathway mutations found in different human cancers (Polakis, 2012).
- d) Wnt signals are known to be most active during the earlier stages of differentiation (Weerkamp *et al.*, 2006b). At the DN1-2 stages, cells go through massive proliferation to expand thymocyte pool before undergoing TCR rearrangements (Mori *et al.*, 2001). Wnt activity is known to support this proliferation. Further, expression of Wnt pathway inhibitors like APC and Axin start to increase at the DN3 stage, which is mirrored by a reduction in β -catenin expression that begins in DN3 stage and lessens even more at the DN4 stage.

Whereas, in the *Ctnnb*^{Δex3} CD4-Cre mice, the β-catenin expression begins at DN4 stage and is established only at DP stage. The R26-β-cat mice show activated β-catenin expression that begins at the DN3 stage and is established only at DN4 stage of T-cell development. In accordance with flow cytometry and RT-qPCR results of Weerkamp *et al* (Weerkamp *et al.*, 2006b), we observed that intracellular β-catenin level begin to reduce in WT thymocytes at DN3 stage and is eventually almost lost. The R26-β-cat mice maintain the high expression of intracellular β-catenin from the DN3 stage, to levels similar to those normally occurring in DN1-2 cells.

Therefore, the above mentioned differences suggest that R26-β-cat mice are different from *Ctnnb*^{Δex3} model and studying R26-β-cat model may add to understanding Wnt pathway in T-cell leukaemogenesis.

IV.II R26-β-cat leukaemogenesis

IV.II.1 What are the possible alternative roles of CD44 in R26-β-cat leukaemia?

CD44 plays an important role in the migration of normal and tumour cells (Siegelman *et al.*, 2000). CD44 is involved in a plethora of physiological functions including cancer metastasis (Naor *et al.*, 2008; Ponta *et al.*, 2003). In T-cells, the isoform CD44v6 allows intrathymic expansion of immature thymocytes and circulation of mature thymocytes (Rajasagi *et al.*, 2009). Further, HCELL (hematopoietic cell E-/L-selectin ligand), a specialised form of CD44 is expressed not only in normal human HSCs, but also in AML, colon and breast cancer cells (Burdick *et al.*, 2006; Hanley *et al.*, 2005; Hanley *et al.*, 2006). Moreover, CD44 is known to confer metastatic behaviour to rat carcinoma cells (Gunthert *et al.*, 1991; Hertweck *et al.*). The above mentioned reasons justify the importance to further investigate the role of CD44 in metastasis. Our results demonstrated that R26-β-cat Wnt-dependent T-ALL does not require CD44 for initiation, progression and homing. This result in fact, is quite contradictory to studies on AML and other cancers that target CD44 to eradicate tumour

progression (Jin *et al.*, 2006a; Zeilstra *et al.*, 2008a). Although the R26- β -catCD44^{-/-} mice develop thymic tumour with the same latency and phenotype as that of R26- β -cat alone, these mice do not show obvious transmigration of leukaemic cells into peripheral organs like spleen. One of the characteristic features of CD44^{-/-} mice is that T-cells, although develop normally, display impaired re-entry of SP cells into thymus (Protin *et al.*, 1999b). Additionally, when R26- β -catCD44^{-/-} tumours are injected into new hosts, transplanted mice do not show any defect in circulation in the periphery because intravenous injection allows cells to access all organs via blood. This result excludes the role of CD44 in homing of R26- β -cat tumours, but not in metastasis. To determine if CD44 is involved in metastasis of R26- β -cat tumours; thymic tumour cells from R26- β -cat and R26- β -catCD44^{-/-} could be transplanted into the thymus by intrathymic injection into WT recipients. Considering that R26- β -cat and R26- β -catCD44^{-/-} mice acquire lymphomas at the same time, recipient mice are expected to develop thymic tumours at a similar time frame. However, while mice that received R26- β -cat tumour cells may show the presence of these cells in peripheral organs like spleen, mice that received R26- β -catCD44^{-/-} tumour cells are expected not to transmigrate into peripheral organs if CD44 is involved in metastasis. These assays may help determine if CD44 plays a role in the metastasis of R26- β -cat leukaemias.

In addition to metastasis, the role of CD44 in resistance to apoptosis has been extensively studied. CSCs are notorious for surviving cancer therapies because of this property, conferring them resistance to irradiation and drugs. There is accumulating evidence that CD44 renders CSCs resistant to apoptosis (Allouche *et al.*, 2000). Additionally, there is increasing evidence that it is predominantly the interaction between the CD44 variant isoforms (CD44v) and hyaluronic acid (HA) which renders CSCs resistance to apoptosis (Allouche *et al.*, 2000; Fujita *et al.*, 2002; Stern, 2008; Toole, 2004; Yu *et al.*, 1997). On the contrary, there are also studies which show that CD44 cross-linking (CD44 receptor binding to its ligands) initiates apoptosis (Marhaba *et al.*, 2008b). However, such effects are largely

restricted to immature lymphocytes (Fanning et al., 2005). Thus it is hypothesised that CD44 over-expression along with oncogenic transformation and cross-linking of CD44-HA initiates signals to promote survival, making the tumour cells resistant to apoptosis (Marhaba *et al.*, 2008b). Additionally, CD44-HA cross-linking confers apoptosis resistance by induction of cyclooxygenase through the PI3K pathway and β -catenin (Misra et al., 2008, Bates, 2001 #267). We showed that pre-leukaemic R26- β -cat DP thymocytes were more resistant to apoptosis than WT thymocytes by apoptosis induction using dexamethasone. We also demonstrated that some DP cells of pre-leukaemic and leukaemic thymus persisted after irradiation while in the WT DP cells were killed. This evidence suggests that CD44 may have a role in conferring resistance to apoptosis of R26- β -cat LSCs. This could be determined by dexamethasone administration to pre-leukaemic (6 weeks old) R26- β -cat and R26- β -catCD44^{-/-} mice. The rate of apoptosis can be estimated by annexin-V staining. Our studies show that R26- β -cat pre-leukaemic DP thymocytes exhibit resistance to apoptosis. If CD44 has a role in this resistance, the R26- β -catCD44^{-/-} thymocytes should display an apoptotic activity that is normal or higher to R26- β -cat tumours (Fig 4.3). These experiments may shed light on the roles of CD44 in T-ALL sub-groups that are not very well understood.

[IV.II.2 How does the loss of PTEN contribute to R26- \$\beta\$ -cat leukaemia?](#)

Activation of mTOR (mammalian target of Rapamycin) signalling pathway (essential for growth and survival of lymphoid malignancies (Ruggero et al., 2004)), has been reported to be associated with GC-resistance (Beesley et al., 2009). PTEN, the negative regulator of PI3K pathway, is seen frequently mutated or deleted in T-ALL (Gutierrez et al., 2009). Inhibition of this pathway may allow the glucocorticoid resistant ALL cells sensitive to chemotherapy agents. Therefore, it is important to investigate if loss of PTEN renders T-ALL cells glucocorticoid resistant via the mTOR pathway. Our results show that 50% of R26- β -cat tumours have Pten deletion, suggesting that Pten deletion is one of the most favoured

secondary events that lead to this leukaemia. Additionally, we report that R26- β -catPten^{F^l/+} mice display accelerated leukaemogenesis.

Additionally, microarray analysis of pre-leukaemic DP cells in R26- β -cat mice showed up-regulation of B cell lymphoma-2 (Bcl-2)-like 2 (Bcl-w), an anti-apoptotic protein of the Bcl-2 family. Bcl-w is reported to send pro-survival signals in CLL (Chen *et al.*, 2010). Moreover, accumulation of stabilised β -catenin is known to extend thymocyte survival by over-expressing Bcl-x_L (a Bcl-2 family anti-apoptotic protein) (Xie *et al.*, 2005). Moreover, administration of dexamethasone (glucocorticoid (GC)) to R26- β -cat pre-leukaemic mice showed abnormal persistence of DP cells.

Given that loss of PTEN is favoured in R26- β -cat tumours, signalling by the downstream PI3K target Akt may be increased. Loss of PTEN is known to up-regulate anti-apoptotic protein myeloid cell leukaemia (Mcl-1) in HSCs (Perry *et al.*, 2011). The anti-apoptotic Mcl-1 expression by PI3K pathway and pro-survival signals (Bcl-x_L) by Wnt/ β -catenin pathway accompanied by up-regulation of Bcl-w may co-operate to confer R26- β -cat thymocytes apoptosis resistance. To determine if mTOR pathway is involved in this process, R26- β -cat cell lines could be treated with adequate concentrations of rapamycin (mTOR inhibitor). Inhibition of mTOR results in lesser activation of Mcl-1 allowing cells to apoptose. Here, cell lines derived from Pten^{F^l/+} tumours can be used as control. Further, xenografts of human T-ALL cell lines display increased tumour latency when treated with rapamycin (Zhang *et al.*, 2011). To check for such mTOR-mediated apoptosis resistance, rapamycin could be administered in mice transplanted with R26- β -cat tumour cells. If the tumour latency of the rapamycin treated, transplanted mice is increased, it is likely that high Akt activity along with increased Bcl-w expression confers R26- β -cat tumour cells resistance to apoptosis. In such case, inhibition of mTOR by rapamycin in pre-leukaemic R26- β -cat mice may be likely to delay the tumour development in these mice (Fig 4.3).

Myc is a target of most oncogenic pathways (Hoffman et al., 2002, Dang, #288) including the Wnt pathway. Targeting Myc in $Pten^{-/-}$ mice ($Pten^{-/-}Myc^{-/-}$), completely abolished the recurrence of acute leukaemia and lymphoma but not lymphadenopathy (enlargement of lymph nodes) (Zhang *et al.*, 2011). Over-expression of Myc has been reported to activate several microRNAs (miRNAs) (Aguda *et al.*, 2008; Dews *et al.*; Mestdagh *et al.*; O'Donnell *et al.*, 2005). Particularly miRNA17-92 activation induced by c-myc over-expression is known to suppress PTEN in neuroblastomas and lymphocytes (Mestdagh *et al.*; Xiao *et al.*, 2008). Furthermore, miRNA 17-92 is known to confer chemoresistance to mantle cell lymphoma via the PI3K pathway (Rao *et al.*, 2011). We report in our studies that Myc expression in pre-leukaemic thymocytes of R26- β -cat mice remain normal while it is upregulated eventually in leukaemic stage. Additionally, the CGH array analysis showed genomic rearrangements of the 3' region of c-Myc regions. Several studies have shown that T-ALL induced by loss of PTEN is associated with TCR α/δ -c-Myc translocations (Guo *et al.*, 2008; Liu *et al.*). We will attempt to detect the occurrence of TCR α/δ -c-Myc translocations by fluorescence *in situ* hybridisation (FISH) in R26- β -cat leukaemias.

To determine the sequential order of events, i.e. if PTEN loss is dependent on Myc over-expression, we could cross R26- β -cat mice with $Myc^{fl/fl}$ mice. Guo *et al* have demonstrated that Myc over-expression is necessary for β -catenin-driven T-ALL in $Ctnnb^{\Delta ex3}CD4$ -Cre mice (Guo *et al.*, 2007). Firstly, we will be able to show if Myc over-expression would also be necessary in case of R26- β -cat leukaemia. And secondly, by investigating the PTEN expression at later stages, it is possible to identify if PTEN loss is Myc expression-dependent. These experiments may reveal the successive secondary genetic events that lead to R26- β -cat leukaemia.

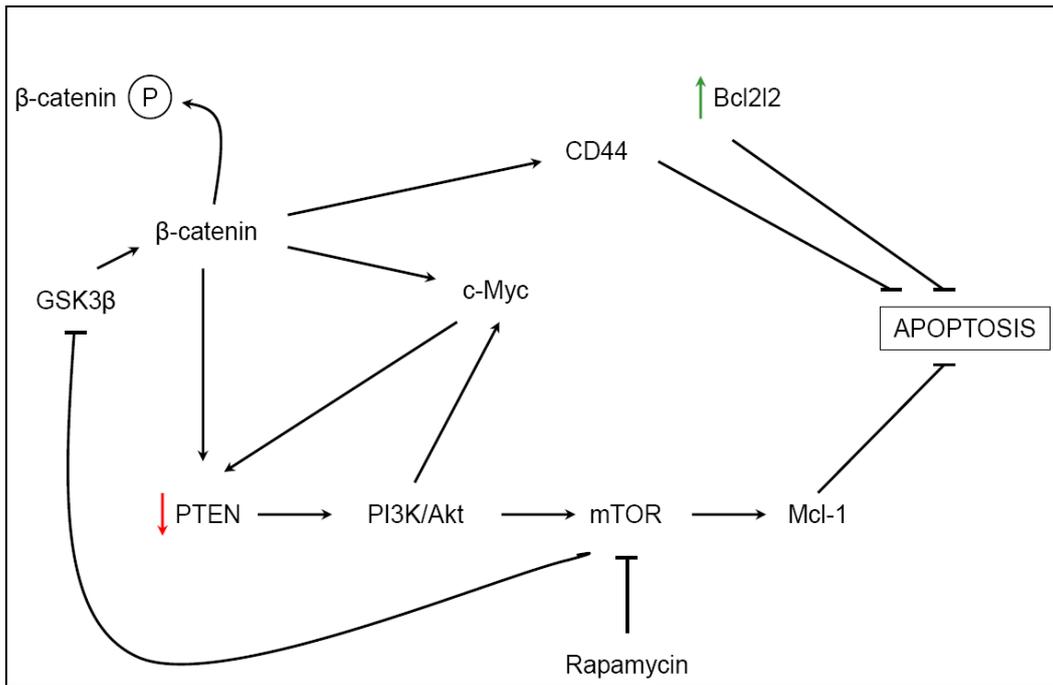


Figure 4.3. Schematic representation of pathways that may confer apoptosis resistance to LSCs of Wnt-dependent T-ALL. Wnt activity via CD44 over-expression may inhibit apoptosis. Additionally, high signalling can be the reason for c-Myc over-expression. These collective events may lead to up-regulation of anti-apoptotic factors like Mcl-1 and inhibit apoptosis. The co-operation between these pathways may largely inhibit apoptosis leading to the persistence of R26-β-cat cells.

IV.III R26-β-cat LSC activity

CSCs have been recently proposed to be responsible for tumour expansion. These cells are known to share some properties with normal stem cells and to be resistant to cancer treatments (Reya et al., 2001). Therefore, to increase event-free survival (EFS), it is important to identify these cells.

We demonstrated that R26-β-cat tumours are malignant and that LSCs were mainly found in DP cells. Furthermore, we showed that LSCs were present in a frequency of 1/580-1/1187 cells and that LSCs capacity to self-renew reduced through serial transplantations. Moreover, R26-β-cat tumours were phenotypically different from wild type thymocytes and contained Sp cells.

IV.III.1 Are the R26- β -cat tumour DN and CD4⁺ populations non-leukaemic?

- a) One of the standards to call a tumour heterogeneous, with respect to LSC activity, is that only one or few sub-populations making up the tumour can re-initiate tumours (Reya et al., 2001). By transplantation assays of different R26- β -cat tumour sub-populations (10000 of DN, CD4⁺ and DP), we found that DP cells alone can re-initiate tumours. These transplantation assays were done by intravenous injection of cells. This method allows cells to flow into the blood stream thereby obligating cells to 'find' the favourable niche. All cells may not succeed in finding niches or may not be subjected to the same fate (Fig 1.12). For these reasons, it becomes important to exclude the fact that DN and CD4⁺ sub-populations did not have such defects and that they can in fact not re-initiate tumours because the LSCs are not enriched in these sub-populations. To do so, we have begun assays where we have sorted DN, DP and CD4⁺ sub-populations and transplanted them into lethally irradiated recipients by intrafemoral injections. Such an assay will provide the DN and CD4⁺ with a favourable niche (bone marrow). We have also transplanted total tumour cells as control. In this experiment, some mice transplanted with total and DP cells have begun to develop the disease and have been analysed. However, some others are yet to develop leukaemia. Results from this experiment will provide evidence that i) DN and CD4⁺ cells do not have homing defects and ii) that LSCs are indeed enriched in DP cells and not in DN or CD4⁺ cells.
- b) Furthermore, if DN and CD4⁺ cells have no homing defects, it is necessary to investigate if these cells are leukaemic. Unless provided with evidence, a sub-population (although arising from a tumour) cannot be termed tumorigenic. It is necessary to show that DN and CD4⁺ cells of R26- β -cat mice are leukaemic to

reinforce the fact that DP cells alone (among the three tumorigenic populations) are enriched in LSCs and can re-initiate leukaemia.

To show that DN and CD4⁺ cells of R26- β -cat thymic tumour are leukaemic, we have already demonstrated that these sub-populations may consist of cells that have or have not undergone excision of neo-cassette. This implies that some of these cells express stabilised β -catenin while some others do not. Since the excision of neo-cassette does not directly imply that Wnt pathway is active in these cells, we sought to adopt another method to show that the cells are tumorigenic.

T-cells normally undergo TCR β rearrangements and exhibit polyclonality. We have shown by PCR analyses that unlike in the WT, R26- β -cat thymocytes are mono/oligoclonal in TCR rearrangements (manuscript fig S2 B). Additionally, we sorted DN, CD4⁺ and DP cells of WT and R26- β -cat thymus and checked for TCR β rearrangements individually. The DN and CD4⁺ cells are mono/oligoclonal, this implies that these cells have undergone similar rearrangements as leukaemic DP cells. These results provide evidence that DN and CD4⁺ are leukaemic. This strengthens the fact that R26- β -cat tumours contain a group of clonogenic cells, a hallmark for the presence of CSCs. However, the rearrangement patterns were not identical between subpopulations and differed from one tumour to another. Therefore, we plan to repeat this experiment by sorting these sub-populations from both WT and R26- β -cat tumours and confirm this result.

[IV.III.2 Self-renewal of R26- \$\beta\$ -cat tumours](#)

The hallmark of normal and cancer stem cells lies in their property of self-renewal. Several studies have reported that HSCs and LSCs have self-renewability (Luo and Han, 2006; Reya et al., 2003; Reya et al., 2001).

We determined the self-renewal potential of R26- β -cat leukaemic cells by serial transplantation assays. We observed that with every transplantation, these cells required more time to re-initiate tumour which was reflected in delay to develop the disease. This was true of four independent tumours. Our results suggested that R26- β -cat tumours gradually lose their capacity to self-renew. These results surprised us for two reasons; i) LSCs by definition are thought to possess self-renewal potential at least through quaternary transplantations (Guo et al., 2008; le Viseur et al., 2008) and ii) LSCs are thought to self-renew and enrich themselves through transplantations such that the tumour latency through serial transplantations is reduced (Luo and Han, 2006; McCulloch, 1983; Reya et al., 2001).

To our knowledge, in CSC biology, no accounts of reduced self-renewability have been reported. There are several explanations as to how and why we observe reduced self-renewal in R26- β -cat leukaemia.

- a) Wnt activity is known to be indispensable for HSC proliferation and function in a dosage-dependent fashion (Luis *et al.*, 2011a; Luis *et al.*, 2011b). While mildly high levels (2 fold increase) of Wnt are known to enhance HSC proliferation and function, moderately high (4-22 fold increase) activity is reported to impair HSC function and enhance T-cell proliferation. Very high levels (>72 fold increase) of Wnt activity to impair HSC self-renewal and differentiation.

The hypothesis that Wnt signalling can promote self-renewal of normal stem cells may also support LSCs because they share some properties. And with high Wnt activity, we may expect in R26- β -catenin leukaemia that LSCs have impaired self-renewability.

In addition to the functionality of HSCs, Wnt pathway has been reported to have an impact in the differentiation of HSCs. Haematopoietic-specific activation of β -catenin in two different mice models showed impaired differentiation of HSCs (Kirstetter et al., 2006; Scheller et al., 2006). Although the mechanism of the block happens in as-

yet-unknown manner, we may hypothesise that high Wnt activity may cause blockade of differentiation in LSCs of R26- β -cat leukaemias.

- b) On a different note, recent studies have demonstrated that mice deficient in β -catenin (β -cat^{-/-}) have short telomeres and that mice with activated β -catenin (β -cat ^{Δ ex3/+}) have long telomeres. It was also established that β -catenin directly regulates *Tert* (an enzymatic subunit of the telomerase complex which controls the telomerase length) (Hoffmeyer *et al.*, 2012). Telomerase directly modulates Wnt signalling by serving as a co-factor in a β -catenin transcriptional complex (Park *et al.*, 2009). On the other hand, high telomerase activity has been associated with high degree of self-renewal in HSCs (Morrison *et al.*, 1996). Additionally, telomere length decreases with repeated transplantation of HSCs (Allsopp *et al.*, 2001). However TERT-deficient mice (TERT^{-/-}) were shown to exhaust self-renewability 'prematurely' while WT HSCs maintain self-renewability through four transplantations (Allsopp *et al.*, 2003a). This implies that telomere length is critical for self-renewal. Although, neither TERT over-expression in mice which maintained their telomere lengths, nor WT HSCs could self-renew beyond four transplantations suggesting that telomere length alone is not sufficient to extend self-renewal capacity (Allsopp *et al.*, 2003b).

Collectively, these studies open a possibility of R26- β -cat LSCs to have long telomeres due to high Wnt activity. However, like the TERT^{-/-} mice, R26- β -cat LSCs appear to lose self-renewability. This may imply that telomere lengths are shortened by some other factors and high Wnt activity cannot rescue this shortening.

- c) Furthermore, one other study has shown that constitutive activation of β -catenin alone results in apoptosis and inhibition of differentiation of HSCs; activation of PI3K pathway enhances anti-apoptotic factors to promote differentiation of the same. Together, the Wnt and PI3K pathway co-operate to promote self-renewal of HSCs

(Perry *et al.*, 2011). This implies that activation of either of the pathways is not sufficient for HSC function but in combination, they drive expansion and self-renewal. Moreover, PTEN is known to inhibit telomerase activity (Zhou *et al.*, 2006) and Akt kinase enhances human telomerase activity through phosphorylation of hTERT subunit (Kang *et al.*, 1999).

Collectively, these studies suggest that in R26- β -cat telomere lengths may be maintained with high Wnt activity and loss of PTEN. However, due to some other factors, they gradually lose their capacity self-renew.

Taken together, there appears to be a vicious cycle of events that work together to reduce the self-renewing potential of R26- β -cat LSCs (Fig 4.4).

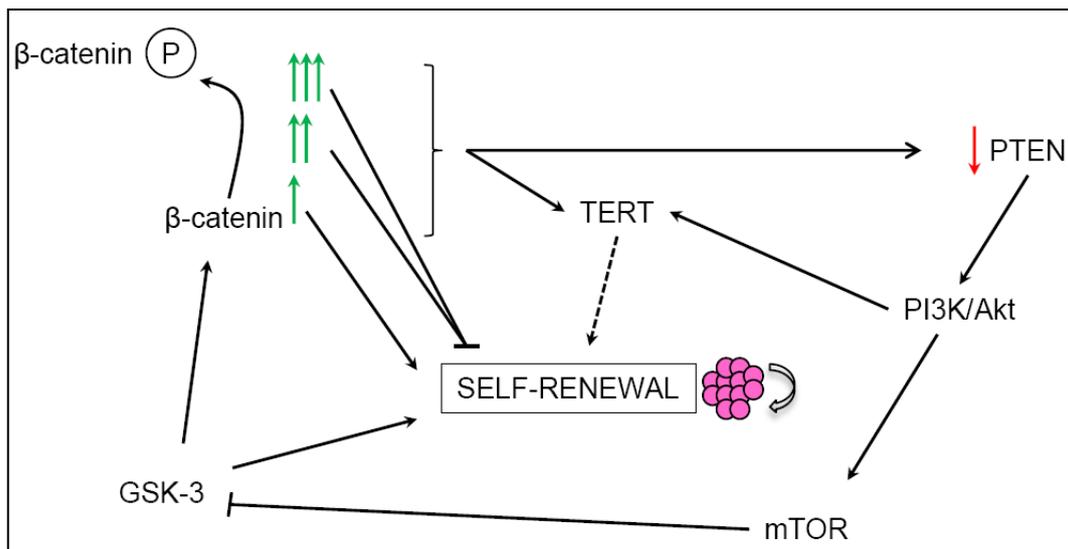


Figure 4.4. Schematic representation of possible co-operations of pathways in self-renewal. While mildly high Wnt activity can enhance self-renewal, higher levels of Wnt activity may impair self-renewal in R26- β -cat LSCs. On the other hand, loss of PTEN resulting in high PI3K/ Akt signalling will lead to elevated mTOR activity which will inhibit GSK3. Meanwhile, TERT expression may be increased because of high Wnt and Akt signalling; but it may not be sufficient to induce self-renewal.

Assessment of long-term potential can be done by serial transplantation assays. Firstly, R26- β -cat tumours with or without the loss of PTEN can be serially transplanted. If there is any difference in the self-renewal trends of these two sub-groups of R26- β -cat tumours, we

can hypothesise that PI3K pathway is involved self-renewal behaviour. Alternatively, PI3K pathway inhibitor Wortmannin administration in serially transplanted mice may help assess if this pathway is involved in reducing self-renewal potential of R26- β -cat tumours.

Furthermore, to identify dominant clonogenic mutations, we can perform CGH array with tumours acquired through serial transplantations. This will allow us to determine the mutations that are favoured over the others that make leukaemic cells 'dominant'.

These studies will allow us to identify various targets that may help to eradicate LSCs within Notch-independent T-ALL.

IV.III.3 Candidate LSC populations

We found Sp cells among R26- β -cat thymocytes, while such cells are not normally present in the WT thymus. Although these cells did not appear to be enriched after treatment with 5-FU, this population is known to contain stem-like cells (Zhou et al., 2001). Therefore, it interests us to investigate if these cells are enriched in LSCs. To determine this, we plan to sort Sp and non-Sp cells from R26- β -cat tumours and transplant these cells by intravenous injection into lethally irradiated hosts. If mice transplanted with Sp cells develop leukaemia in a reduced latency than those transplanted with non-Sp cells, it will imply that Sp cells are enriched in LSCs. Further, if this is the case, a limiting dilution assay can be performed to determine the frequency of LSCs in Sp. Additionally, we can analyse the transcriptome of these cells to determine which pathways are involved in conferring these cells LSC potential.

Furthermore, the results of immuno-phenotyping and sub-population assays suggested that some cell-surface markers (CD133, CD150, Sca-1, and CD200) may mark the LSC population of R26- β -cat tumours. To determine which markers define the LSC population, R26- β -cat tumours can be stained with each of the markers individually; cells positive and negative for these markers can be sorted and transplanted into lethally irradiated hosts. If

cells expressing certain markers can develop leukaemia in mice at decreased tumour latency than some others, these cell surface markers may help characterise LSCs.

IV.IV β -catenin as a potential target for leukaemia regression

We demonstrated that R26- β -cat cell lines could not proliferate when Wnt pathway was inhibited using ICAT (catenin- β interacting protein). We have confirmed this with three independent cell lines *in vitro*. Further, we have also begun transplantation assays where we have injected R26- β -cat cell lines infected with MiG ICAT-GFP or MiG-GFP (control). These transplantation assays will reveal if the R26- β -cat cells fail or show a delay to re-initiate tumours when Wnt pathway is inhibited. These assays will justify that β -catenin may be used as a potential target to inhibit Wnt-dependent T-cell leukaemias.

Overall, we report that Notch-independent T-ALL can fall into a new sub-group of Wnt-dependent T-ALLs. Secondary genetic events like loss of PTEN and Myc activation through genomic rearrangements can co-operate with Wnt activity to contribute to the progression of this leukaemia progression, possibly because these cells become resistant to apoptosis. Further, the R26- β -cat tumours exhibit heterogeneity and may contain several candidate LSC populations. In addition, we provide evidence to show that β -catenin may be used as a potential target to regress Notch-independent T-cell leukaemias.

V. Materials and methods

V.I Mice and *in vivo* experimental procedures

R26 β -catenin mice were generated in the lab of Dr. Claus Nerlov by inserting a cDNA encoding Myc-tagged stable form of activated β -catenin (human- S33Y) into the ubiquitously expressed Rosa26 locus by homologous recombination. This was done in stem cells E14.1 downstream of a lox-flanked neomycin stop cassette. Expression of Cre leads to removal of neo cassette and expression of β -catenin.

β -catenin CD44^{-/-} and β -catenin PTEN^{fl/+} double knockout mice were generated as mentioned in the manuscript.

V.I.1 Irradiation

An X-ray source by CEGLEC was used to irradiate mice with a probe from UNIDOS detecting the dose of irradiation.

To immunologically compromise mice, a lethal dose of 9Gy was used. For sub-lethal irradiations, a dose of 6Gy was used.

V.I.2 Intravenous and intrafemoral injections

Intravenous injections - Before intravenous injections, mice were pre-warmed for 10 minutes. Mice were held in a mouse holder and 500 μ l of IMDM with appropriate amount of cells were then injected slowly through the tail vein by 26G sterile needles. The bleeding was then stopped with applying pressure at the punctured area with a piece of tissue for about 30 seconds.

Intrafemoral injections - All mice were injected with anaesthesia (100 μ l for 6 week old females and 110 μ l for 6 week old males) before intrafemoral injections. Using a 26G needle, a hole was drilled manually by applying gentle pressure into the bone at the femur and tibia junction. The needle was then withdrawn and a smaller syringe with 29G needle was then inserted into the same aperture. 30 μ l of IMDM with cells was injected slowly in the marrow and the needle was gently withdrawn.

V.I.3 Transplantation assays

Black6 Ly5.1 or Ly5.1/.2 mice were used as recipient mice.

For Black6 Ly5.1 mice, Black6 Ly5.1/.2 mice' BM cells were used as competitor and vice versa. For all injections, 2×10^5 competitor cells were used.

6-7 weeks old Black6 Ly5.1 or Black6 Ly5.1/.2 mice were used as recipient mice. These mice were lethally irradiated with an X-ray source at 9Gy. Within 24 hours after irradiation, the mice were injected with cells as per experiments' demands.

V.I.3.1 Tumour amplification

To amplify primary tumours, 10×10^6 or 5×10^6 tumour cells were injected intravenously with 2×10^5 WT BM cells as competitor cells in IMDM. These mice were sacrificed before 20 days to proceed with an experiment for which the tumour was amplified.

V.I.3.2 Bone marrow transplantation assays

The recipient mice were injected intravenously (500 μ l) with a mixture of 10000 tumour cells (Ly5.2) and WT competitor BM cells (Ly5.1/.2 or Ly5.1) in IMDM. The mice were bled after 3 weeks to check for tumour competency. Sick mice were analysed for different markers by flow cytometry.

V.I.3.3 Limiting dilution assays

Reducing number of cells, i.e. 10000, 2000, 500 and 100 tumour cells were injected intravenously (500 μ l) with competitor WT BM cells in IMDM. These mice were bled first at 3 weeks and then at monthly intervals to track the growth of the tumours. Mice were sacrificed for further analysis just as the health of the mice deteriorated.

V.I.3.4 Serial transplantation assays

An amplified tumour sorted for donor-derived cells or bulk primary tumour cells (10000) were injected intravenously (500 μ l) injected with WT BM cells (2×10^5) into recipients. When these primary recipients developed tumours, donor-derived cells were sorted and were re-injected (10000) with WT competitor BM cells into lethally irradiated secondary recipients. Similarly, tertiary recipient mice were injected intravenously with tumours and WT competitor BM cells as the secondary recipients developed tumours.

V.I.3.5 Homing assay

To perform homing assays, primary β -cat tumours were sorted to obtain $CD44^+CD3^-CD8^-CD4^-$, $CD44^+CD3^-CD8^+CD4^+$, $CD44^+CD3^+CD8^+CD4^+$ and $CD44^+CD3^+CD4^+$ cells. These cells were injected directly into the BM along with WT BM competitor cells using 26G and 29G needles.

V.I.4 5-Fluorouracil administration

β -cat mutant mice, WT mice or mice with amplified tumours were injected with 5-Fluorouracil (Sigma F 6627) at a concentration of 150 mg/kg by intraperitoneally. 24 or 48 hours later, mice were sacrificed for analyses. Cells derived from BM, spleen and thymus collected from these mice was stained for several cell surface markers. The BM cells were stained for side-

population; the spleen and the thymus were stained with T-cell and cancer stem cell markers.

V.II Staining for flowcytometry analyses

V.II.1 Extracellular cell-surface staining

2×10^6 to 5×10^6 cells were stained for analysis. The cells were pelleted in a 96 well plate. Purified/ unconjugated antibodies were first added in a volume of 50 μ l (diluted) and incubated on ice for 15 minutes. The cells were washed with 200 μ l wash buffer (1X PBS+1%FCS) at 1200 rpm for 2 minutes. The plate was then briskly flipped to remove the supernatant. Cells were resuspended by vortexing. Secondary antibodies or a cocktail of directly conjugated antibodies were added in a volume of 50 μ l and incubated for 15 minutes on ice. The cells were washed with wash buffer and resuspended in fresh wash buffer. The stained cells were analysed by BD LSRII.

V.II.2 Staining for sorting

$250-800 \times 10^6$ cells were pelleted in a FACS tube and a cocktail of different antibodies was prepared to stain cells at a concentration of 250×10^6 / 500 μ l. Cells were stained by rocking at 4°C for 30 minutes and washed twice with wash buffer. Cells were then filtered by 0.45 μ filter to avoid clumps and were sorted on BD Aria with a 70 μ nozzle. The cells were diluted at 60×10^6 /ml to sort with wash buffer. Sorted cells were collected in tubes coated with wash buffer overnight at 4°C.

V.II.3 Peripheral blood analysis

Mice were pre-warmed for 10 minutes. Mice were put in a mouse holder and the tip of the tail was cut with a pair of sharp sterile scissors. Approximately two drops of blood were collected in heparin-coated tubes or in 1.5 ml eppendorf tubes with sterile 1X PBS with 2mM EDTA (200 μ l). 500 μ l of 2% Dextran (Sigma 31392-50G diluted in 1XPBS) was then added to each of the tubes. The tubes were incubated at 37°C for 40 minutes. After 40 minutes the upper phase in the tube was pipetted into a fresh 1.5 ml tube. 1 ml of wash buffer (1X PBS + 1% FCS) is added into the collected upper phase. The tubes were centrifuged at 2000 rpm for 5 minutes. The supernatant was discarded and 500 μ l of ammonium chloride (0.83g/100 ml in water) is added. The tubes are incubated at room temperature for 8 minutes. The samples are then washed with 1 ml of wash buffer and centrifuged at 1200 rpm for 5 minutes. The red blood cell (RBC) lysis was repeated if necessary. The samples are then stained with respective antibodies and passed on BD LSR II for further analysis.

V.II.4 Side-population staining

Staining media – DMEM + 2% FBS + 10 mM HEPES

Hoechst 33342 (Sigma B-2261) at 5 mg/ml

Verapamil hydrochloride (Sigma 4629) at 100 mg/ml

$1-5 \times 10^6$ cells were resuspended in 0.9 ml media without the dye and incubated at 37°C water bath for 20 minutes. 10x working concentration of the dye Hoechst was incubated in the water bath at 37°C for 20 minutes. 100 μ l of 10X dye was added into cells in 0.9mL medium. This was incubated for 90 minutes at 37°C. The cells in the dye were mixed every 15 minutes to ensure homogeneity of the solution throughout. After 90 minutes, the cells were plunged into ice to rapidly stop the staining process. The cells were centrifuged at 1200

rpm at 4°C and further extracellular staining was carried out. Hoechst stained cells were passed on BD ARIA with violet laser and TOPRO3 (1 µM) was added just before passing cells on the BD ARIA to eliminate dead cells from analyses.

Conjugated antibodies used:

Lineage cocktail (from supernatant) for Thymus

CD4 (GK1.5) – 200 µl

CD8 (YTS169.4) – 200 µl

CD3 (KT3) – 100 µl

B220 (RA3.6B2) – 40 µl

Mac-1 (CD11b – M1/70) – 40 µl

Gr1 (H30b) – 0.5 µl

Nk1.1 (PK136) – 25 µl

Used at 100 µl per sample.

CD2 FITC (1:100), CD3 FITC (1:50), CD3 Cy5 (1:50), CD3 V500 (1:100), CD4 PE (1:200), CD4 APC-Cy7 (1:100), CD8 FITC (1:100), CD8 APC (1:100), CD8 PECy5 (1:300), CD8 PerCPCy5.5 (1:100), CD24 APC (1:100), CD44 Biotin (1:100), CD44 FITC (1:100), CD44 AlexaFluor 647 (1:100), CD44 PECy7 (1:100), CD44 Cychrome (1:100), CD45.1 PECy7 (1:100), CD45.2 FITC (1:100), CD45.2 Alexafluor 700 (1:100), CD45.2 PerCPCy5.5 (1:100), CD45.2 PECy5.5 (1:100), CD62L Biotin (1:100), CD71 Biotin (1:100), CD71 FITC (1:100), c-kit APC (1:100), CD133 Biotin (1:100), CD150 PerCPCy5.5 (1:100), CD150 PE (1:100), CD200 PE (1:200), Notch-1 Biotin (1:100), Sca-1 FITC (1:100), Sca-1 PECy7 (1:100), TCRαβ biotin (1:100), Valpha2 biotin (1:100), Valpha11.1, 11.2 biotin (1:100)

All antibodies were ordered from BD Pharmingen, eBiosciences or Biolegend.

Secondary antibodies used

Goat anti-rat TRITC (1:100), Streptavidin PE (1:200), Streptavidin AlexaFluor 405 (1:100), Streptavidin Cy5 (1:300)

V.III Cell Culture

V.III.1 Cell lines

149 cell lines and T-29

Cell lines derived from R26 β -catenin tumours (named in 149 series) were cultured in RPMI with 25 mM HEPES with 10% FCS, 1 mM Sodium pyruvate, 1% Gentamycin and penicillin-streptomycin. The cells were split every 3 days and re-plated at a concentration of 1×10^6 /ml for optimum growth.

Eco Phoenix cells

The cells were grown in DMEM (4.5 g/L glucose) medium. This medium was supplemented with 10% FCS, 1 mM Sodium pyruvate and penicillin-streptomycin. The cells were split every 4 days. To split cells, the medium was aspirated and the cells were washed with 1X PBS. Cells were then detached with diluted trypsin. The cells were washed with medium, centrifuged at 4°C at 1200 rpm and re-plated at 1/10 dilution.

293T

These cells were grown in DMEM (1g/ L glucose), 10% FCS, 1% Gentamycin, 1 mM sodium pyruvate and penicillin-streptomycin. The cells were split every 4 days by using diluted trypsin to detach them, washed and re-plated at 1/10 dilution.

V.III.2 Transfection for retrovirus production

24 hours prior to transfection, 4×10^6 EcoPhoenix cells were plated on 10 cm BIOCOAT dishes with 10 ml medium. Before transfection, the medium was aspirated and 8 ml of fresh medium containing 25 μ M chloroquine was added. 20 μ g of plasmid DNA was diluted in 875 μ l H₂O in a 14 ml polypropylene tube. 125 μ l of 2 M Calcium chloride (CaCl₂) was added. Using 1 ml tip, add 1 ml of 2X HBS quickly. This mixture was vigorously vortexed and the solution was made to bubble using 1 ml tips. This mix was added dropwise on cells. 10 hours after transfection, the medium was replaced with fresh 10 ml medium. 24 hours post-transfection, the EcoPhoenix cells were shifted into virus room (P2) and 6 ml of fresh medium was added after aspirating the medium into the dishes. On the 3rd day, the supernatant from all the dishes were pooled and filtered to remove floating cells. This medium was then aliquoted in 1.5 ml eppendorf tubes. The retroviruses were always titrated on NIH3T3 cells.

V.III.2.1 Titration of retrovirus on NIH3T3 cells

To infect 3T3 cells, they were plated in 6-well plates at 1×10^6 per well a day before transduction. 24 hours after plating 3T3 cells, 1.1 μ l of 4 mg/ml stock polybrene and 100 μ l of virus was added directly onto the cells. 24 hours after the infection, 3 ml of fresh medium was added into the old medium. 48 hours after transduction, the cells were trypsinised, washed and checked for GFP expression under a fluorescence microscope.

V.III.2.2 Spinfection of 149 cell lines with retroviruses

149 cells are plated at a concentration of 1×10^6 per ml in 6 well plates. The day after the cells are split and plated at a concentration of 2×10^6 per 1.5 ml medium. The plates are shifted to virus room (P2). 4 μ l of polybrene was added in all wells and 500 μ l of viruses was pipetted directly into the wells. The plate was centrifuged for 90 minutes at 2600 rpm. The plates are

then incubated at 37°C. 8 hours later, the cells were counted and re-plated in 2 ml of medium. An aliquot of cells was checked for GFP expression by FACS. The next day, cells were counted and re-plated again. The cells re-plated at 1×10^6 per ml each day to keep the efficiency of the infection optimum and constant.

V.III.3 Transfection for Lentivirus production

293T cells were grown in T175 flasks in DMEM (1 g/L glucose), 10% FCS and penicillin-streptomycin medium. Each type of lentivirus to be produced required 3 T175 flasks of 293T cells. The cells were plated at 1×10^6 / flask concentration. Four days later, when the flask achieved 70-80% confluency, medium was changed with 12 ml of DMEM (4.5g/L glucose) + 10% FCS, 1 mM sodium pyruvate, penicillin-streptomycin and Glutamax. 3-4 hours after changing medium make the following mix: pVSVG (70 µg) + pLp8.9 (100 µg) + plasmid of interest (100 µg) + CaCl_2 (2 M) + H₂O to make up to 5 ml. The DNA was first mixed with water and CaCl_2 was added to the DNA mix. 1 ml of this DNA cocktail was added drop-wise while continuously vortexing a 15 ml tube with 1 ml of HBS (2X). This mix was pipetted and mixed vigorously to ensure homogeneity. This mix was let stay at room temperature for about 30 minutes. The mix was equally distributed in 3 T175 flasks. 4 hours later, 8 mL of fresh medium was added to the flasks. The next day, medium was changed and fresh 25 ml of DMEM 4.5 g/L glucose was added. 48 hours after adding transfection mix, supernatant from 3 flasks (containing one type of lentivirus) was pooled into two 50 ml falcon tubes. The supe was centrifuged at 1200 rpm for 5 minutes to eliminate cell debris. The supernatant was then filtered with a 0.45 µm sterile filter with 20 ml syringes into a new 50 ml falcon tube. To this mix, 10 µg/ml of DNaseI and 1 mM of MgCl_2 was added and incubated at 37°C in the water bath. Meanwhile, SW-28 ultracentrifuge tubes were UV sterilized for 15 minutes under the hood. 75 ml of collected supe for each lentivirus type was distributed among 2 SW-28 ultracentrifuge tubes. The supernatant was centrifuged at 22000 rpm for 90 minutes. The

supernatant was aspirated and the tubes were inverted to allow excess liquid residue to seep down. The inner walls of the centrifuge tubes were wiped with paper and 70 μ l of cold PBS was added directly on the pellet and this was incubated at 4°C for 20 minutes. The viruses were then resuspended with an additional 60 μ l of 1X PBS and aliquoted. These were stored at -80°C.

V.III.3.1 Spinfection of 149 cell lines with lentiviruses

149 cells are plated at a concentration of 1×10^6 /ml in 6 well plates. The day after the cells are split and plated at a concentration of 2×10^6 per 1.5 ml medium. The plates are shifted to P2. 4 μ l of protamine (5 mg/ml) and 20 μ l of concentrated viruses were pipetted directly into the wells. After spinfection of cells for 90 minutes at 2600 rpm, the plates at 37°C. 8 hours later, the cells were counted and re-plated in 2 ml of medium. An aliquot of cells was check for GFP expression by FACS. The next day, cells were counted and re-plated again. The cells re-plated at 1×10^6 /ml each day to keep the efficiency of the infection optimum and constant.

V.III.4 Genomic DNA extraction

$0.5-1 \times 10^6$ cells were lysed overnight at 55°C in 400 μ l lysis buffer (50mM Tris pH 8.0, 100mM EDTA, 100 mM sodium chloride, 1% SDS made up to 100mL. Phenol-chloroform (see below) extraction of lysed cells was performed the next day.

V.III.5 Western blotting

Cells were lysed with 1X Laemmli buffer (4X solution – 250 mM 1M Tris pH-6.8, 8% SDS, 40% glycerol, 0.01% bromophenol blue, 1 ml beta-mercaptoethanol at 20000 cells/ 10 μ l

buffer. Samples were heated at 100°C (7 minutes) for denaturing proteins and vortexed thoroughly before use. 12% SDS PAGE were casted using Biorad western blotting apparatus. $0.5-1 \times 10^6$ cells were loaded and was run at 40mA in 1X running buffer (10X – 151.65 g Tris, 720 g Glycine, 50 g SDS made up to 5L with water and stored in dark). Proteins were transferred on PVDF membranes using semi-dry transfer apparatus from Amersham biosciences using Anode buffer I (0.3M Tris pH10.4, 10% Methanol), Anode buffer II (25 mM Tris pH 10.4, 10% Methanol) and Cathode buffer III (25 mM Tris pH 9.4, 40 mM Glycine, 10% Methanol) as instructed in the user manual. Membranes were blocked with Blotto (5% milk in 0.1% PBS Tween) for an hour after which they were incubated with primary antibodies (Anti-PTEN (1:1000) – Cell Signalling #9552, Anti- β -actin (1:10000) clone AC-15 SIGMA A-1978). Secondary antibodies conjugated horseradish peroxidase (HRP) were used at 1:10000 concentration. Proteins were detected by chemiluminescence by ECL reagents from Pierce/ thermoscientific (β -actin) and Millipore (PTEN).

V.VI Cloning

V.VI.1 Competent bacteria preparation

An aliquot of 10 ml of Luria broth medium (Invitrogen 12780-052) was inoculated with a single bacterial colony and was incubated overnight at 37°C. 0.2 ml of this culture was then used to inoculate 200 ml of LB in a litre large conical flask. This culture was incubated at 37°C for 3 hours on a shaker till the optical density (OD) of the culture reaches 0.6-0.8. Bacteria were is transferred into 50 ml falcon tubes and incubated on ice for 15 minutes. The bacteria were centrifuged at 3000 rpm for 8 minutes at 4°C. The pellets were pooled and resuspended in 24 ml of 0.1M ice cold CaCl_2 and incubated in ice for 1 hour. The tubes are then centrifuged at 3000rpm at 4°C for 8 minutes and resuspended in $1/10^{\text{th}}$ the volume of 0.1M CaCl_2 + 17% glycerol (ice cold). 500 μl aliquots were stored at -80°C.

V.VI.2 Phenol-chloroform purification

The volume of DNA solution was made up to 500 µl using PCR grade water. Equal volumes of water saturated phenol and chloroform is added into the DNA. The tube is vortexed well and centrifuged for 5 minutes at room temperature (RT) at 13000 rpm. The upper phase was transferred into a fresh tube and again, equal volumes of phenol-chloroform was added, mixed well and centrifuged. The upper phase was transferred to a fresh tube and washed with equal volumes of chloroform thrice. After 3 washes, the DNA was precipitated with 35 µl of 5M sodium chloride (NaCl) and two volumes of 100% ethanol. 1 µl Glycogen (Roche) was added if necessary. The tubes were placed at -80°C for 20 minutes and centrifuged at 4°C for 20 minutes at 13000rpm. The pellets were washed with 70% ethanol and dried at RT. Dried pellets were resuspended in sterile water.

V.VI.3 Transformation

Competent bacteria were thawed on ice. Approximately 50-100 ng of DNA was mixed with competent bacteria and incubated on ice for 30 minutes. The bacteria were then given a heatshock at 42°C for 45 seconds and transferred into ice for 10 minutes. 600 µl of LB base was added into each tube and was incubated at 37°C, shaking for an hour. The samples were then centrifuged to pellet transformed bacteria at 2000 rpm for 5 minutes. 400 µl of the supernatant was discarded and the pellet was resuspended in the remaining medium. The resuspended bacteria was pipetted onto an Ampicillin LB agar plate, which were warmed at 37°C for an hour, dropwise and was spread using a glass spreader till the bacteria was thoroughly soaked into the LB agar. The plates are then incubated at 37°C incubator overnight.

V.VI.4 Ligation

Ligations were performed overnight at 16°C using NEB T4 ligase in 20µL reaction mix.

V.VI.5 Digestion

All digestions which required further purifications were incubated for 3 hours at 37°C in 50µL reaction mixes. Digestions that were used to confirm positive clones were incubated at 37°C for an hour in 20µL reaction mixes.

V.VI.6 Midi and mini preps

For midi preps, 200mL LB was inoculated with starter culture. For mini preps, colonies were picked using toothpicks or tips and dropped into polystyrene tubes containing 3mL LB medium. All midi and mini preps were performed using Machery Nagel Nucleospin DNA purification kits (740410-10 and 740588-50).

V.VI.8 Gel extractions and PCR purifications

All gel extractions and PCR purifications were done using Qiagen gel purification and PCR purification kits (28704 and 28104).

V.VI.7 Cloning strategies

V.VI.7.1 Dominant-negative Tcf4 in pTRIP MND-GFP lentiviral vector

cDNA of dn-Tcf4 (1229 bp) was amplified by PCR. BamHI restriction site was introduced in the primers to favour ligation into the lentiviral plasmid pTRIP MND-GFP. Amplified PCR product was then cloned into TOPO TA vector using TOPO cloning kit (Invitrogen TOPO TA cloning – Five minute cloning of Taq polymerase-amplified PCR products K4500-01, K4500-

40, K4510-20, K4520-01, K4520-40, K4550-01, K4550-40, K4560-01, K4560-40, K4500-02, K4510-02 pCR2.1-TOPO) as per provided protocol. TOPO-TA-dnTcf4 was sequenced with M13 TOPO forward and reverse primers to ensure no mutations have been acquired during PCR. The selected positive clone was amplified. TOPO-BamHI-dnTcf4-BamHI and pTRIP MND-GFP (11000bp) were digested with BamHI (Fermentas). Digested TOPO BamHI-dnTcf4-BamHI mix was purified by gel extraction. Digested pTRIP MND-GFP was phenol chloroform purified. Vector and insert were ligated overnight. The ligation reaction was transformed on Ampicillin containing LB plates the next day. Colonies were screened for positivity by digestion with BamHI. The selected positive clone was amplified and digested with enzymes XhoI (NEB) and KpnI (NEB) to open the plasmid. Plasmid pLKO.1 was also double digested with enzyme XhoI and KpnI to excise the hGPK-puromycin cassette. The digested insert (gel extracted) and vector pTRIP MND-dnTcf4 GFP (phenol-chloroform purified) were ligated and transformed the next day. Clones were randomly picked for mini-preps and confirmed for positivity by single digestion with BamHI and double-digestion with XhoI and KpnI. The plasmid was then sequenced for dnTcf4 using oligos 5'-TTCTGTTCGCGCGCTTCTGC-3' and 5'-TACGTCGCCGTCCAGCTCGA-3' and puro cassette using oligos 5'-CATGGTCCTGCTGGAGTTTCG-3' and 5'-GTGAATTAGCCCTTCCAGTC-3'.

[V.VI.7.2 TOP /FOP in pTRIP MND-GFP lentiviral vector](#)

TOP (3 times Tcf binding site with Tk promoter) and FOP (3 times mutated Tcf binding site with Tk promoter) were amplified by PCR (from TOP/FOP luciferase plasmid – Upstate biotech (Cycle: 94°C-30 seconds, 62°C-30 seconds, 72°C-45 seconds)) using primers 5'-AGGCGCGCCTCACGACGTTGTGTA-3' and 5'-GAAGATCTTTAAGCGGGTTCGCTG-3'. Restriction sites Ascl (compatible cohesive with MluI) and BglII (compatible cohesive with BamHI) were introduced at 5' and 3' ends respectively. The vector pTRIP MND-GFP was

digested with MluI and BamHI to get rid of the promoter MND and was purified by phenol-chloroform extraction. The purified PCR products (TOP and FOP) were digested with AscI and BglII to give sticky ends. Both the digested products were ligated and transformed. Obtained colonies were screened for the presence of TOP and FOP by digestion with enzymes MluI and BamHI. After sub-cloning, the selected clones were confirmed by sequencing using oligos 5'-GTTGGGAGTGAATTAGCCCT-3' and 5'-CGCGCCTCACGACGTTGTAA-3'. hGPK-blasticidin cassette was also sub-cloned into this construct from pLKO.1 blast plasmid in exactly the same way puro cassette was sub-cloned. Blasticidin sequence was confirmed using the same oligos.

[V.VI.7.3 Catenin beta interacting protein 1 \(Icat\) in MiG GFP retroviral vector](#)

Icat from *Mus musculus* was amplified by PCR from cDNA obtained from total RNA extracted from wild type thymus using primers 5'-GAAGATCTGCCACCATGAACCGCGAGGGAGCA-3' and 5'-GGAATTCGAAGCTTGCTGCCTCCGGTCTTCCGT-3' (Cycle: 94°C-30 seconds, 60°C-30 seconds, 72°C-45 seconds). The PCR product was purified and sub-cloned into MigR1 linker-eGFP vector by using enzymes BglII and EcoRI via TOPO-TA cloning. Positives clones were tested for the presence of Icat using oligos 5'-TGACCTGGGAAGCCTTGGCT-3' and 5'-GGACACGCTGAACTTGTGGC-3'.

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VII.I Appendix I

ETUDE D'UN MODELE MURIN DE LAL-T WNT DEPENDENT

INTRODUCTION

La voie de signalisation Wnt joue un rôle crucial dans le développement de différents types cellulaires. Par des approches de perte et gain de fonction, il a été montré que la voie Wnt intervient dans le développement et la fonction des cellules hématopoïétiques (Luis *et al.* 2012). Cependant, alors que certaines études montrent que la β -caténine, une molécule clé de la voie Wnt, est nécessaire au développement des cellules T, d'autres données supportent le contraire (Cobas *et al.*, 2004; Xu *et al.*, 2003b). Ainsi, le rôle de cette voie de signalisation au cours de la différenciation des cellules T est controversé. Les mutations affectant la voie Wnt contribuent à l'émergence d'un grand nombre de cancers dont le cancer du sein, du cerveau, du colon, de la cavité buccale, les mélanomes, les carcinomes hépatiques, les tumeurs gastro-intestinales et les leucémies (Klaus and Birchmeier, 2008). Précédemment, il a été montré que l'activation de la β -caténine dans les cellules T conduit au développement de leucémies aiguës lymphoblastiques T (LAL-T) (Guo *et al.*, 2007). L'ensemble de ces données suggère que la dérégulation de la voie Wnt est impliquée dans les LAL-T.

Les LAL-T comprennent 15-20% des cas de LAL chez l'adulte. Actuellement, chez les adultes les LAL-T sont traitées avec 30-40% de survie à long terme sans rechute. La majorité des thérapies utilisées en oncologie visent à tuer les cellules prolifératives sans forcément éliminer les cellules souches cancéreuses (CSC) ou leucémiques (LSC) qui sont quiescentes et résistantes aux drogues. Afin d'augmenter le nombre de rémissions à long

terme, il est important d'apporter de nouvelles thérapies visant à éliminer également les LSCs.

Au cours de mon travail de thèse, je me suis intéressée 1) aux mécanismes moléculaires de leucémogénese et 2) à l'identification des LSCs en utilisant un modèle murin de LAL-T.

RESULTATS

La stabilisation de la β -caténine engendre des défauts de différenciation des cellules T

Nous avons généré une lignée de souris appelée R26- β cat exprimant une forme stable de la β -caténine sous le contrôle du promoteur du gène CD4. L'analyse de l'expression intracellulaire de la β -caténine dans des thymocytes contrôles et R26- β cat montre que l'expression de celle-ci diminue après le stade immature double négatif (DN)² chez les souris contrôles alors que celle-ci reste élevée tout au long du développement des cellules T R26- β cat. L'analyse phénotypique des cellules T par cytométrie de flux révèle que les souris R26- β cat âgées de 6 semaines présentent des défauts de différenciation avec une accumulation de cellules DN4, CD4⁺CD8⁺ doubles positives (DP) et une réduction du nombre de cellules matures simples positives (SP) dans le thymus et la rate. Par ailleurs, nous avons observé une persistance des cellules DP après une irradiation sub-létale des souris R26- β cat jeunes. De plus, l'injection de dexaméthasone chez ces souris montre que les cellules DP sont plus résistantes à l'apoptose induite que les cellules DP normales. D'autre part, il a été démontré que les cellules DP exprimant une forme stable de la β -caténine sont plus résistantes à l'apoptose en partie à cause de la sur-expression du gène

anti-apoptotique Bcl-xl (Xie *et al.*, 2005). L'ensemble de ces données montre que l'expression d'une forme stable de la β -caténine conduit au blocage de la différenciation des cellules T au stade DN4 et DP et augmente la survie des cellules DP générant un contexte favorable à l'acquisition des mutations supplémentaires dans cette population.

La stabilisation de la β -caténine conduit au développement de LAL-T Notch indépendantes

Les souris R26- β cat âgées de 24 semaines développent des LAL-T et succombent avec 98% de pénétrance. Ces LAL-T sont constituées majoritairement de cellules DP. Nous avons montré par des expériences de transplantation que ces tumeurs sont malignes.

L'activation de la voie Notch a été détectée dans plus de 50% des cas de LAL-T (Aster *et al.*, 2008). Cependant l'analyse du transcriptome des cellules leucémiques R26- β cat a mis en évidence que la voie Notch ainsi que certains gènes cibles de cette voie sont sous-exprimés dans les tumeurs R26- β cat. De plus, l'analyse par cytométrie de flux et par RT-qPCR a montré que l'expression de Notch est diminuée dans les cellules tumorales R26- β cat suggérant que ces tumeurs sont Notch indépendantes. Pour tester cette hypothèse, nous avons inhibé la voie Notch par transduction rétrovirale d'une forme dominante négative de mastermind-like 1 dans des lignées cellulaires issues de tumeurs R26- β cat. L'inhibition de la voie Notch dans ces cellules n'affecte pas leur prolifération. Les souris R26- β cat constituent donc un bon modèle pour l'étude de LAL-T Notch-indépendantes et définissent peut être un nouveau sous-groupe de LAL-T.

Similairement, nous avons inhibé la voie Wnt par transduction rétrovirale de la protéine ICAT dans les lignées cellulaires R26- β cat. Ces expériences démontrent que la voie Wnt est requise pour le maintien de la prolifération de ces cellules suggérant que l'activation de cette voie est requise pour l'initiation et le maintien de la leucémie.

CD44 n'affecte pas la leucémogénèse dépendante de l'activation de Wnt

De manière intéressante, nos données transcriptomiques montrent que la protéine CD44 est sur-exprimée dans les cellules préleucémiques, suggérant que cette dérégulation est peut être un évènement précoce responsable de la transformation tumorale des cellules DP. CD44 est un gène cible connu de la voie Wnt et est impliqué dans un grand nombre de cancers (Wielenga *et al.*, 1999b). Des études réalisées sur le cancer du colon montrent que l'absence de CD44 peut retarder et réduire l'apparition de ce cancer (Kemper *et al.* 2010). Pour tester le rôle de CD44 dans les leucémies R26- β cat, nous avons croisé ces souris avec des souris déficientes pour CD44 (Protin *et al.*, 1999b). De façon surprenante, les souris R26- β cat/CD44^{-/-} développent des tumeurs avec la même latence et le même phénotype que les souris R26- β cat. D'autre part, il a été montré que la molécule de surface CD44 joue un rôle important dans la greffe des cellules souches leucémiques (Krause *et al.*, 2006). Nous avons délimité le rôle de CD44 dans le processus de greffe de cellules leucémiques en transplantant les cellules leucémiques de deux tumeurs R26- β cat/CD44^{-/-} dans des souris hôtes. Les cellules tumorales R26- β cat/CD44^{-/-} sont capables de ré-initier des leucémies de façon similaires aux cellules R26- β cat. Ces données montrent que CD44 n'intervient pas dans le processus de leucémogénèse des leucémies Wnt dépendantes.

La perte de PTEN coopère avec l'activation de la β -caténine dans les LAL-T

L'analyse globale du profil d'expression des cellules préleucémiques et leucémiques R26- β cat montre que seuls 142 gènes sont dérégulés au stade préleucémique alors que 1800 gènes sont dérégulés dans les tumeurs, suggérant que l'activation de la voie Wnt a elle

seule n'est pas suffisante pour induire le programme tumoral mais que des évènements supplémentaires sont requis pour la transformation. Afin d'identifier les mutations secondaires pouvant contribuer à leucémogénèse, nous avons réalisé des expériences d'hybridation génomique comparative (HGC) avec dix tumeurs R26- β cat. Ces expériences montrent que 50% des tumeurs R26- β cat portent une délétion dans le gène suppresseur de tumeur PTEN. Nous avons confirmé l'expression réduite de PTEN dans ces tumeurs par des expériences de RT-qPCR et de Western blot. De plus, l'analyse de l'expression de Pten dans les lignées cellulaires R26- β cat montre que celle-ci ont des niveaux d'expression de Pten plus faible que les tumeurs dont elles sont dérivées, suggérant que la perte de Pten est également sélectionnée dans les cellules in vitro. Afin d'étudier la synergie entre l'activation de la voie Wnt et la perte de Pten, nous avons croisé les souris R26- β cat avec des souris Pten^{F/F} (Suzuki *et al.*, 2001). La perte monoallélique de Pten (R26- β cat/Pten^{F/+}) accélère la leucémogénèse T comparée aux souris R26- β cat. De façon intéressante, peu ou pas de protéine Pten a été détecté dans les tumeurs R26- β cat/Pten^{F/+} suggérant que dans ces tumeurs la perte du second allèle est sélectionnée. Ces résultats montrent que la perte de Pten coopère avec la voie Wnt dans le processus de leucémogénèse.

Sur-expression de c-myc dans les leucémies Wnt dépendantes

Par ailleurs, l'analyse par CGH des tumeurs R26- β cat a montré que toutes les tumeurs R26- β cat possèdent des aberrations génétiques au niveau de l'enhancer du TCR α /d et de la région 3' du locus de c-myc. Des translocations entre les régions similaires TCR α /d et c-myc ont été identifiées dans les LAL-T humaines et murines (Erikson *et al.*, 1986; Finger *et al.*, 1986). De plus, les leucémies murines engendrées par la délétion de Pten portent des

translocations similaires et possèdent une activation de la voie Wnt (Guo *et al.*, 2008). Nous proposons que des translocations similaires se produisent dans les cellules T exprimant une forme stable de la β -caténine. En outre, nous avons observé par RT-qPCR que l'expression de l'oncogène c-myc est normale dans les cellules préleucémiques et est augmenté uniquement dans les cellules leucémiques dans lesquelles prennent place ces translocations. Ces données suggèrent que la translocation rapprochant l'enhancer de $TCR\alpha/\delta$ avec la région 3' de c-myc entraîne une sur-expression de cet oncogène et contribue aux processus de transformation des cellules R26- β cat.

Identification des CSL dans les tumeurs R26- β cat

Afin d'identifier les CSLs de ces tumeurs, nous avons montré par des expériences de transplantation de dilution limite de cellules que les tumeurs R26- β cat sont hétérogènes et que seule 1/580 à 1/1,187 cellule est capable de ré-initier la leucémie, suggérant que ces tumeurs possèdent des CSLs. Nous avons utilisé différentes méthodes afin d'identifier ces CSLs.

-les marqueurs de surface : Nous avons transplanté différentes sous populations de cellules T (DN, DP, $CD4^+$) dans des souris hôtes. Ces expériences ont montré que seules les cellules DP sont capables de ré-initier des tumeurs, suggérant que les CSLs sont contenue dans la fraction DP des tumeurs R26- β cat. De plus, afin d'identifier une population de cellules enrichie en CSLs, nous avons examiné l'expression de marqueurs de surface de cellules souches et de CSC. Bien que les marqueurs Sca-1, CD138, CD133 et CD200 sont exprimés anormalement sur les cellules tumorales R26- β cat, ils n'ont pas permis d'identifier une population enrichie en CSLs. Parallèlement, nous avons utilisé le marquage des cellules de la « Side Population » (SP) permettant d'identifier les cellules quiescentes et résistantes

aux drogues pour identifier les CSLs dans nos tumeurs. Ces expériences ont montré que les tumeurs R26- β cat possèdent un certain nombre de cellules SP suggérant que cette population peut contenir les CSLs.

-traitement au 5-Fluorouracil (5-FU) : Nous avons traité les souris R26- β cat au 5-FU, puisque cette technique a permis dans des études précédentes d'identifier les CSCs (Zhang *et al.* 2010; Zhou *et al.*, 2001). Cependant, l'analyse des marqueurs de surface et de la SP de souris traitées au 5-FU n'a pas permis de mettre en évidence une population de cellules pouvant être enrichie en CSLs.

L'ensemble de ces données montre que les tumeurs R26- β cat possèdent des CSLs dans la fraction DP ainsi qu'une population de cellules SP.

CONCLUSIONS ET PERSPECTIVES

Nous avons généré un modèle murin de LAL-T Notch indépendant, exprimant une forme stable de la β -caténine dans les cellules T. Les souris préleucémiques présentent un blocage de la différenciation des cellules T au stade DN4 et DP. Ces cellules DP préleucémiques possèdent une survie accrue générant un contexte favorable à la transformation de celles-ci. La perte de Pten ainsi que la sur-expression de c-myc constituent des événements secondaires coopérants avec l'activation de la voie Wnt dans le processus de leucémogénese. Par ailleurs, nous avons montré que les tumeurs R26- β cat sont hétérogènes et que les CSL sont contenues dans la fraction DP. De plus, les tumeurs possèdent une population de cellules SP.

Cette étude apporte des éléments nouveaux sur le mécanisme de leucémogénese des LAL-T Notch-indépendantes. Nos expériences d'identification des CSLs encore préliminaires

montrent que des populations pouvant contenir des CSLs existent dans les tumeurs R26- β cat. Nos données suggèrent que la β -caténine constitue une cible thérapeutique potentielle pour les LAL-T.

Ces données font l'objet d'un manuscrit en cours de préparation. Nous espérons soumettre ce manuscrit avant ma soutenance de thèse en Septembre.

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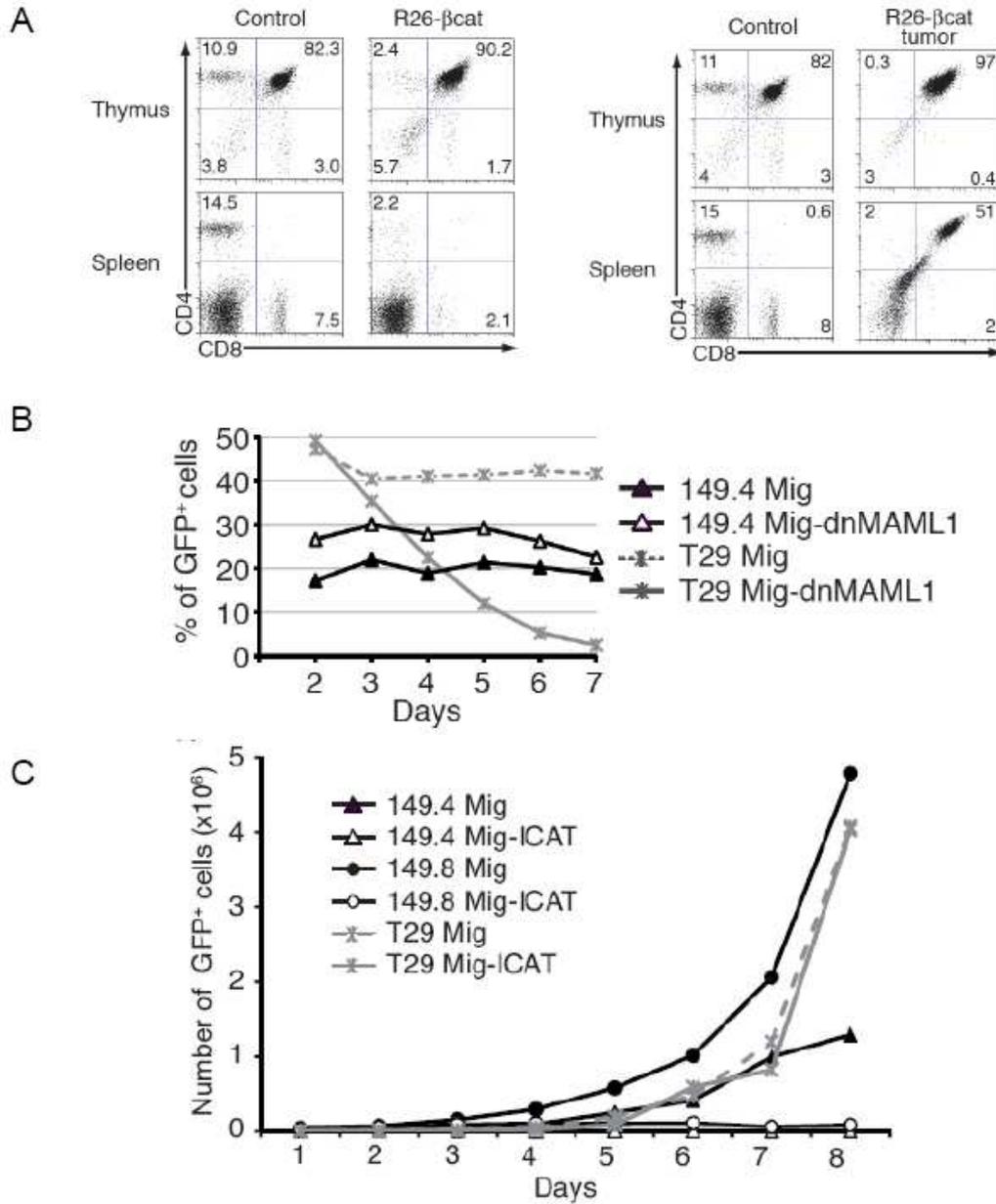


Figure 6.1 Les souris R26- β -cat développent des leucémies indépendamment de l'activation de la voie Notch. A) Analyses de souris contrôles, pré-leucémiques et leucémiques R26- β -cat par cytométrie en flux de thymocytes et de cellules de la rate marquées à l'aide d'anticorps dirigés contre les marqueurs de cellules T, CD4 et CD8. B) Prolifération des cellules des lignées cellulaires R26- β -cat 149.4 et T29 (dérivées de tumeurs Ikaros^{L/L}) après infection à l'aide du retrovirus Mig-dn-MAML-GFP et Mig-GFP. C) Prolifération des cellules des lignées cellulaires R26- β -cat 149.4 et 149.8, et T-29 infectées avec le retrovirus Mig-ICAT-GFP et le control Mig-GFP.

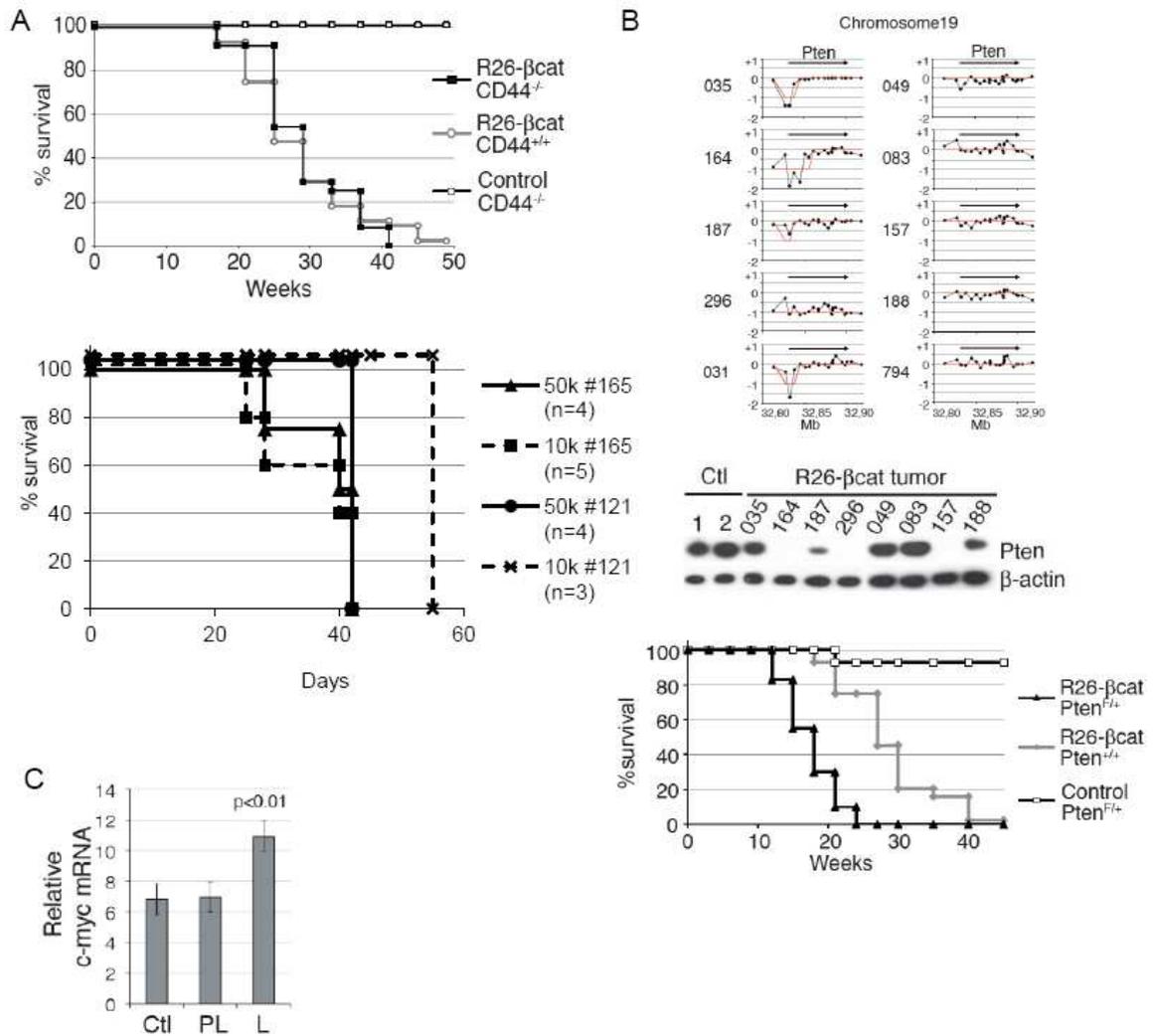


Figure 6.2 Les voies moléculaires impliquées dans la leucémogénèse R26-β-cat. A) Courbe Kaplan-Meier de survie des souris R26-β-catCD44^{-/-} (Haut) et des souris transplantées avec des cellules tumorales issues de deux tumeurs indépendantes R26-β-catCD44^{-/-} (Bas). B) Analyse du locus Pten de dix tumeurs R26-β-cat par CGH (Haut) et de l'expression de Pten dans les tumeurs R26-β-cat et les thymocytes contrôles par western blot (Milieu); Courbe Kaplan-Meier de survie des souris R26-β-catPten^{fl/+}, R26-β-catPten^{+/+} et des souris contrôles Pten^{fl/+} (Bas). C) Analyse par RT-qPCR de l'expression du RNAm de Myc dans des thymocytes pré-leucémiques (PL), leucémiques (L) et contrôles (Ctl).

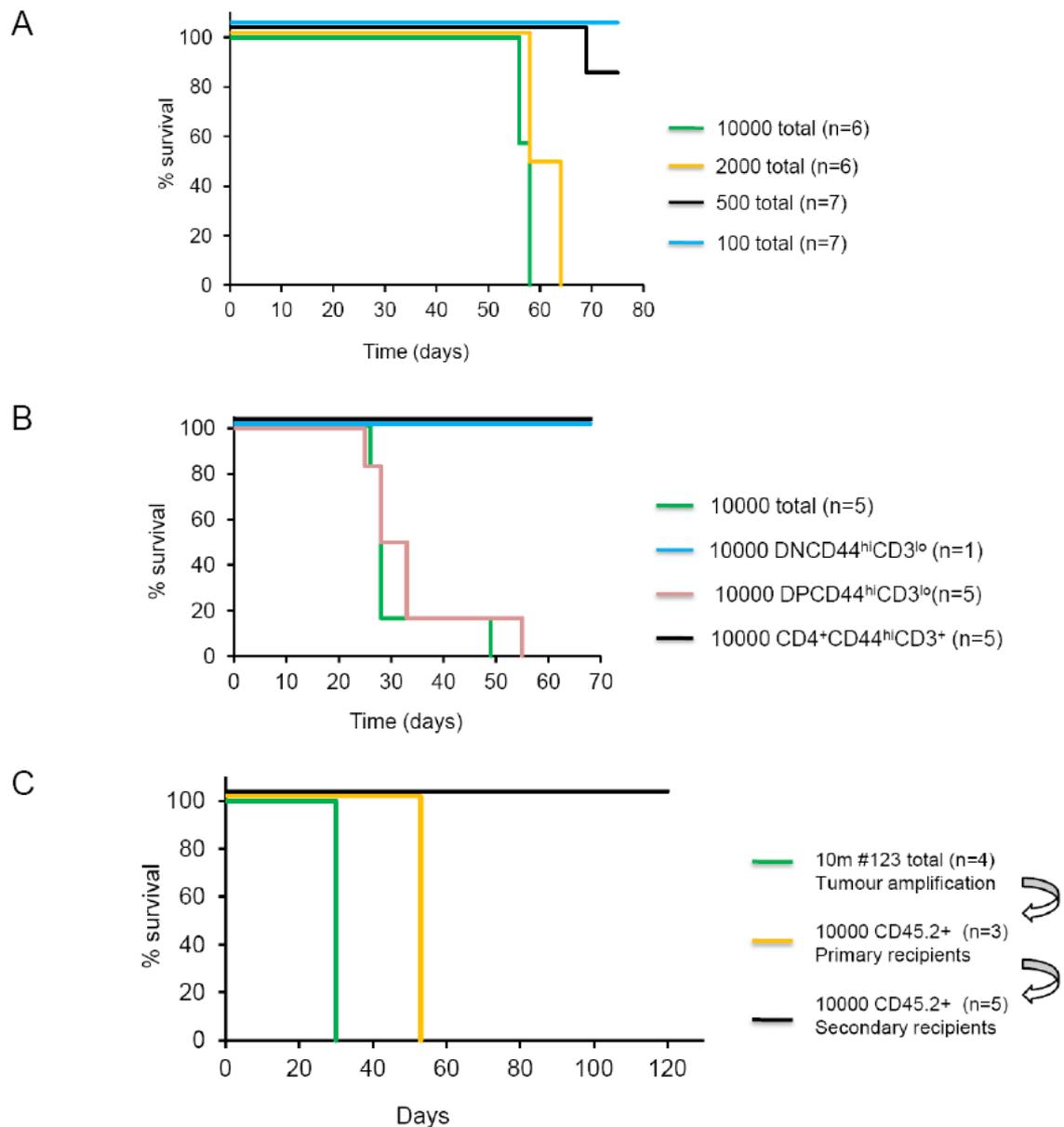


Figure 6.3 **Activité des cellules souches leucémiques dans les tumeurs R26-β-cat.** Courbe Kaplan-Meier de survie : A) de souris irradiées à des doses létales et transplantées avec des cellules tumorales R26-β-cat DPCD44^{hi}CD3^{lo} en nombre limite (10000, 5000, 2000, 500 and 100). B) de souris transplantées avec 10000 cellules issues de sous-population de thymocytes isolées à l'aide des marqueurs de surface CD4, CD8, CD44 et CD3. C) représentative de souris transplantées en série avec des cellules tumorales R26-β-cat. Les cellules tumorales R26-β-cat de la moelle osseuse des souris hôtes primaires ont été purifiées à l'aide du marqueur CD45.2+ puis transplantées en série dans les souris hôtes irradiées.

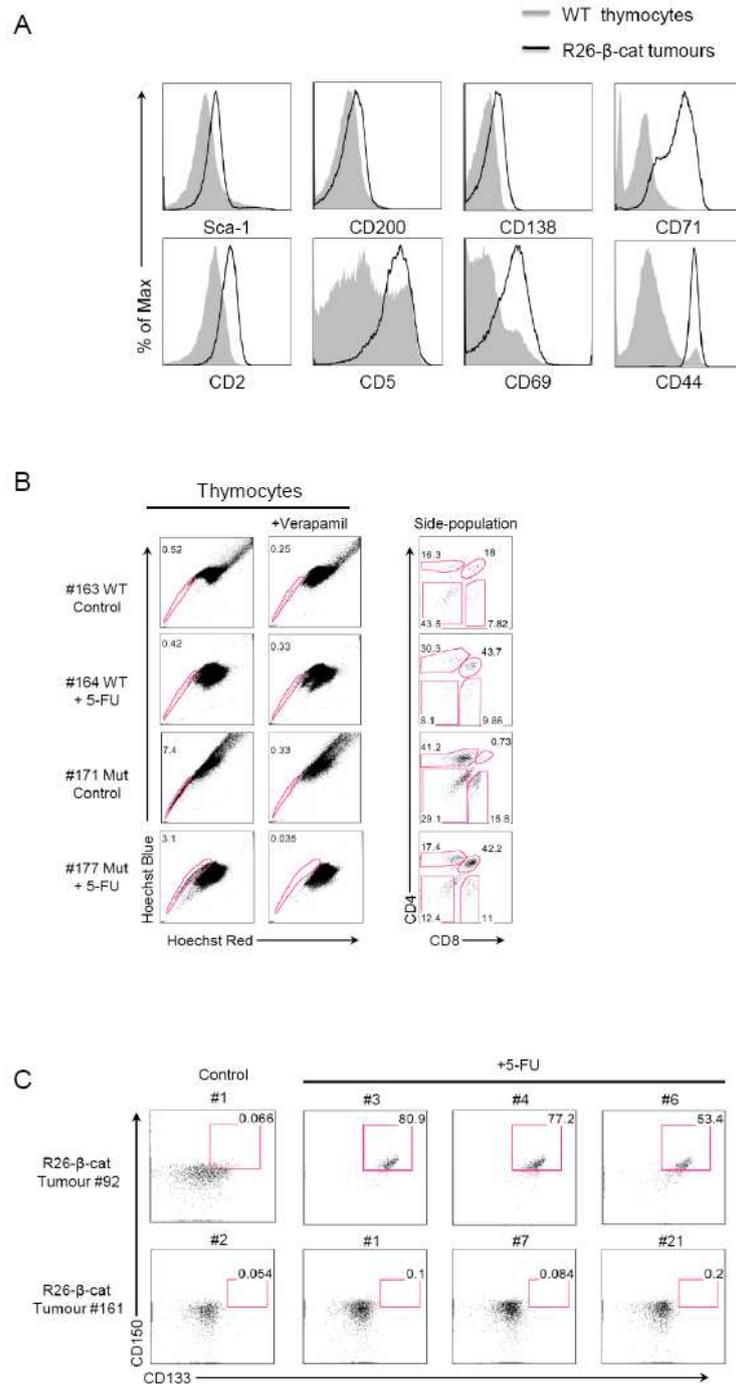


Figure 6.4 Caractérisation des CSLs dans les tumeurs R26- β -cat. A) Analyse par cytométrie en flux de tumeurs R26- β -cat (ligne noire) et de thymocytes contrôles (courbe colorée grise) de l'expression de Sca-1, CD200, CD138, CD71, CD2, CD5, CD69 et de CD44. B) Marquage au colorant Hoechst de cellules de souris R26- β -cat et contrôles traitées au 5-FU. Les cellules de la « Side-Population » sont identifiées à l'aide des cellules contrôles traitées au Verapamil. C) Immuno-phénotype de cellules de la moelle osseuse de souris hôtes traitées au 5-FU après transplantation de cellules tumorales R26- β -cat.

VII.II Appendix II

Role of Ikaros in plasmacytoid dendritic cell (pDC) differentiation

(October 2008 – July 2009) Deepika Kaveri, Susan Chan and Philippe Kastner

Introduction

Plasmacytoid dendritic cells (pDCs) represent a distinct subset of dendritic cells that are specialised in rapid type I interferon (IFN) secretion in response to viruses. Earlier studies from the lab show that mice with diminished Ikaros activity ($Ik^{L/L}$) have a block in pDC differentiation (1). These mice have reduced pDCs but other DC subsets were seen unaffected. Interestingly, it was also observed that the mutant mice have no mature pDCs ($120G8^+ CD11c^+ B220^+$) in the spleen but, do contain immature pDCs ($120G8^+ CD11c^+$) in the bone marrow. In this study, we attempted to decipher the role of Ikaros during pDC differentiation.

Results

A) Notch pathway in pDC differentiation

We first standardised pDC cultures in vitro from whole bone marrow cells by using the cytokine Flt3 ligand. Because our transcriptome data from the $Ik^{L/L}$ pDCs showed an upregulation of Notch target genes, we checked if inhibiting Notch pathway by using γ -secretase inhibitor (GSI) would affect pDC differentiation. GSI was added at different times during culture to check if Notch activity is required at a particular stage during differentiation. We observed that adding GSI reduced pDC numbers in cultures, but this effect was not consistent and this could be because GSI does not specifically target Notch. We therefore infected pDCs with a retrovirus against MAML1 (mastermind-like 1, a Notch co-activator) to specifically block only

the Notch pathway. These infections did not affect pDC cultures in vitro. The results from these experiments were contradictory and hence not conclusive.

B) I k ^{L/L} mice have more dendritic cell progenitors

Because the I k ^{L/L} pDCs fail to mature, we checked for the presence of dendritic cell progenitors in the control wild type (WT) mice and I k ^{L/L} mice. Dendritic cell progenitors, characterised by Lin⁻Flt3⁺c⁻kit^{int}CD115⁺, were indeed found in greater numbers in the I k ^{L/L} mice. This could mean that the pDCs are blocked at this stage and further differentiation would probably require Ikaros activity.

C) Generation of a 'pDC-less' mouse model

To check if Ikaros is absolutely required for the development and differentiation of pDCs, we planned to generate a mouse model which would be 'pDC-less' by specifically abolishing Ikaros activity. We inserted Ikaros6 (Ik6), a dominant negative form of Ikaros to block Ikaros activity, in the SiglecH locus (a pDC specific gene) by homologous recombination in embryonic stem (ES) cells to achieve the expression of Ik6 specifically in pDCs. The chimeras were tested for the presence of pDCs. We found that the all mice expressed normal amounts of pDCs from Sv129-derived ES cells. Therefore, the Siglec-H-IRES-Ik6 construction did not inhibit pDC differentiation.

Résumé français

Les leucémies aiguës lymphoblastiques T (LAL-T) proviennent de la transformation maligne des cellules T. Les LAL-T représentent 15% des LALs pédiatriques et 20% des LALs chez l'adulte. Les LAL-T humaines sont causées par la voie de signalisation Notch dans plus de 50% des cas. Cependant, les acteurs moléculaires responsables des LAL-T Notch-indépendantes restent en grande partie méconnus. La β -caténine, un régulateur clé de la voie de signalisation Wnt, a été décrite comme étant superflue et nécessaire pour le développement des cellules T, laissant le rôle de cette molécule controversée dans ce processus. Nous avons généré une lignée de souris R26- β -cat exprimant une forme stable de la β -caténine sous le contrôle du promoteur de CD4. La stabilisation de la β -caténine conduit au blocage de la différenciation des cellules T au stade DN4 et DP ainsi qu'au prolongement de la survie des cellules DP générant probablement un contexte favorable à l'acquisition de mutations génétiques secondaires dans ces cellules. De plus, nous n'avons pas détecté d'activation de la voie Notch dans le transcriptome de ces tumeurs et nous avons montré que la prolifération des lignées cellulaires R26- β -cat issues de tumeurs primaires est indépendante de l'activation de Notch. Ainsi, les souris R26- β -cat constituent un modèle *in vivo* pour l'étude des LAL-T indépendantes de la voie Notch et définissent peut être un nouveau sous-groupe de LAL-T. L'inhibition de la voie Wnt dans les lignées cellulaires R26- β -cat a montré que l'activation de cette voie est requise pour la prolifération et l'expansion de ces cellules. De surcroît, CD44, un gène cible de la voie Wnt surexprimé dans les thymocytes R26- β -cat ne contribue pas à la leucémogénèse, ni au potentiel de greffe de ces leucémies. De plus, l'analyse par hybridation comparative d'ADN génomique (CGH) des tumeurs R26- β -cat a révélé des aberrations chromosomiques persistantes dans le locus du TCR α/δ et en 3' du gène Myc. Par ailleurs, nous avons observé par analyse RT-qPCR une augmentation de l'expression de Myc dans les thymocytes uniquement au stade leucémique, suggérant que la surexpression de cet oncogène contribue à la leucémogénèse. De façon intéressante, l'analyse par CGH a également mis en évidence que 50% des tumeurs R26- β -cat possèdent une délétion du gène Pten (phosphatase and tensin homologue on chromosome 10). Nous avons montré que la perte du gène Pten coopère avec la voie Wnt et contribue au processus de leucémogénèse R26- β -cat.

Actuellement, les thérapies conventionnelles permettent une rémission à long terme pour seulement à 30-40% des LAL-T des adultes. Ces thérapies visent essentiellement à éradiquer les cellules cancéreuses prolifératives sans éliminer les cellules quiescentes et résistantes aux drogues telles que les cellules souches cancéreuses ou leucémiques (CSC ou CSL). Afin d'augmenter le taux de rémission à long terme il est important de définir de nouvelles thérapies ciblant spécifiquement les CSLs. Nous avons cherché à déterminer l'activité des CSLs dans les tumeurs R26- β -cat. Afin d'identifier les CSLs dans ces tumeurs, nous avons d'abord montré que ces tumeurs sont hétérogènes par des essais de transplantations de cellules en dilution limite. Puis nous avons démontré que les CSLs sont contenues dans la fraction CD4⁺CD8⁺ puisque seules ces cellules sont capables de ré-initier des tumeurs dans des souris hôtes. L'analyse par cytométrie de flux a montré que les cellules leucémiques R26- β -cat sont phénotypiquement différentes des cellules T sauvages. D'autre part, le marquage au colorant Hoechst des cellules leucémiques R26- β -cat a mis en évidence la présence d'une « Side Population » (SP) connue pour avoir une activité de CSL. Afin d'identifier les CSLs de la masse tumorale, nous avons tenté d'isoler les cellules résistantes aux drogues par traitement au 5-fluorouracil (5-FU). L'administration de 5-FU à des souris R26- β -cat leucémiques a montré que les cellules leucémiques ainsi traitées ne sont pas enrichies en CSLs. En résumé, nous avons généré un modèle murin de LAL-T exprimant une forme stable de la β -caténine dans les cellules T. Cette étude a contribué à la compréhension des mécanismes moléculaires impliqués dans les LAL-T induites par l'activation de la β -caténine et supporte l'hypothèse que la β -caténine constitue une cible thérapeutique potentielle dans les LAL-T.

Résumé

Mots-clés : Leucémie aiguë lymphoblastique T, Voie de signalisation Wnt, Cellules souches leucémiques

Nous avons généré une lignée de souris, R26- β cat, qui exprime une forme stable de la β -caténine dans les cellules T. Les souris R26- β cat présentent un blocage de la différenciation des cellules T aux stades DP du à leur résistance accrue à l'apoptose. De façon intéressante, les souris R26- β cat développent des leucémies T indépendantes de la voie Notch. Nous avons montré que la perte du suppresseur de tumeur Pten et la sur-expression de Myc sont favorisées dans ces leucémies et constituent peut être des événements secondaires contribuant à cette leucémogénèse. Nous avons également mis en évidence que les tumeurs R26- β cat sont malignes, hétérogènes et que les cellules souches leucémiques (CSL) sont enrichies dans la fraction DP. De surcroît, l'auto-renouvellement des CSL R26- β cat est affaibli.

Nous proposons que le modèle R26- β cat définit un nouveau sous-groupe de leucémie aiguë lymphoblastique T et que la β -caténine pourrait constituer une cible potentielle pour traiter ces leucémies.

Résumé en anglais

Key words: T-cell acute lymphoblastic leukaemia, Wnt pathway, Leukaemia stem cells

We report a murine model, R26- β cat, expressing a stable form of β -catenin in T cells. R26- β cat pre-leukemic mice show a developmental block in T-cell differentiation and exhibit increased resistance to apoptosis. Interestingly, the mice develop T cell lymphomas independent of the Notch pathway. Furthermore, we showed that loss of the tumour suppressor Pten and over-expression of Myc was favoured; and may constitute the secondary events contributing to this leukemogenesis. We also demonstrated that R26- β cat tumours are malignant, heterogeneous and that leukaemia stem cells (LSC) were enriched in DP cells. Furthermore, the self-renewal capacity of R26- β cat LSCs can to be exhausted.

We propose that the R26- β cat model defines a new sub-group of Notch-independent T-ALL and the β -catenin may serve as a potential therapeutic target for these tumours.