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By

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Abstract

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Professor Mark S. Schlissel, Chair

Apparently, science has fought a long war on cancer with a myriad of efforts. Nonetheless cancer is still one of the leading causes of death, and our understanding of cancer remains very narrow. Many studies have recognized that one of the hallmark features of cancer is the complex involvement of many cellular components and events throughout cancer development.

In 1970, Abelson-Murine Leukemia Virus (A-MuLV) was accidentally discovered as a replication-defective mutant retrovirus that causes acute leukemia in mice. A-MuLV can infect mouse bone marrow, and generate so-called Abelson transformed cell lines. However, A-MuLV infected cells exhibit two distinct stages of transformation with stringent clonal selection during the transformation process. This suggests that in addition to expression of the viral oncogene v-Abl, cellular factors also play an important role in the transformation process.

Here, we studied this v-Abl transformation system to ask some questions about how cellular factors are involved in leukemic transformation. 1) Are there a unique subset of cell populations susceptible to v-Abl transformation? With bone marrow fractionation experiments, we confirmed that pro-B cells are the major target cells of v-Abl transformation. 2) In which stages of the process is tumor suppressor p53 involved in the transformation? p53 knock-down studies clearly validated that p53 is also involved in early v-Abl transformation, as well as later stages. 3) Are there any other cellular factors important in v-Abl transformation? A loss-of-function screen identified Dpf2, a zinc finger transcription factor, as a protein that can affect v-Abl transformation in a p53-dependent manner.

In the meanwhile, we have developed a 96 well round-bottom plate assay that can measure initial v-Abl transformation frequency reliably and accurately. Also we have implemented a sequential infection method, which could provide a good platform to allow genetic manipulation of cells during the transformation process. These assays should facilitate answering many more questions about the v-Abl transformation process.

For Eunjung, Sharon and Janie.

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I am very blessed to have all these people around. Without them, I would be much different person than I am. In my future career, I wish to be a person like them, supporting and helping others.

But he knows the way that I take; when he has tested me, I will come forth as gold.
(Job 23:10)

Chapter1. An Introduction to Tumorigenesis, v-Abl Transformation, and B Cell Development

Tumorigenesis – a complex multi-step process

Undoubtedly cancer has become a leading cause of death worldwide, and scientists have made every effort to fight and win the "war on cancer" (Ferlay et al. 2010, Tiwari et al. 2012). Particularly, it has been a great interest on how tumor develops, or tumorigenesis. To obtain an uncontrollable growth and malignancy, normal healthy cells need to disrupt and alter their cellular pathways of growth control and defense mechanisms. Thus, It has been thought that tumorigenesis is a complex multi-step process with critical multiple events (Vogelstein et al. 1993). In 1971, Knudson analyzed retinoblastoma and suggested 'two-hit' hypothesis, which is two mutation events are necessary in tumorigenesis (Knudson. 1971). Since that, cancer researchers have identified many other 'hits' required for different phase of various tumors, such as activation of oncogenes and inactivation of tumor suppressor genes (Bombonati et al. 2011; Fearon et al. 1990; Jin et al. 2012).

Moreover, Hanahan and Weinberg conceptually proposed that cancer has multiple hallmarks of biological capabilities that is acquired during the tumor development (Hanahan et al. 2011). These hallmarks include sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, activating invasion and metastasis, reprogramming of energy metabolism, evading immune destruction, disrupting genome stability, and inducing tumor promoting inflammation. Overall, tumor formation is a multi-stage process with progressive acquisition of many genetic and epigenetic changes in the normal cells to obtain these necessary traits of cancer hallmarks.

Although myriads of tumor research have discovered many tumor-specific alterations during tumorigenesis, still our understanding of tumor development is very limited. And it is essential to fully understand the progression of tumors for development of therapeutic measures to cancers.

A-MuLV and oncogene v-Abl

In 1970, Abelson and Rabstein accidently found an acute transforming virus mutant from Moloney Murine Leukemia Virus (M-MuLV) studies (Abelson et al. 1970a,b; Baltimore et al. 1979). They were testing M-MuLV-induced tumors in athymic mice, to validate T cell tropism of M-MuLV. And one of the hundreds of infected mice, developed unusual lymphosarcoma 37 days after virus infection. A distinguished virus was isolated from this tumor, which is called Abelson Murine Leukemia Virus (A-MuLV), named after the discoverer. Further studies showed that A-MuLV is a replication defective virus that causes B cell lymphoma in mice with a short

latency period, unlike M-MuLV that generates T cell lymphoma with a long latency (Scher et al. 1975; Premkumar et al. 1975).

After the large efforts to reveal the molecular identity of A-MuLV, it turned out that A-MuLV encodes the oncogene v-Abl. v-Abl is the fusion protein resulting from recombination between viral gag from M-MuLV and truncated c-Abl, the murine cellular homolog of v-Abl (Goff et al. 1980; Reddy et al. 1983) (Figure 1.1.). c-Abl is a ubiquitously expressed non-receptor tyrosine kinase with various functions in cell differentiation, cell division, cell adhesion, and stress response (Van Etten. 1999; Pendergast. 2002). SH3 domain in N-terminal region of c-Abl negatively regulates its kinase activity. However fusion of viral gag in v-Abl disrupts this regulatory region, which confers v-Abl constitutively elevated kinase activity, and hence transforming ability (Wang et al. 1984; Oppi et al. 1987). In addition, this gag region targets v-Abl protein to the plasma membrane by myristoylation (Yi et al. 2007).

Another variant of c-Abl, BCR-Abl, is also a fusion gene of c-Abl and the breakpoint cluster region (BCR) from a specific chromosomal translocation, which is called the Philadelphia chromosome (Daley et al. 1990). Importantly, BCR-Abl is related to human diseases, such as Chronic Myelogenous Leukemia (CML) and Acute Lymphocytic Leukemia (ALL) (Drexler et al. 1999). Therefore, understanding v-Abl transformation can provide invaluable insights to human diseases - CML and ALL.

v-Abl transformation of B cells

As A-MuLV is discovered as an acute B cell lymphoma causing mutant virus, oncogene v-Abl can transform early B lineage cells *in vitro* and *in vivo* (Abelson et al. 1970b; Alt et al. 1981). There also have been reports that A-MuLV can transform many other types of cells, including fibroblast, mast cells, macrophage, erythroid cells, T cells and mature B cells in certain conditions (Chung et al. 1986; Scher et al. 1975; Cook et al. 1982; Waneck et al. 1981; Raschke et al. 1978; Serunian et al. 1986). However, B cell transformation is the most common and prevalent form of v-Abl transformation *in vivo*. Infection of bone marrow cells with A-MuLV induces the transformation of B lineage cells in culture that resemble cells at the early pre-B cell stage (Chen et al. 1994; Rosenberg. 1994; Alt et al. 1981; Hardy et al. 1991). Transformed cells halted their Ig light chain gene rearrangement, and exhibits high cell proliferation just like early pre-B cells, but they are blocked for further differentiation. Therefore, Abelson transformed pre-B cells have been widely used as a useful tool to study B cell development and V(D)J rearrangement (Chen et al. 1994; Rosenberg. 1994).

Like other tumorigenesis, *in vitro* v-Abl transformation is also a multi-step process (Green et al. 1989; Radfar et al. 1998; Unnikrishnan et al. 1999). Typically, v-Abl transformation process exhibits distinctive two steps. The first step is the early proliferation stage where infected cells show rapid cell growth over a period of two weeks. The second step is an apoptotic crisis that can continue several weeks to months, during which the cultures show erratic growth and high

apoptosis among the infected cells. Finally, Abelson transformed cell lines are established from few survival cells after this high levels of apoptosis, mostly resulting in mono-clonal or oligo-clonal cell lines (Green et al, 1987). This clonal selection suggests that there is a pressure of stringent selection during the transformation. However the v-Abl expression level and its kinase activity do not change in this multi-step process (Whitlock et al. 1983; Zimmerman et al, 2008). Therefore it raises an idea that, in addition to v-Abl, other cellular factors and events are involved in the Abelson transformation process.

Elucidating how such cellular factors modulate tumorigenesis will help better understanding the v-Abl transformation. Importantly, it is very likely that cellular factors modulated in v-Abl transformation could be a novel tumor suppressor and therapeutic targets for lymphoid malignancies or other tumor systems.

p53 pathway and v-Abl transformation

p53 transcription factor is one of the most critical tumor suppressor genes in tumorigenesis (Rivlin et al. 2011, Jacks et al., 1994; Levine et al. 2009). As p53 is also called as "guardian of genome", it has roles in many cellular stress response including but not limited to cell cycle arrest, DNA repair, senescence and apoptosis (Lane et al. 1992). Although p53 is not required for normal development, it has been implicated in most of cancers (Donehower et al. 1992).

In normal non-stressed condition, p53 is kept inhibited by negative feedback loop with Mdm2 (Levine et al. 2006; Levine et al. 2009) (Figure 1.2.). Mdm2 binds and ubiquitinates p53, which results in degradation by proteasome. When cells are under the various stress, upstream sensors such as ATM, ATR or p19 ARF relay stress signals and activate p53 by breaking the p53 - Mdm2 core pathway. Then, p53 turns on downstream signals by inducing transcription of many downstream target genes and/ or by direct protein-protein interactions. Finally, the outcome of the p53 pathway contributes to manage the input cellular stress.

Not surprisingly, p53 also plays an important role in v-Abl transformation. More than 50% of BCR-Abl induced human lymphomas have p53 mutation (Imamura et al. 1994; Prokocimer et al. 1994). A Study with *In vitro* Abelson transformed pre-B cells found 50% of the cell lines acquired mutant form of p53 with strong tumorigenicity (Thome et al. 1997). Recently, more evidence came out to emphasize this role for the p53 pathway in v-Abl transformation process (Radfar et al. 1998; Unnikrishnan et al. 1999; Zou et al. 2000). Bone marrow cells from p53 null mice and p19ARF null mice showed increased transformation by bypassing the apoptotic crisis step. Further, it was reported that p19ARF, one of the upstream sensors in p53 pathway, is modulated to increase its expression level and change localization to the nucleolus during the transformation process (Zimmerman et al. 2008). Thus, p53 pathway appears to be one of the important cellular factors modulated in Abelson transformation.

However, rather than p53 pathway, other cellular factors implicated in v-Abl transformation are still largely unknown. Particularly, screening experiments using library has never been tried in this system yet, which might fish out uncharted factors in the tumorigenesis.

Early B cell development in mouse bone marrow

B cells are important players in adaptive immune system and they are responsible for production of antibody (Cooper et al. 2006). It is very critical to generate diverse antibody repertoire to be able to defend from myriads of pathogens. And it is V(D)J recombination that made this diverse antibody repertoire possible, which is processed during B cell development (Schatz et al. 2011).

As the B cell's 'B' stands for Bursa or Bone marrow, mice adult B cells develop in bone marrow (Hardy et al. 1991). There have been many efforts to define and characterize B cell development by many groups. And Osmond, Melchers and Rolink, and Hardy each had suggested their own models and nomenclatures of B cell development, which do not overlap completely (Osmond et al. 1998; Hardy et al. 2001; Hardy et al. 2007). In addition, on-going studies revealed that there are alternative routes to B cell development (D'Amico et al. 2003). In this paper, we will use Philadelphia nomenclature, also called Hardy fraction system, to describe major stages of early B cell development (Hardy et al. 2001).

Hematopoietic stem cells (HSC) have ability to generate all the cells of blood lineage (Figure 1.3.). They are expressing c-kit (CD117) but no other known lineage specific proteins. Following the HSCs, Common lymphoid progenitors (CLP) have potentials to become B cells, T cells and NK cells, and express IL-7Rα (CD127) in addition to c-kit (Kondo et al., 1997). V(D)J rearrangements of Ig Heavy chain locus begins from this CLP stage. To be developed as a B cell lineage, CLPs become fraction A cells. They are the first early B lineage specific cells, expressing B220 (one isoform of CD45R), a representative marker of B cell lineage. These fraction A cells also exhibit low levels of Heat-stable antigen (HSA, or CD24). Next stage is fraction B, where CD19, another famous marker for B cell lineage, starts to be expressed. Fraction B also exhibits intermediate levels of HSA. Then, fraction C cells show BP-1 (glutamyl aminopeptidase or Enpep) expression additionally. If Ig heavy chain is successfully rearranged at this point, cells halt rearrangements and express pre-B cell receptor (pre-BCR) with surrogate light chains. This is fraction C' or early pre-B stage, where cells undergo high cell proliferation. Fraction C' shows High levels of HSA. In fraction D, or late pre-B, Ig light chain rearrangement begins. Fraction D cells do not express CD43. After rearrangements of Ig light chain, cells with functional B cell receptor become immature B cells, corresponding to fraction E. Fraction E cells display IgM on their surface. Later, Fraction E cells leave the bone marrow and migrate to the spleen, where they become Fraction F, mature B cells, with co-expressing IGM and IgD.

Target cells of v-Abl transformation

Since phenotypes of v-Abl transformed cell line resembles early pre-B cells in B cell development, it has been largely assumed that this early pre-B cells are the target cells of Abelson transformation (Alt et al. 1981). On the other hand, there is also an attractive concept that cancer cells might originate from stem cells (Sell. 2004). Cancer cells resemble self-renewal characteristics of stem cells, and they are associated with similar signaling pathways in stem cells (Reya et al. 2001). Particularly, studies in various leukemias reported that HSCs and early progenitor cells as their cells of origin (Kelly et al. 2002; Jamieson et al. 2004; George et al. 2001; Hope et al. 2004). Thus, it is still questionable about the origin cells of v-Abl transformation. However, the true target cell population of the transformation has never been directly tested in experiments, to the extent of my knowledge. Undoubtedly, the origin of the tumorigenesis is very critical to understand the full aspects of Abelson leukemia.

Purpose

As briefly described earlier, v-Abl oncogene expression initiates Abelson transformation process. However, throughout the process, transforming cells undergo proliferation burst and apoptotic crisis stages of tumorigenesis with high selection pressure. This points out that many cellular components and events must be modulated and regulated to complete the v-Abl transformation. In this study, we intend to identify and characterize these cellular factors in the multi-step process of the v-Abl transformation. For this, we performed Flow cytometry sorting experiments to answer **1) what is the target cell population of v-Abl transformation**. Then, shRNA against p53, a well-known cellular factor involved in Abelson transformation, was applied to establish **2) a better method to generate Abelson transformed cell lines**. Finally, **3) what other cellular factors are involved in the transformation process** has been investigated by performing loss-of-function shRNA library screening.

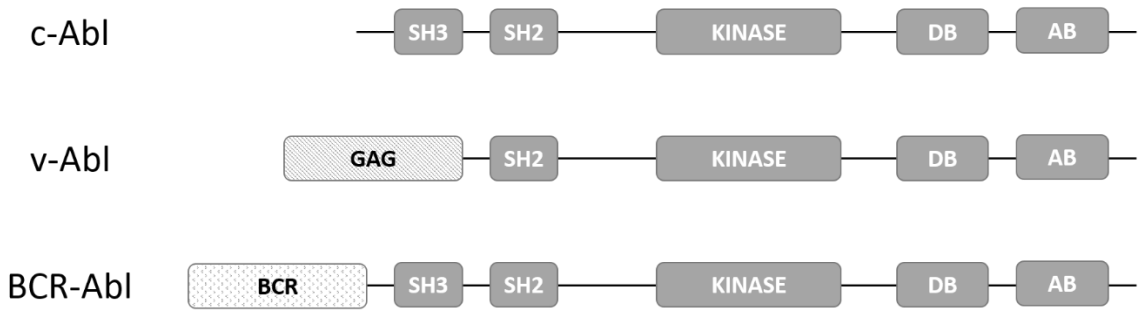


Figure 1.1. Diagram of v-Abl and its homologs

Adapted from (Sirvent et al. 2008). v-Abl oncogene is fusion protein of c-Abl and viral GAG, with disrupted SH3 regulatory domain. Similarly, BCR-Abl is formed by recombination of c-Abl and BCR.

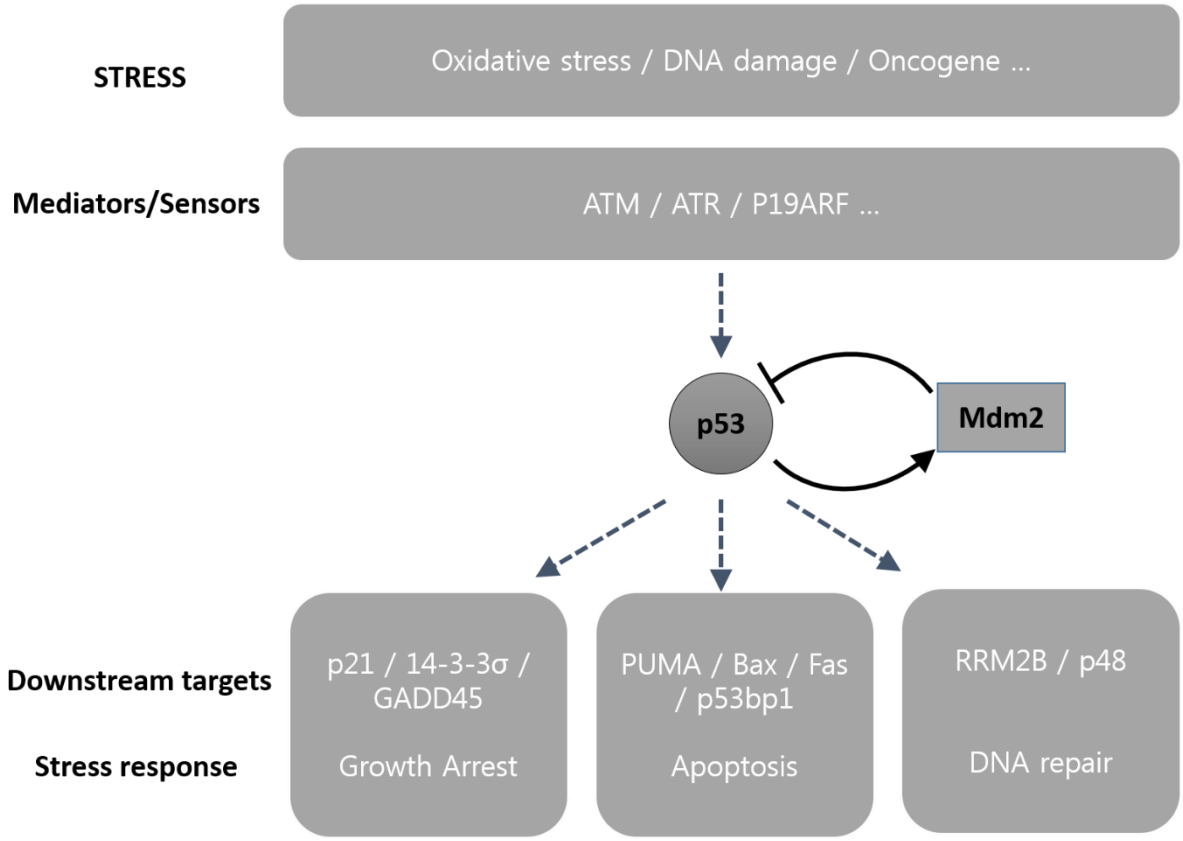
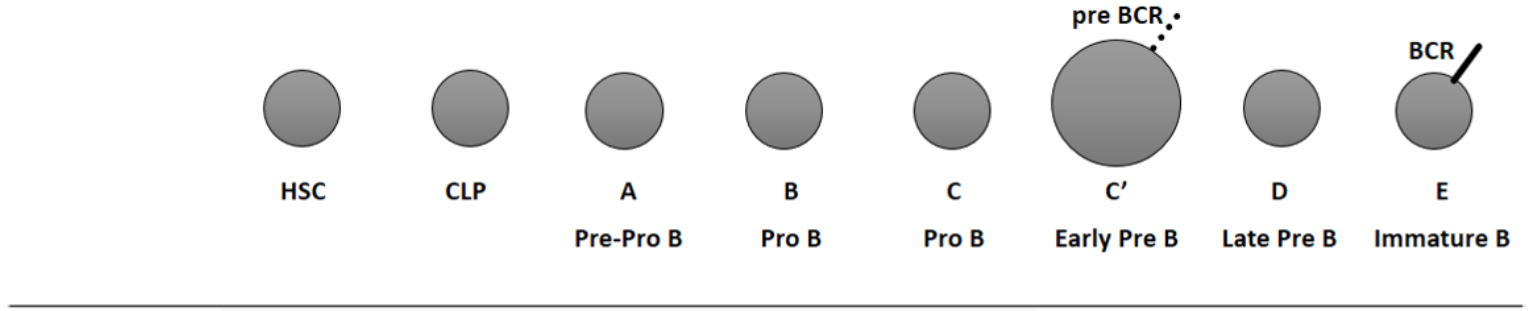


Figure 1.2. Simple scheme of p53 pathway

Adapted from (Levine et al. 2009). In normal cells, p53 is down-regulated by Mdm2. Cellular stress including environmental stress and oncogene activation leads to activation of p53 through many stress sensors. Then, activated p53 relay signals to downstream targets, resulting in appropriate stress response in the cell.



	HSC	CLP	A Pre-Pro B	B Pro B	C Pro B	C' Early Pre B	D Late Pre B	E Immature B
C-Kit	+	+	+	+	+			
B220			+	+	+	+	+	+
CD43	+	+	+	+	+	+		
HSA				+	+	++	++	++
Bp-1					+	+		
CD19				+	+	+	+	+
CD25						+	+	
Heavy chain	GL	GL	DJ	DJ	DJ	VDJ	VDJ	VDJ
Light chain	GL	GL	GL	GL	GL	GL	VJ	VJ

Figure 1.3. Diagram of early B cell development

Adapted from (Hardy et al. 2001). From HSC, B cell develops through fraction A, B, C, C', D and E in bone marrow according to Hardy fraction system. Cell surface marker expression and BCR gene rearrangement is indicated for each B cell developmental stage. GL: Germ line.

Chapter2. Identification of Target cells in v-Abl Transformation

Backgrounds:

In *in vitro* v-Abl transformation of bone marrow, very low numbers of cells are induced to proliferate after A-MuLV infection. It has been reported that usually about 10 – 100 cells among the 10^6 bone marrow cells start a proliferative burst and make colonies in the v-Abl transformation assay (Rosenberg et al. 1976). This number means only 0.001%-0.01% cells are initiating transformation at the very beginning, which raise the question of why such a low percentage of the cells begin transformation. However, it is hard to decipher the real transformation frequency, until the actual infection rate of a true target population for v-Abl transformation is considered.

As described in introduction, A-MuLV was discovered as a mutant virus that has B cell tropism rather than T cell tropism of M-MuLV (Abelson et al. 1970b). Further studies confirmed that resulted Abelson transformed cell lines resembles pre-B cell phenotypes. Transformed cells have rearranged Ig heavy chain but rearrangement of light chain is blocked (Chen et al. 1994; Rosenberg. 1994; Alt et al. 1981). They show high rates of cell cycling with no further differentiation. Since Abelson transformation results in pre-B cell like cell lines, it has been largely assumed that target cells of the transformation could be also pre-B cells.

Whitlock and colleagues investigated v-Abl transformation with cloned non-transformed B lineage cells (Whitlock et al. 1983). In this study, cultured pre-B cell lines were able to undergo v-Abl transformation. Interestingly, they also found that earlier stage B cells, which is prior to the pre-B cells can produce pre-B cell like Abelson transformed cell lines. In other study, pristane treated mice developed Ig-producing plasmacytomas with A-MuLV infection (Potter et al. 1973). These studies clearly indicate that v-Abl transformation can target various stages of B lineage cells in certain conditions. However, it is still vague what major target cell population in bone marrow results in Abelson transformed pre-B cell line in common *in vitro* v-Abl transformation. And many of these experiments have a limitation in using cultured cell lines rather than fresh bone marrow cells. To the best of my knowledge, direct experiment to uncover the real target cell population has never been reported.

It has been more than a century that people postulated cancers arise from stem cells (Sell. 2004). This concept is very attractive, since cancer cells have characteristics of stem cells such as the ability to self-renew (Reya et al. 2001). Moreover, many cancer associated pathways such as Wnt, Shh, and Notch pathway are also important in self-renewal of normal stem cells, particularly in HSCs (Austin et al. 1997; Bhardwaj et al. 2001; Varnum-Finney et al. 2000). Recently, HSCs and bone marrow progenitors have been demonstrated as the origin cells of various leukemias (Kelly et al. 2002; Jamieson et al. 2004; George et al. 2001; Hope et al. 2004).

For the same reason, we also like to test whether the target cell populations in v-Abl transformation are HSCs or very early B lineage cells.

Rosenberg and Baltimore developed a soft agar assay to quantify initial transformation frequency (Rosenberg et al. 1976). In this assay, virus infected cells were plated with semisolid agar to check cytokine independent cell growth. Others began to use a 96-well plate culture assay (Chen et al. 2008). A certain number of A-MuLV infected cells are seeded in 96-well plates, and the number of wells with cell growth is monitored later. However, these methods are cumbersome, difficult, and subjective, while providing limited information. So, a better method to quantify initial transformation frequency is necessitated, and we have developed a new assay, improvising round-bottom 96-well plates with Fluorescence-activated cell sorting (FACS) technology.

In this study, we hope to test whether the very low initial transformation frequency of v-Abl is caused by low infection rate of virus and/or unique and rare target cell population. First, we developed a new colony quantification method, a round-bottom 96-well plate assay, to evaluate the accurate transformation frequency of v-Abl transformation. Then, v-Abl transformation initiating cell populations were investigated using this method. Applying FACS sorting and Magnetic-activated cell sorting (MACS) depletion technique, each stage of early B cell development in Hardy fraction system was analyzed.

Results:

Round-bottom 96 well plate assay as a better quantitative method for measuring initial transformation

To develop a better quantitative assay to measure initial transformation, we tried to sort virus infected cells into round-bottom 96 well plates (Figure 2.1.). Sorting only virus infected cells by FACS machine, can eliminate the problem of uninfected population in the sample. In the round-bottom plate, cells precipitate to the very bottom of the well. Consequently, this allows proliferating cells to make a colony-like cell mass just like the colony in the soft agar assay, but unlike the diffused cell culture in a flat-bottom plate (Figure 2.1., Figure 2.2.). And this cell colony from the round-bottom plate assay were very easy to be counted and measured by low magnification microscopy.

In order to test the experiments using single cell sorting into 96 well plate by FACS, we first tried to exam with already established highly proliferating Abelson cell lines. After plating a single cells into round-bottom 96 well plates, we confirmed that more than 80% of the wells in the plate showed cell proliferation and formation of a large cell colony after about 7-10 days (data not shown). In fact, single cell sorting into plates for culture has been widely used (Battye et al. 2000). Overall, this indicates that single cells sorted into round-bottom 96 well plate can grow into colonies which can be possibly used as a quantitative measures.

To validate the new assay, one v-Abl transformation sample was analyzed with three different transformation frequency assays in parallel (Table 2.1.). Mouse bone marrow cells were infected with v-Abl GFP retrovirus from the two-promoter vector that expresses v-Abl oncogene and green fluorescence protein (GFP) as a marker. Then, traditional soft agar assay, 96 well plate culture assay and round-bottom 96 well plate assay were performed with this sample. Since single cell sorting resulted in too low colony formation frequency, about 0 - 2 colonies per 96 well plate, 20 cells/well sorting was used for all subsequent experiments (data not shown). At 14 days after the plating, all three assays displayed cell colonies. Although their specific number is somewhat different, overall they were in similar ranges of transformation frequencies as a result. This confirms that new assay using round-bottom 96 well plate can reproduce the initial transformation frequency in Abelson transformation system as other assays.

Initial transformation frequency of v-Abl transformation among infected cells is very low.

Observed low frequency of transformation initiating cells might come from low infection rate of A-MuLV. Thus, we investigated the initial transformation frequency of v-Abl transformation among the virus infected cells with the newly developed round-bottom 96 well plate assay. Retrovirus infection rate of total bone marrow cells usually ranged from 2 – 15 %

including this experiment, depending on the batches of the virus and experiments (data not shown). This number is similar to the common MSCV retrovirus infection rate with mouse bone marrow cells (Kanbe et al. 2004). We observed that less than 1% of the successfully A-MuLV infected cells are initiating transformation process (Table 2.2.). This is still a small proportion of the infected cells, suggesting that oncogene v-Abl expression itself is not sufficient to drive the very first step of the transformation. And it raise an idea that there might be a specific target population among bone marrow cells susceptible to the v-Abl transformation.

Stem cells and early progenitor cells are not the major target cells of v-Abl transformation.

In order to test whether there is a unique target cell population in v-Abl transformation, we first hypothesize that stem cells might be the origin cells of Abelson transformation. Uncontrollable proliferation of the cancer is highly reminiscent of the self-renewal property of stem cells (Reya et al. 2001). Recently, increasing findings are corroborating the attractive concept that cancer arises from stem cells (Sell. 2004). In the same way, rare stem cells in bone marrow might be the key population for v-Abl transformation, resulting in the very low colony numbers in transformation frequency assays.

To test this idea, we used FACS techniques to fractionate bone marrow cells into various developmental stages of B cells, which were later assayed for initial transformation frequency (Figure 2.3.). First, wild-type bone marrow cells were sorted with respect to c-Kit and CD19 markers (Figure 2.4.) As depicted in Figure 1.3., c-Kit+ CD19- population represents very early B lineage cells including HSCs, multi-lineage progenitor cells and pre-pro B cells. Each sorted population was infected with v-Abl GFP retrovirus, then the round-bottom 96 well plate assay was performed among the infected cells. We observed c-Kit+ CD19- population did not show many colonies upon the plate assay (Figure 2.4.(D)). Instead, most of the colonies were originated from later B lineage cells, c-Kit+ CD19+ population, which includes pro B cells (Fraction B, Fraction C). Virus infection rate of all four population was acceptable number with c-Kit+ CD19+ population had highest infection rate (Figure 2.4.(C)). This result demonstrated that stem cells and early progenitor cells are not the major target population of v-Abl transformation. The interesting result that pro-B cells (c-Kit+ CD19+) showed majority of colony formation in the assay, made us to verify what specific fraction of B cell development is responsible for the initiation of v-Abl transformation upon A-MuLV infection.

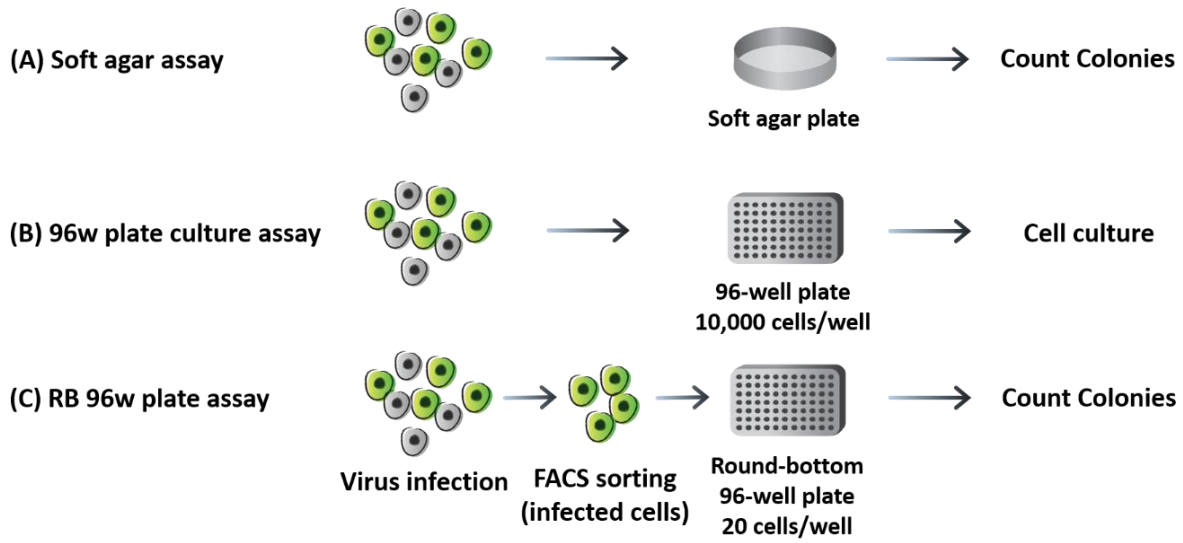
pro-B cells are the target cell population of initial v-Abl transformation.

To directly test which B cell stage is the target for v-Abl transformation, we sorted Bone marrow cells according to the classical Hardy fraction system of B cell development (Figure 2.5.) (Hardy et al. 2001). Then each fractionated population was subjected to v-Abl transformation and the round-bottom 96 well plate assay to test initial transformation frequency. Among Fraction A, B, C +C', and D, majority of the colony formation were observed in Fraction B

population (Figure 2.5.(D)). Less but some colonies were counted in Fraction C + C'. Thus, this result suggests that main target cells of v-Abl transformation are Fraction B rather than Fraction C + C', which is contrary to the widespread assumption that Abelson transformed pre-B cells are derived from infected pre-B cells.

The MSCV retrovirus, used as a A-MuLV, requires host cell division to complete its transduction (Yamashita et al. 2006). As you can see in Figure 2.5.C., fast cycling Fraction C + C' exhibited higher infection rate compared to Fraction B. However, Fraction B with less virus infection rate actually resulted in more transforming colonies among virus infected cells. So, this indicates that proliferation and virus infectivity are not the critical factor of v-Abl transformation initiation.

Regarding this Hardy fractionation experiments, we were concerned about handling too small number of sorted cells, particularly in Fraction C + C', during the virus infection and the plate assay. To avoid this problem and validate further, we decided to perform depletion experiments utilizing MACS technique (Figure 2.6.). This time, bone marrow cells were stained with antibodies against either IgM, CD19, CD25, or Bp-1 with phycoerythrin (PE) fluorochrome. Then, anti-PE magnetic beads were used as a secondary antibody for MACS depletion to get rid of marker positive populations (Figure 2.7.). Subsequently, each depleted samples were subjected to v-Abl GFP virus infection and initial transformation frequency assay. As in Figure 2.7.(C), depletion of CD19 + cells abrogated the colony formation in the assay, while depletion of other marker positive cells did not have any effects. According to the B cell development and its markers, this results confirms that Fraction B, rather than Fraction C and C', is important for v-Abl transformation initiation (Figure 1.3.; Hardy et al. 2001). Altogether, FACS sorting and MACS depletion experiments demonstrate that Fraction B cells in bone marrows are the target cell population for v-Abl transformation.



(D)

	Cell source	Assay plat form	Culture medium	Readout
Soft agar assay	All population	Plate	Semi solid agar	Cell colnies
96 well plate culture assay	All population	96 well plate	Liquid media	Cell culture density
Round-bottom 96 well plate assay	Only infected cells by FACS	96 well plate	Liquid media	Cell colnies

Figure 2.1. Comparison of three transformation frequency assays: Soft agar assay, 96 well plate culture assay, and round-bottom 96 well plate assay.

(A) Soft agar assay uses whole infected pools to plate on plate with semi-solid agar media. Cell proliferation is measured by counting colonies (Rosenberg et al. 1976).
(B) 96 well plate culture assay divides whole infected pools into 96 well plate as 10,000 cells/well. The readout of the assay is density of cell culture.
(C) Round-bottom 96 well plate assay utilize FACS sorting to plate only virus infected cells into round-bottom 96 well plate as 20 cells/well. Round-bottom allows cell colony formation, which can be easily counted and measured.

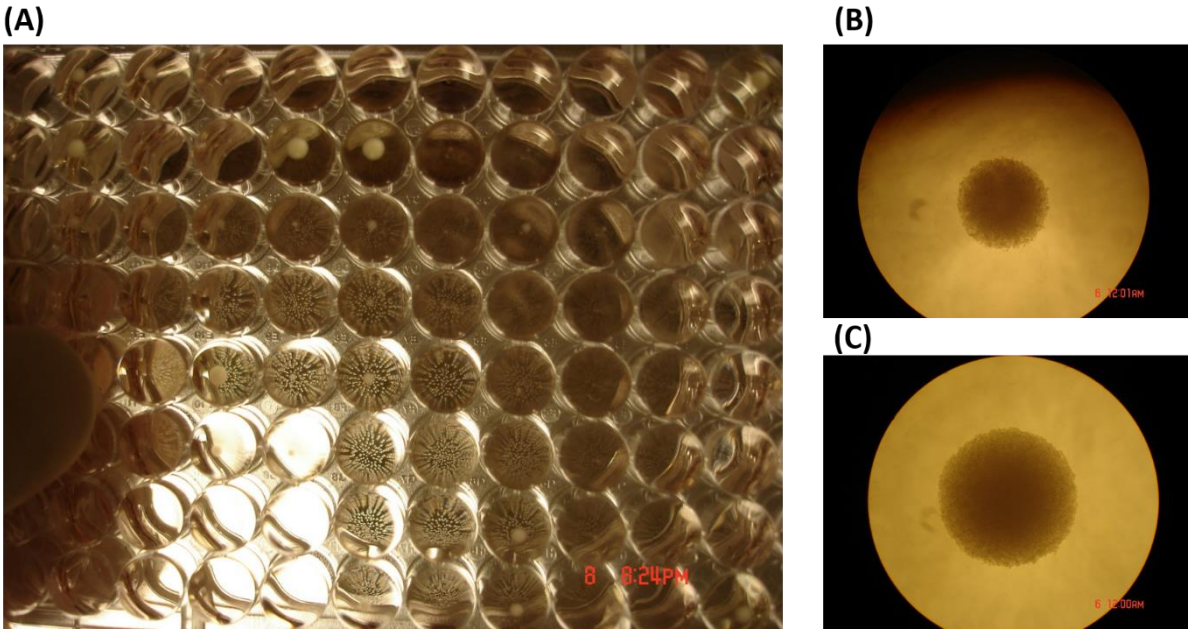


Figure 2.2. Representative picture of colonies from the round-bottom 96 well plate assay

(A) Picture taken from the bottom of the round-bottom plate after the assay
(B,C) Representative picture of a small size colony(B) and a large size colony(C), taken by 40X magnification microscopy.

	Soft Agar Assay	96 well plate culture	RB 96 well plate
Total cell number	1 million cells	1 million cells 10,000 cells/well	1 million cells 1% infection rate 20 cells/well
colony count	31	29 / 96 wells	12 / 96 wells
colony formation frequency	0.0031 (%)	0.0030 (%)	0.0063 (%)

Table 2.1. One representative data of the parallel experiment using three transformation frequency assays: Soft agar assay, 96 well plate culture assay, and round-bottom 96 well plate assay

	colony growing wells/ 96 wells	frequency of colony formation among infected cells
Experiment 1	**14 ±1**	**0.73 (%)**
Experiment 2	**14.75 ±2.75**	**0.77 (%)**
Experiment 3	**15.75 ±1.11**	**0.82 (%)**

Table 2.2. Initial v-Abl transformation frequency among virus infected cells

C57BL/6 wild-type mice total bone marrow cells were infected with vAbl GFP virus. Retrovirus infected, GFP marker positive cells were sorted into round-bottom 96 well plate as 20 cells/ well. 14 days after infection, macroscopic colonies were counted.

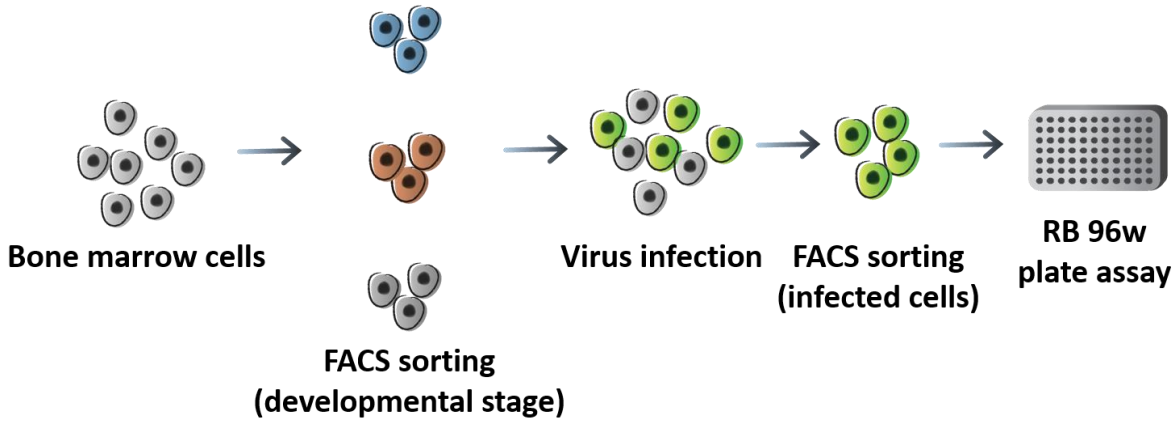
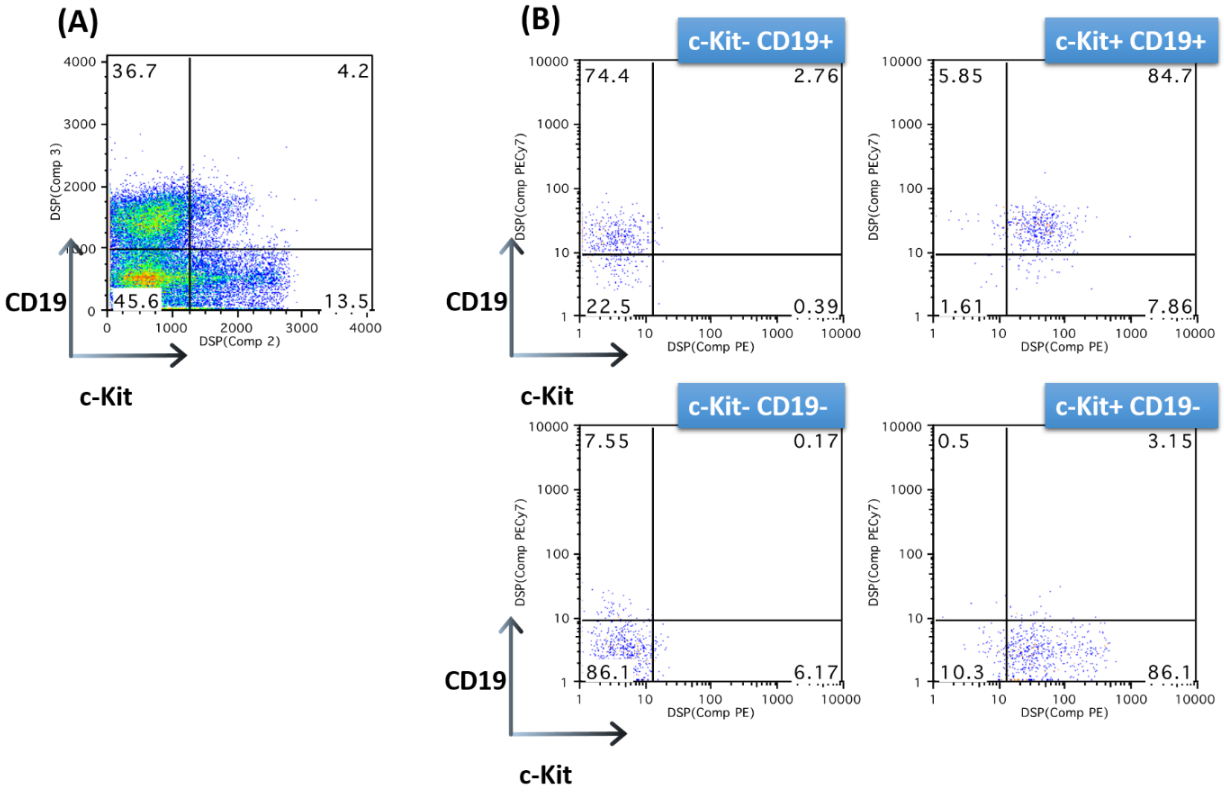


Figure 2.3. Scheme of initial transformation frequency assay with FACS sorted cells

Wild-type bone marrow cells were stained with antibodies against B-cell development surface markers, and FACS sorted into various developmental stages. Each sorted population was infected with v-Abl GFP retrovirus, then subjected to round-bottom 96 well plate assay.

Figure 2.4. c-Kit+ CD19+ cell population but not c-Kit+CD19- cells, were susceptible to v-Abl transformation (See next page).
(A) FACS plot of bone marrow cells stained with CD19 and c-Kit antibodies
(B) FACS plots after sorting of c-Kit- CD19+, c-Kit+ cD19+, c-Kit- CD19-, and c-Kit+CD19- populations
(C) v-Abl GFP virus infection rate of four population and ungated control population (All sorted)
(D) Results of Initial transformation frequency assay
Four populations and the control population were infected with v-Abl GFP virus, then subjected to the round-bottom 96 well plate assay. 20 infected cells/well sorted.
This data is the representative of three experiments



(C)

	Virus infection rate
cKit+ CD19+	14.7 (%)
cKit+ CD19-	4.97
cKit- CD19+	4.96
cKit- CD19-	6.66
All sorted	5.34

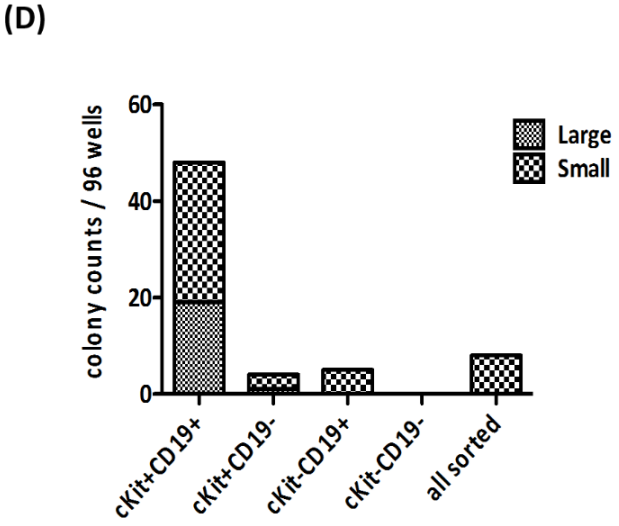
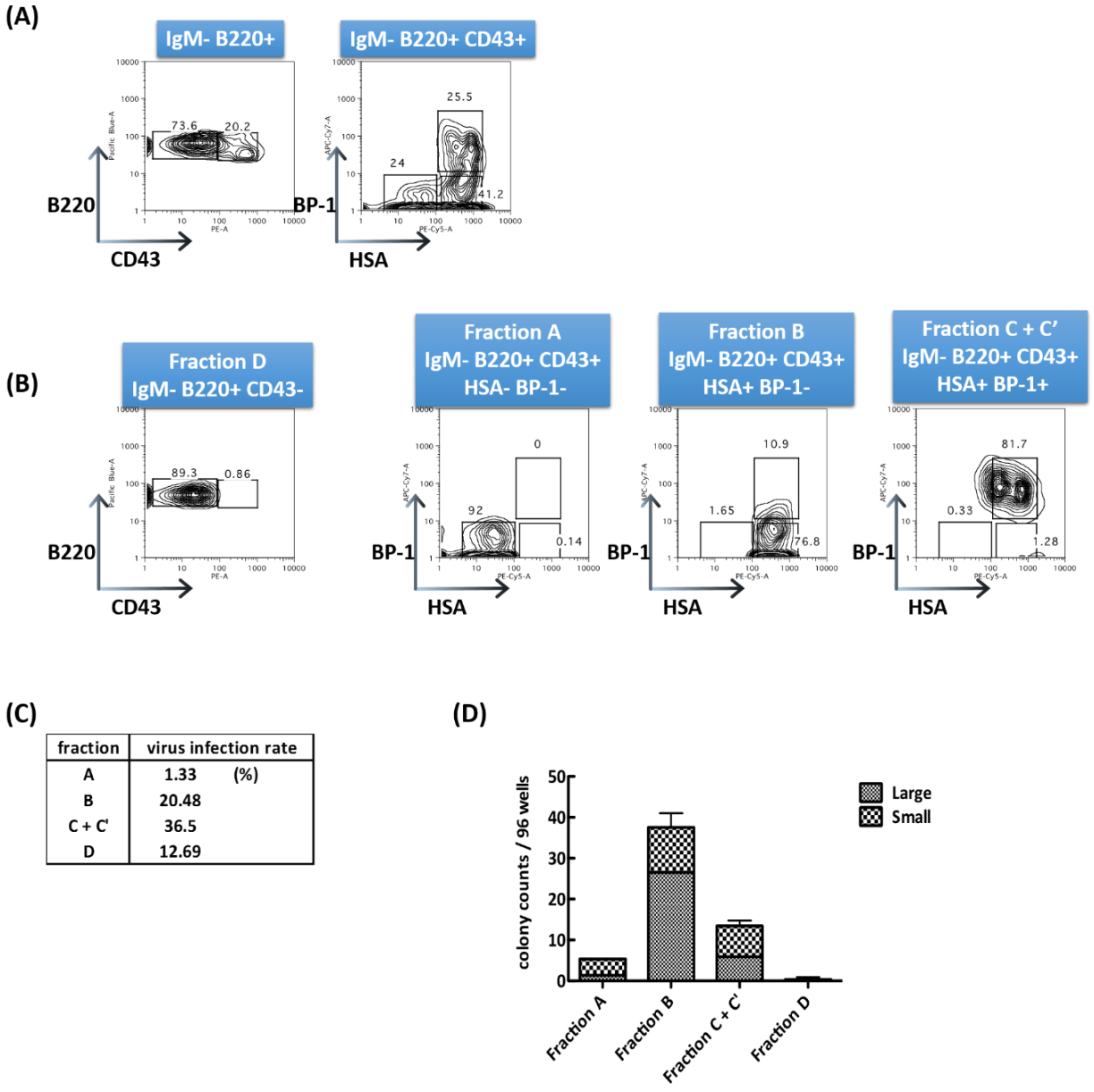


Figure 2.4. (See before page)



fraction	virus infection rate
A	1.33 (%)
B	20.48
C + C'	36.5
D	12.69

Figure 2.5. Fraction B cells were the major target population in v-Abl transformation.

(A) FACS plots of bone marrow cells stained with IgM, B220, CD43, BP-1 and HSA antibodies
(B) FACS plots after Hardy fractionation: Fraction D, A, B, and C + C' (Hardy et al. 2001)
(C) v-Abl GFP virus infection rate of each population
(D) Results of initial transformation frequency assay
Each sorted population was infected with v-Abl GFP, then subjected to the round-bottom 96 well plate assay. 20 infected cells/plate sorted. This data is the representative of three experiments

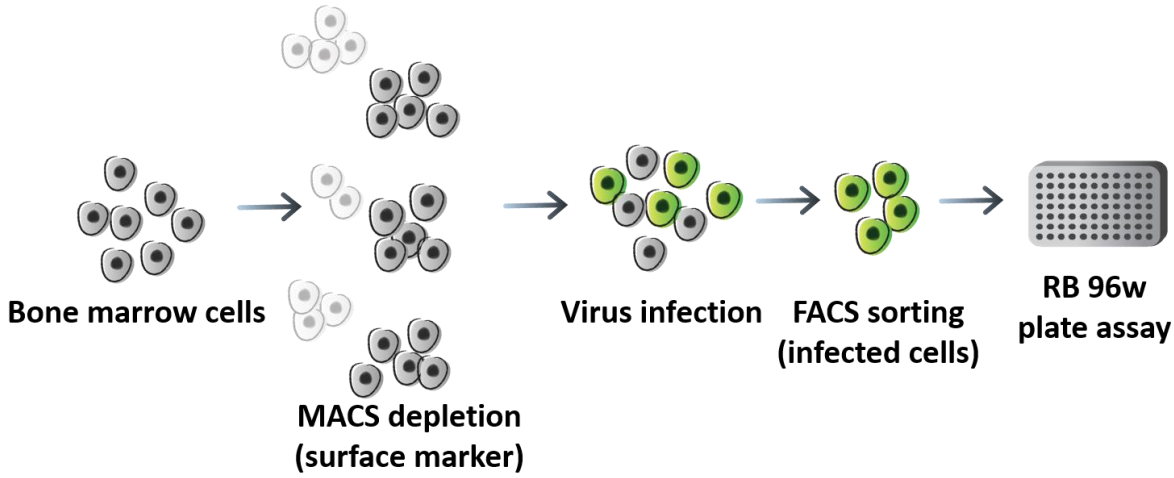
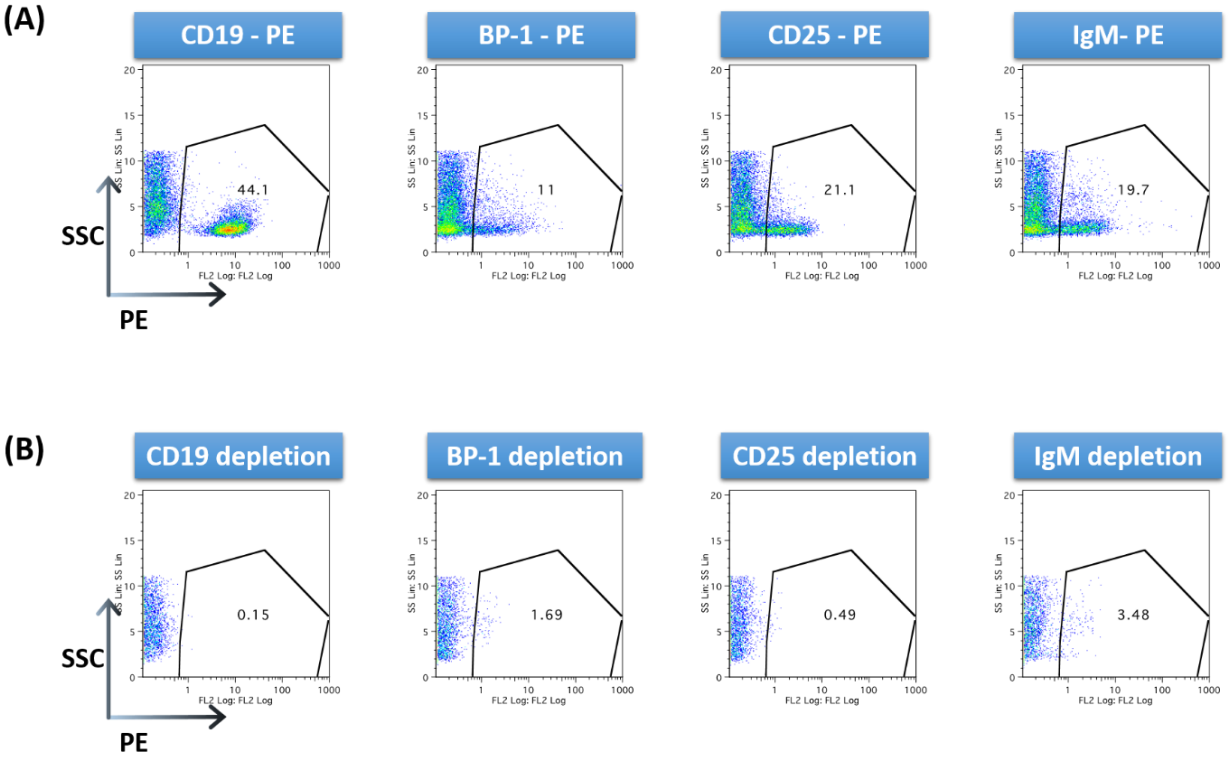


Figure 2.6. Scheme of initial transformation frequency assay with MACS depleted cells

Wild-type bone marrow cells were stained with antibodies against CD19, BP-1, CD25, and IgM surface markers, then subjected to MACS depletion. Each depleted population was infected with v-Abl GFP retrovirus, then subjected to the round-bottom 96 well plate assay.

Figure 2.7. v-Abl transformation was restricted by depletion of CD19 positive cells in bone marrow cells (See next page).

(A) FACS plots of Bone marrow cells stained with CD19, BP-1, CD25, or IgM antibodies conjugated with PE.
(B) Post sort FACS plots of MACS depleted populations
Surface marker antibody stained samples were MACS depleted using anti-PE antibody conjugated with magnetic beads.
(C) Results of initial transformation frequency assay
Each depleted population and undepleted control cells were infected with v-Abl GFP virus, then subjected to the round-bottom 96 well plate assay. 20 infected cells / plate sorted. This data is the representative of two experiments



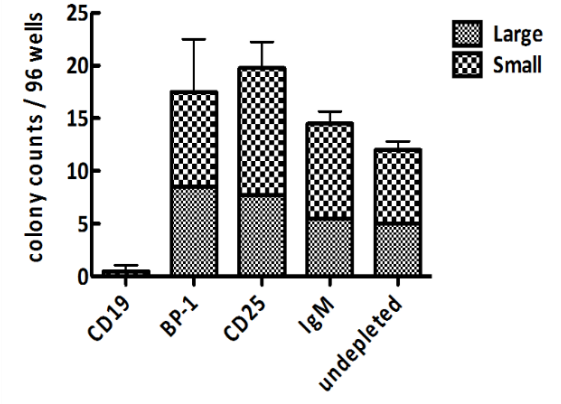


Figure 2.7. (See before page)

Discussion:

In v-Abl transformation, cells initiating tumorigenesis and undergoing proliferative burst appear to be a very low fraction of bone marrows. In order to figure out accurate initial transformation frequency, infection rate of the true target cell population in v-Abl transformation has been investigated. First, we have developed a new quantitative method using round-bottom 96 well plates and FACS techniques. With this method, we have confirmed a still very low frequency of cells starting transformation, among the virus infected cells. Then we have tested the idea that this low frequency might be come from unique target cell population of v-Abl transformation in bone marrows. In spite of the popular idea that stem cells are the origin of cancer cells, HSCs and progenitor cells were not the major tumor initiating cells in v-Abl transformation. Hardy fractionation experiments directly proved that pro-B cells are the main population susceptible to v-Abl transformation. This is contrary to the common assumption that v-Abl transformed cell lines displaying early pre-B cell phenotypes might be derived from early pre-B cells.

Soft agar assay has been used long for measuring anchorage-independent cell proliferation of adherent cell transformation (Shin et al. 1975). In non-adherent hematopoietic cells, this assay was used to assess cytokine-independent cell growth (Rosenberg et al. 1976). Since manipulated hot agars might harm cells in unexpected way, we found that this assay is somewhat cumbersome, and frequently resulting in inconsistent results. Some researchers tried plating cells into 96 well plate in liquid culture (Chen et al. 2008). Then, the readout is the density of cell culture, which could be laborious to measure many wells manually or require separate cell proliferation assay which usually ends up killing the cells. In addition, both methods does not account for the infection rate of virus, which could be even variable from sample to sample even in the same batch. After go through these two methods, we have developed new assay using round-bottom plate 96 well plates. Using the liquid media culture eliminates cumbersome agar experiments, and cells are easy to be recovered from the assay for further analysis. Since round-bottom allows colony-like cell mass formation, the readout is simple and straightforward colony counting. These colonies are even easier to be measured, because they are all located in the designated position, and at the same focal plane. With the FACS sorting, the assay could avoid the problem from uninfected population, focusing only on the virus infected cells. Here, we have successfully developed and applied new round-bottom 96 well plate assay for accurate evaluation of initial transformation frequency. Plus, it will be intriguing to try this method in more high-throughput screening format with using 384 or 1536 well plates and with appropriate imaging technologies.

However, there are some shortcomings in this new assay. First, this round-bottom plate inevitably increases cell-to-cell contacts in crowded cell colonies. Interaction with mesenchymal stromal cells plays a crucial role in hematopoiesis and leukemic transformation (Iwamoto et al. 2007). We do not know how these cell-to-cell interaction will affect the transformation process. But single cell sorting of A-MuLV infected cells into 96 well plates generates cell growth in

similar low frequency (data not shown), suggesting that interactions with stromal cells or between cells are not necessary in v-Abl transformation. In subsequent experiments, we tried to use only CD19 + cells by MACS enrichment, to avoid this possible cell-to-cell interaction problems, particularly with stromal cell. Secondly, to do FACS sorting, virus infected bone marrow cells were cultured *in vitro* without cytokine for 36 hours until GFP markers of virus could be fully expressed and detected. It is hard to understand how this artificial condition will affect the transformation. Overall, there are limitations in *in vitro* transformation system, and it will be interesting to compare *in vivo* v-Abl transformation with our findings. Specifically, we would like to test v-abl transformation of Knock-out mice, whose B cell development is blocked at the certain stage.

Surprisingly, pro-B cells turned out to be the major target cells of v-Abl transformation. Unlike the common postulation, HSCs or proliferating pre-B cells were not the origin cells of the Abelson transformed cell lines. Many researchers pointed out that the self-renewal characteristics of stem cells would contribute to the malignant cellular growth of cancer (Reya et al. 2001). In many leukemia studies, HSCs and progenitor cells have been recognized as their origin cells (Kelly et al. 2002; Jamieson et al. 2004; George et al. 2001; Hope et al. 2004). CML patients were frequently found with myeloid and erythroid cells with BCR-Abl translocation, a variant of v-Abl, which initially originated in HSCs (Passegué et al. 2003). Thus, it is quite interesting how pro-B cells turned out to be the target cells of v-Abl transformation. We do not have any answer for this question, but one possible explanation could be recombination activating gene (RAG) activity in pro-B cells. RAG is the main machinery responsible for DNA cleavage of V(D)J rearrangements (Helmink et al. 2012). Its expression is tightly regulated during the B cell development, for instance high in pro-B cells but not in early pre-B cells (Schlissel. 2003). However, RAG activity still generates double strand breaks (DSB) around the genome, and if repaired improperly, this DSB can lead to the tumorigenesis (Helmink et al. 2012). Particularly some chromosomal translocations in B lymphomas arise from aberrant RAG activities (Küppers et al. 2001; Nussenzweig et al. 2010). Therefore, RAG expression in pro-B cell but not in early pre-B cells could provide the driving force of v-Abl transformation with generating lots of mutations in the population. Interestingly, Activation-induced Cytidine Deaminase (AID), responsible for hypermutation of Ig genes, was found to be involved in tumorigenesis of v-Abl transformation and BCR-abl ALL in a similar manner (Gourzi et al. 2006; Gruber et al. 2010).

Our findings essentially open up many questions. How pro-B cells infected with v-Abl virus changes into pre-B cell phenotype during the transformation? Does v-Abl transformation of pro-B cells and pre-B cells end up in transformed cell lines with different phenotypes? Do v-Abl transforming cells originated from pro-B cells have advantage over other transforming cells not from pro-B cells throughout the transformation process? While it is almost impossible to answer these questions now, but we want to emphasize that v-Abl transformation is very dynamic, complex and progressive process just as other cancer. Many studies have found v-Abl transformed weak clones with low tumorigenic potential progress to the malignant tumorigenic clones by clonal selection (Green et al. 1989; Whitlock et al. 1983). This clearly demonstrates

that v-Abl transforming cells keep changing and progressing throughout the whole transformation process. Thus, transformation of target cells might enable and facilitate initiating the first step of tumorigenesis, but the additional cellular events will be also important to complete the v-Abl transformation. For this reason, we have focused on the cellular factors involved in v-Abl transformation for later studies.

Chapter3. The Role of p53 in early v-Abl Transformation

Backgrounds:

In addition to the v-Abl oncogene, cellular factors and events are also important in v-Abl transformation. As described in the introduction, v-Abl transformation exhibits a multi-step process, a common characteristic of cancer. Particularly, Abelson transformation starts with proliferative burst stage and later goes through apoptotic crisis stage (Green et al. 1989; Radfar et al. 1998; Unnikrishnan et al. 1999). Furthermore, the process is under strong selection pressure, so transforming a population of cells results in mono-clonal or oligo-clonal cell lines (Green et al, 1987). However, oncogene v-Abl expression level and kinase activity remain constant during the transformation process (Whitlock et al. 1983; Zimmerman et al, 2008). So, this suggest that, in addition to v-Abl, cellular factors are playing an important role in this v-Abl tumorigenesis. And there have been many efforts to understand these cellular factors responsible for v-Abl transformation.

Not surprisingly, many researchers studying v-Abl transformation have focused on the p53 tumor suppressor and its pathway. More than 50% of the Abelson cell lines obtained mutation or deletion in p53 sequence (Thome et al. 1997). Also, p53 Knock-out mice were more susceptible to A-MuLV induced leukemia compared to wild-type mice (Unnikrishnan et al. 1999). Furthermore, p53 and p19ARF, an upstream activator of p53, null bone marrow cells circumvented apoptotic crisis stage of v-Abl transformation (Zou et al. 2000; Unnikrishnan et al. 1999; Radfar et al. 1998). Recently, p19 ARF was demonstrated to increase its expression level and change localization to the nucleolus during the transformation process (Zimmerman et al. 2008). Altogether, p53 and its pathway are the important cellular factors that are regulated and modulated in Abelson transformation.

Although loss of p53 enhances v-Abl transformation, two studies showed somewhat different results with p53 null mice experiments (Zou et al. 2000; Unnikrishnan et al. 1999). Although both studies agreed on absence of p53 bypassed apoptotic crisis of v-Abl transformation, they reported differently about initial transformation frequency. Zou et al. observed more early transformed colonies in p53 null bone marrow cells infected with A-MuLV, while Unnikrishnan et al. concluded there were no significant difference in transformation frequency. This discrepancy might come from the intrinsic limitation of the difficult soft agar assay method. Since the new round-bottom 96 well plate assay has been developed and evaluated, effects of p53 on initial v-Abl transformation frequency were carefully re-examined.

Abelson transformed pre-B cells are very useful *in vitro* model system to study B cell biology. Transformed cell lines are arrested at pre-B cell like stage (Chen et al. 1994; Rosenberg. 1994; Alt et al. 1981). However, treatment of STI571, the Abl kinase inhibitor, can lead to the

further differentiation of the cells (Muljo et al. 2003). For this reason, Abelson cell line has been widely used for studying early B cell development and Ig gene rearrangement (Amin et al. 2008). Besides, Abelson transformation with various knock-out mice provided invaluable Knock-out pre-B cell lines. However, we frequently observed difficulties in generating Abelson transformed cell lines, because of the severe apoptosis and clonal selection. After about a month of transformation process, the transforming cells suddenly ended up with no survived cells. This low establishment rate of v-Abl transformed cell line has been previously reported, too (Unnikrishnan et al. 1999). Therefore, it would be useful to develop a better v-Abl transformation method, facilitating the whole tumorigenesis process.

RNA interference (RNAi) technology makes use of double-stranded RNAs that are processed by the cellular machinery to down-regulate the expression of a gene with the homologous sequence (Elbashir et al. 2001). In contrast to chemically synthesized short interfering RNA (siRNA), short hairpin RNA (shRNA) can be incorporated into expression vectors to mediate persistent gene silencing (Paddison et al. 2002). To knock-down p53, we took advantage of one well-studied p53 shRNA (Figure3.1.)(p53.1224 from Dickins et al. 2005). This p53 shRNA is embedded on endogenous mir30 shRNA context with polymerase II promoter, to ensure the strong p53 knock-down on target cells (Stegmeier et al. 2005). This p53 shRNA construct was used to test whether p53 knock-down can facilitate v-Abl transformation process.

Cellular factors are implicated in multi-step tumorigenesis. Particularly, p53 has been widely studied in v-Abl transformation process. Since we have observed still low transformation frequency among the virus infected target cell population, cellular factors such as p53 might be involved in early transformation process as well as later apoptotic stage. Here, we made a hypothesis that p53 knock-down can facilitate v-Abl transformation process even in the early transformations stage. To test this idea, new retrovirus with v-Abl and p53 shRNA construct was generated and tested for v-Abl transformation using initial transformation frequency assay and cell competition assay.

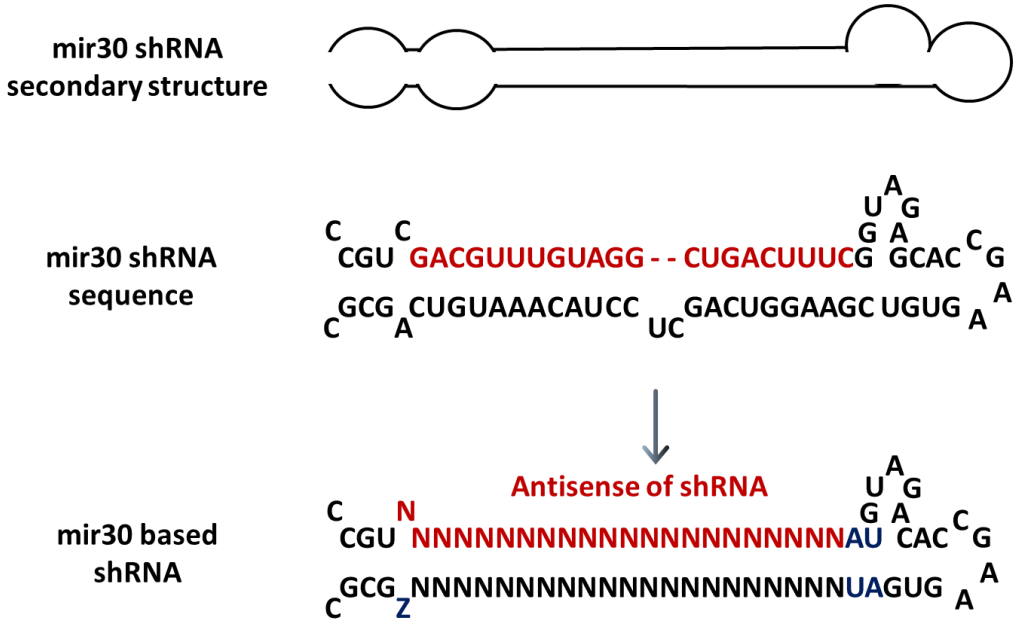


Figure 3.1. Diagram of mir30 based shRNA

mir30 based shRNA mimics endogenous miRNA, mir30, to ensure effective knock-down of target genes. All the shRNAs used in this study, including individual shRNAs such as p53, Dpf2 and Luc shRNAs, and library shRNAs are based on this shRNA.
Adapted from (Stegmeier et al. 2005).

Results:

p53 knock-out enhances initial transformation frequency.

Two studies resulted in different conclusion whether p53 null bone marrow can enhance initial transformation frequency of v-Abl transformation (Zou et al. 2000; Unnikrishnan et al. 1999). In order to clarify this discrepancy, we performed the newly developed round-bottom 96 well plate assay with p53 knock-out mice. Bone marrows from p53 +/+, +/-, and -/- littermates were harvested and, MACS enriched CD19+ cells were infected with v-Abl GFP retrovirus. After the plate assay, we could confirm that p53 null bone marrow transformation exhibited more than 5 fold increased transformation frequency compared to wild-type control (Figure 3.2.(A)). p53 +/- resulted in intermediate levels of colony formation. In addition, p53 null samples contained higher proportion of large sized colonies to small ones. Thus, this experiment recapitulated the result of Zou et al., p53 also plays an important role in early v-Abl transformation (Zou et al. 2000).

It is noteworthy, that the maximum readout of the 96 well plate assay is 96. Since, p53 -/- sample showed high numbers, many of the wells would have double or triple hits among the 20 cells sorted into one well. To assess the correct frequency, separate experiments using single cell sorting rather than 20 cells/well, were performed, which resulted about 10 % of the infected cells were forming colonies from p53 Knock-out mice (Figure 3.2.(B)). This is very high percentage, although it is not 100%, which suggests there might be still other limiting factors existing except the p53.

p53 shRNA successfully down-regulates p53 in mRNA and protein levels.

To test the p53 knock-down on v-Abl transformation, we have utilized the strong p53 shRNA, widely used in other studies (p53.1224 from Dickins et al. 2005). This mir30 based p53 shRNA is incorporated into MSCV v-Abl GFP vector, generating v-Abl GFP p53 shRNA vector (Figure 3.3.(A)). In this vector p53 shRNA is located 3' to the GFP cDNA driven by the PGK promoter.

In order to validate p53 knock-down ability of this shRNA, wild-type mouse bone marrow cells were infected with v-Abl GFP p53 shRNA and control Luc shRNA vector (an irrelevant shRNA designed to target the luciferase transcript not expressed in these cells). After 2 weeks, cells were harvested and p53 mRNA expression levels were measured by qPCR, and p53 protein levels by Western blot with p53 antibody (Figure 3.3.(B) and (C)). It is clearly demonstrated that p53 shRNA down-regulated p53 mRNA expression and protein levels about 4 folds compared to control shRNA.

p53 shRNA knock-down enhances early v-Abl transformation.

To test whether p53 knock-down can facilitate early v-Abl transformation, we performed round-bottom 96 well plate assay with v-Abl p53 shRNA and control Luc shRNA vector. Just as p53 knock-out cells showed more colonies compared to wild-type mice, p53 knock-down also increased colony formation in the assay (Figure 3.4.). However, knock-down exhibited 3 fold enhancement, which is not as much as the p53 knock-out mice experiment. This is probably because of the residual p53 levels remaining in the p53 knock-down samples (Figure 3.3.).

Interestingly, the same experiment was performed with Balb/c wild-type mice, and resulted in the same outcomes: more initial transformation colonies upon p53 knock-down (data not shown). Unnikrishnan et al. argued that their discrepancy in initial v-Abl transformation frequency might come from the different p53 knock-out mice strain, since they used Balb/c backgrounds while Zou et al. used 129/SV × C57BL/6 mixed backgrounds (Unnikrishnan et al. 1999, Zou et al. 2000). But our results clear demonstrated that p53 affects early v-Abl transformation in both mice backgrounds.

To further confirm p53 shRNA effects on v-Abl transformation, we designed mixed virus competition experiments in v-Abl transformation culture (Figure 3.5.). We have prepared a vAbl puro virus that has no fluorescence marker (Figure 3.5.(A)). Titrated v-Abl puro virus and v-Abl GFP p53 shRNA or control v-Abl Luc shRNA virus were mixed as 100 to 1 ratio. Next, this mixed virus was used to infect wild-type bone marrows to initiate v-Abl transformation. Then, GFP virus infected population in the transforming culture was monitored by FACS. If GFP virus infected cells have advantage over puro virus infected ones, GFP+ population should increase over time. The result demonstrated that p53 shRNA virus outcompeted puro virus and became majority of the transforming population, suggesting p53 shRNA facilitates v-Abl transformation including the early time period (Figure 3.6.). However, the control Luc shRNA virus did not enrich but disappear in the mixed population over time even in the higher 10 to 1 ratio experiments.

p53 knock-down enhances v-Abl transformation by protection from apoptosis and cell cycle arrest.

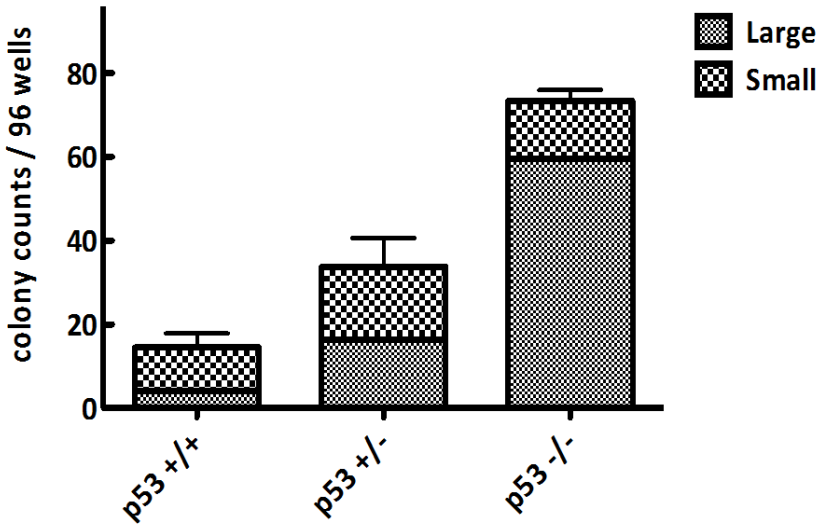
Since, we have confirmed p53 knock-down enhances early v-Abl transformation, we tried to investigate how p53 knock-down facilitates v-Abl transformation. First, we evaluated cell proliferation of transformation culture with v-Abl GFP p53 shRNA or v-Abl GFP Luc shRNA. As expected, total cell numbers of v-Abl GFP p53 shRNA infected cells were higher compared to control infected culture throughout the transformation process (Figure 3.7.). It is also noticeable, that the p53 knock-down culture expanded consistently during the whole time. However, the control culture slowed down proliferation particularly around two to three weeks after virus infection, the time point when the high level of apoptosis begins (see below and

Figure 3.8.). This is well explained by previous findings that apoptotic crisis of v-Abl transformation is dependent on p53 (Zou et al. 2000; Unnikrishnan et al. 1999).

In the p53 pathway, p53 activation induces many downstream responses including apoptosis and cell cycle arrest (Levine et al. 2009) (Figure 1.2.). Considering high cell death and apoptosis levels observed in the transforming culture, we have measured apoptosis levels during v-Abl transformation process by Annexin V staining (Figure 3.8.). Transforming cells infected by v-Abl GFP p53 displayed lower apoptosis levels compared to control cells throughout the whole process. Largely, apoptosis levels in the control transformation increased with time, then maintained high apoptosis, nicely representing the major two steps of v-Abl transformation, the proliferation blast and the apoptotic crisis stage (Green et al. 1989). However, p53 knock-down transforming cells dramatically recovered from the peak of apoptosis, again demonstrating a major role of p53 in apoptotic crisis.

To test whether cell cycle arrest is affected by p53 knock down, we have performed cell cycle analysis by Propidium Iodide (PI) staining. With analysis of transforming cells 14 days after virus infection, p53 knock-down cells resulted slight, but statistically meaningful decrease in G1 phase cells (Figure 3.9.) To confirm further, we have checked p21 mRNA expression in these samples. p21 is cyclin-dependent kinase inhibitor and one of the major downstream targets of p53, which induces G1 cell cycle arrest (Harper et al. 1993). One study showed that p21 is mediating v-Abl induced cell cycle arrest in mouse embryonic fibroblasts (Cong et al. 1999). In p53 knock-down samples, p21 expression was reduced about 4 fold compared to control samples (Figure 3.10.). And p21 mRNA levels of these samples were particularly high compared to established Abelson cell lines, suggesting strong induction during transformation. So, p53 knock-down decreases cell cycle arrest mediated by p21 in early v-Abl transformation. Altogether, v-Abl p53 shRNA vector enhances early v-Abl transformation, by reducing apoptosis levels, and alleviating cell cycle arrest.

(A)



(B)

single cell sorting experiment		
	colonies / 96 wells	frequency
p53 +/+	1.3 ± 0.58	1.4 (%)
p53 -/-	9.7 ± 3.51	10.1 (%)

Figure 3.2. p53 knock-out cells increased initial transformation frequency in v-Abl transformation.

(A) CD19+ cells were enriched by MACS sorting with bone marrows from p53 +/+, +/-, and -/- littermate mice. Then these cells were infected with v-Abl GFP retrovirus and subjected to the round-bottom 96well plate assay to measure initial transformation frequency. This data is the representative of three experiments.
(B) Same experiment was performed with single cell sorting rather than 20 cells/well sorting.

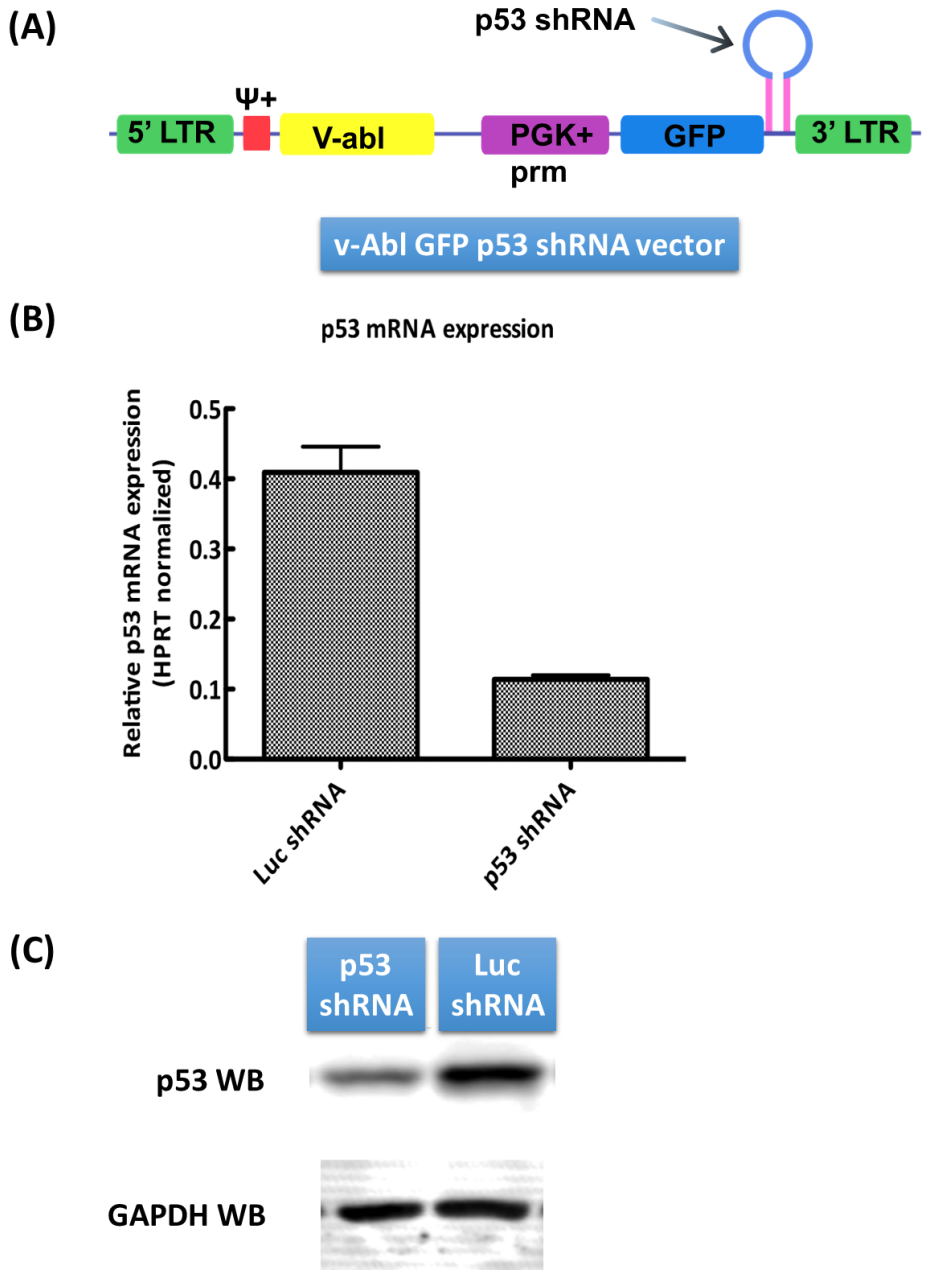


Figure 3.3. p53 shRNA knock-down down-regulated p53 mRNA expression levels and protein levels in the transforming cells.

Bone marrow cells were infected with v-Abl GFP p53 shRNA or v-Abl GFP Luc shRNA virus. Two weeks after virus infection, total RNA and protein were isolated from transforming cells. This data is the representative of three experiments.
(A) Diagram of v-Abl GFP p53 shRNA vector
(B) p53 mRNA expression levels were measured by qPCR with p53 qPCR primers
(C) p53 protein levels were measured by Western blot with anti-p53 antibody

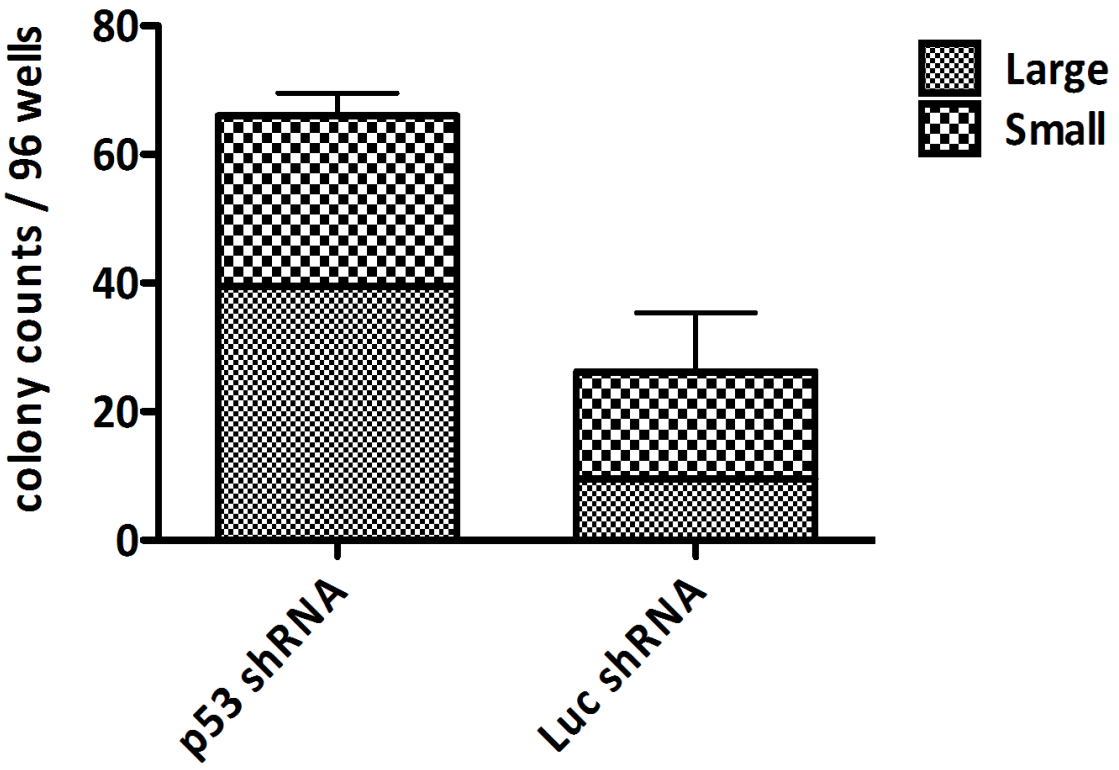


Figure 3.4. p53 shRNA knock-down increased initial transformation frequency in v-Abl transformation.

MACS sorted CD19+ cells from wild-type bone marrow cells were harvested and infected with v-Abl GFP p53 shRNA or control Luc shRNA retrovirus. Then, infected cells were subjected to the round-bottom 96 well plate assay to measure initial transformation frequency. This data is the representative of three experiments.

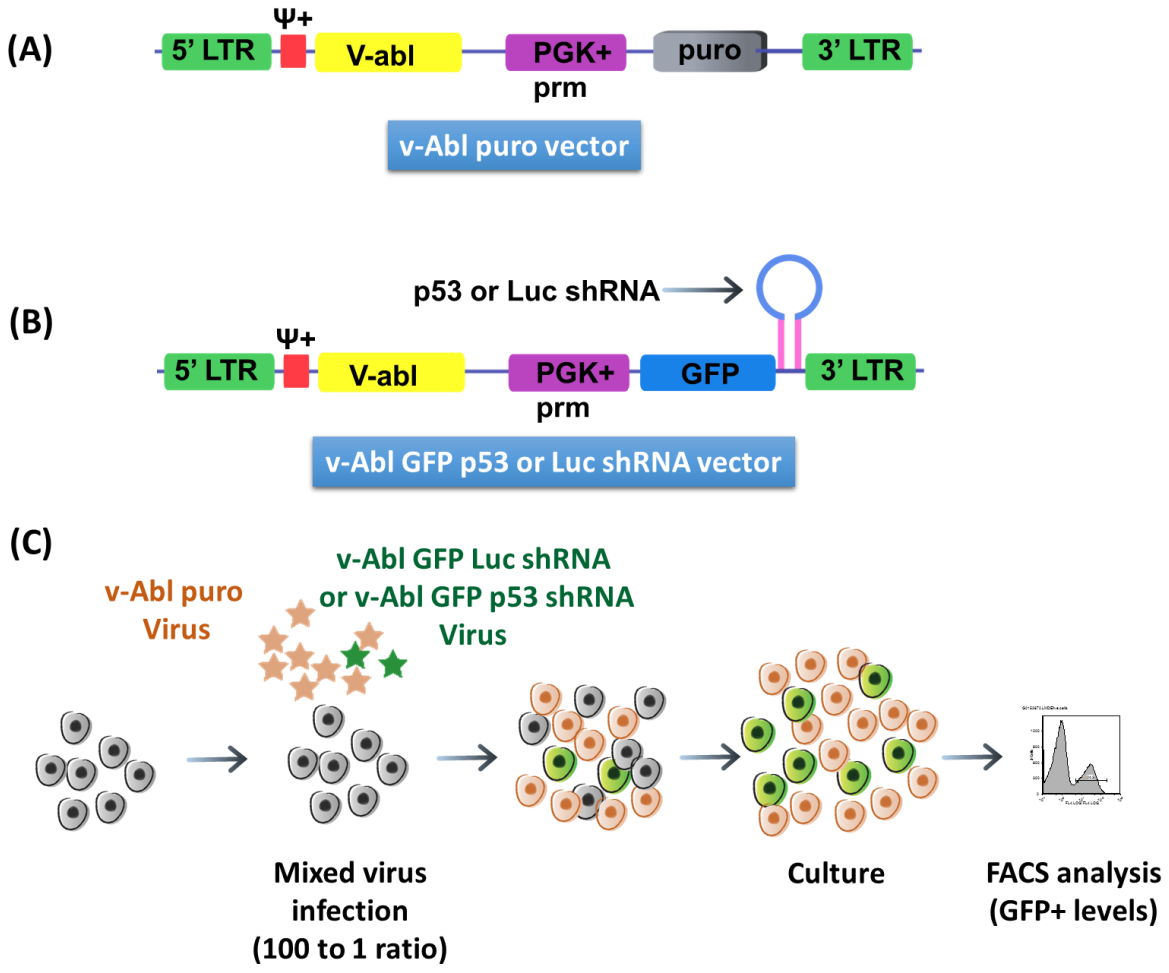


Figure 3.5. Scheme of the mixed virus competition experiment in v-Abl transformation

(A) Diagram of v-Abl puro virus
(B) Diagram of v-Abl GFP p53 shRNA and v-Abl EGFP Luc shRNA virus
(C) Diagram of the mixed virus competition experiment
v-Abl puro virus and v-Abl GFP p53 shRNA or Luc shRNA virus were mixed in 100 to 1 ratio. Wild-type bone marrow cells were infected with these mixed virus to start v-Abl transformation. v-Abl GFP virus infected cells among the transformation population were monitored by FACS throughout the transformation process.

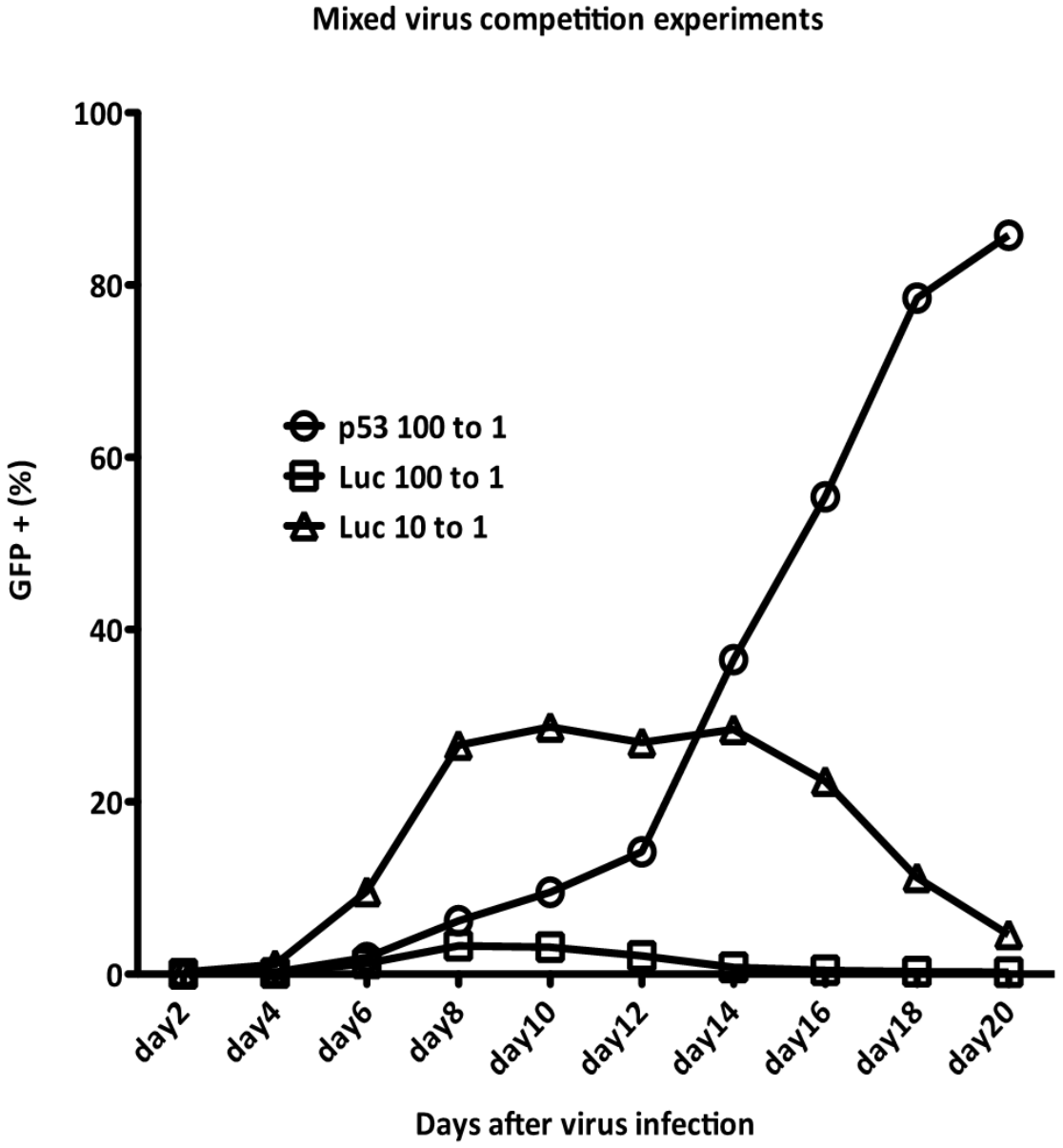


Figure 3.6. v-Abl p53 shRNA virus infected cells outgrew control virus infected cells in v-Abl transformation.

This experiment is explained in Figure 3.5. Cells infected with v-Abl puro virus and v-Abl GFP Lu c shRNA virus mixed in 10 to 1 ratio samples were added as an additional control. This data is the representative of three experiments.

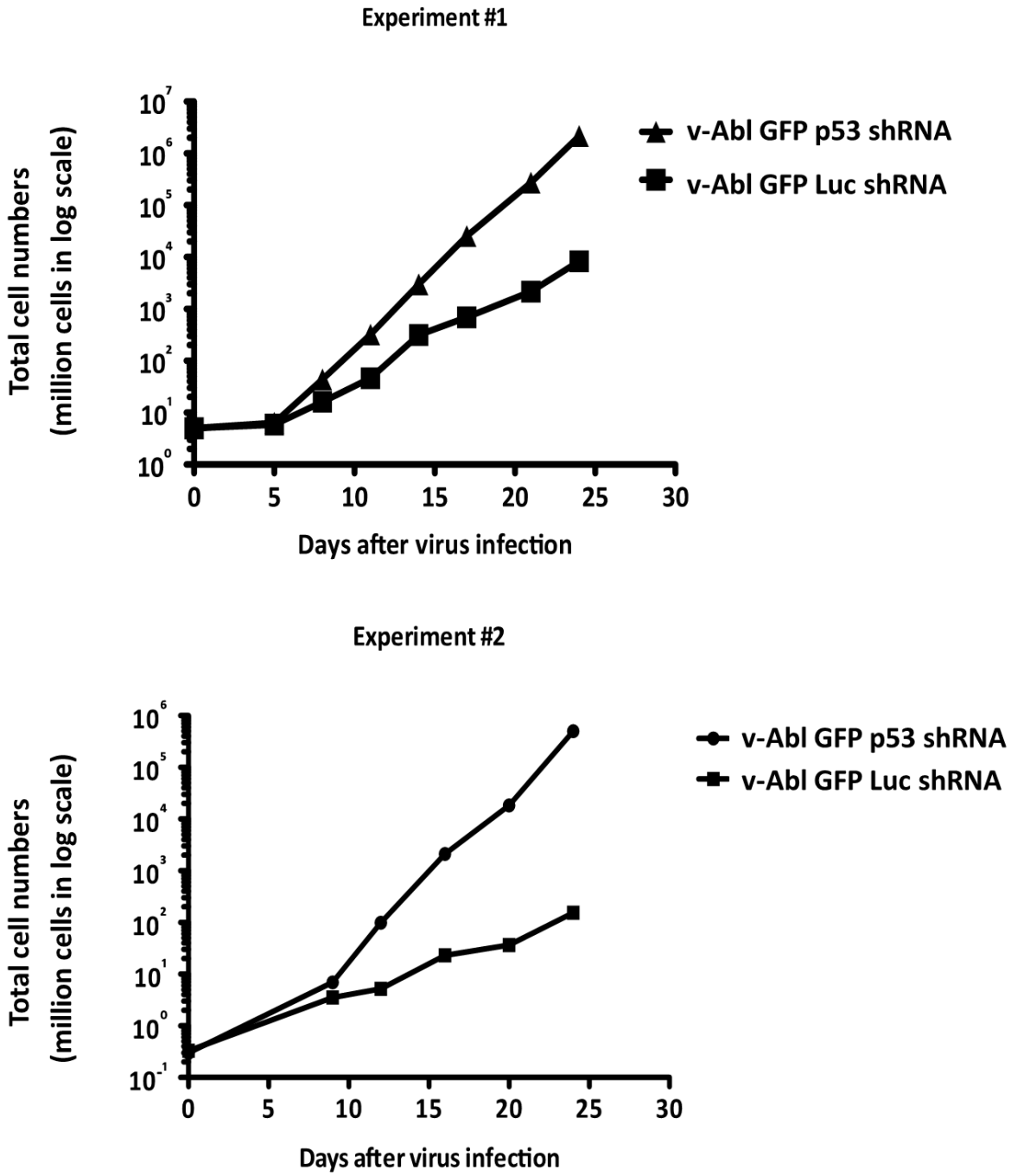


Figure 3.7. p53 knock-down showed enhanced proliferation in both early and late v-Abl transformation.

Wild-type bone marrow cells were infected with v-Abl GFP p53 shRNA or v-Abl GFP Luc shRNA. Total cell number was counted every 3 to 4 days during the transformation. Two independent experiments were shown here.

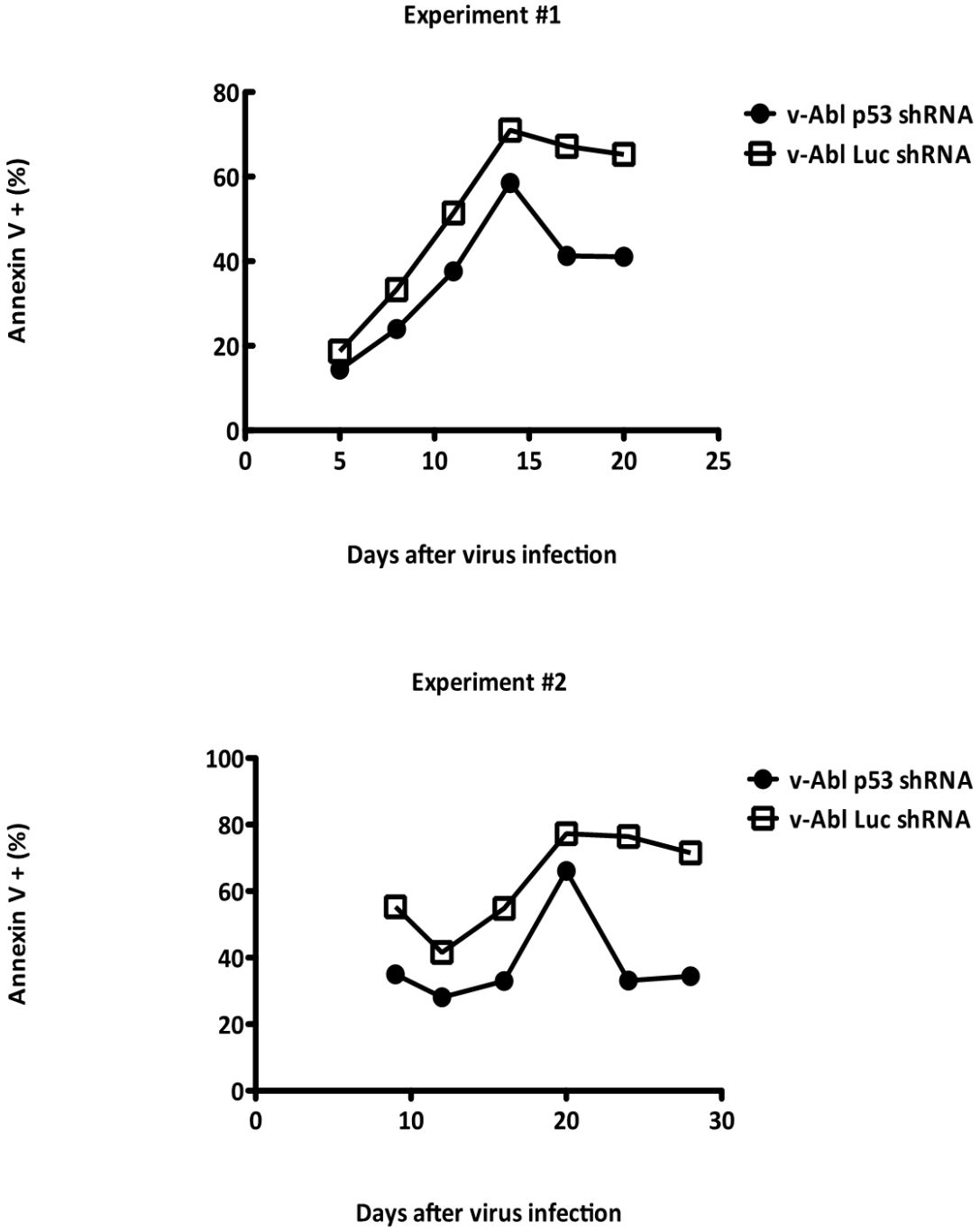
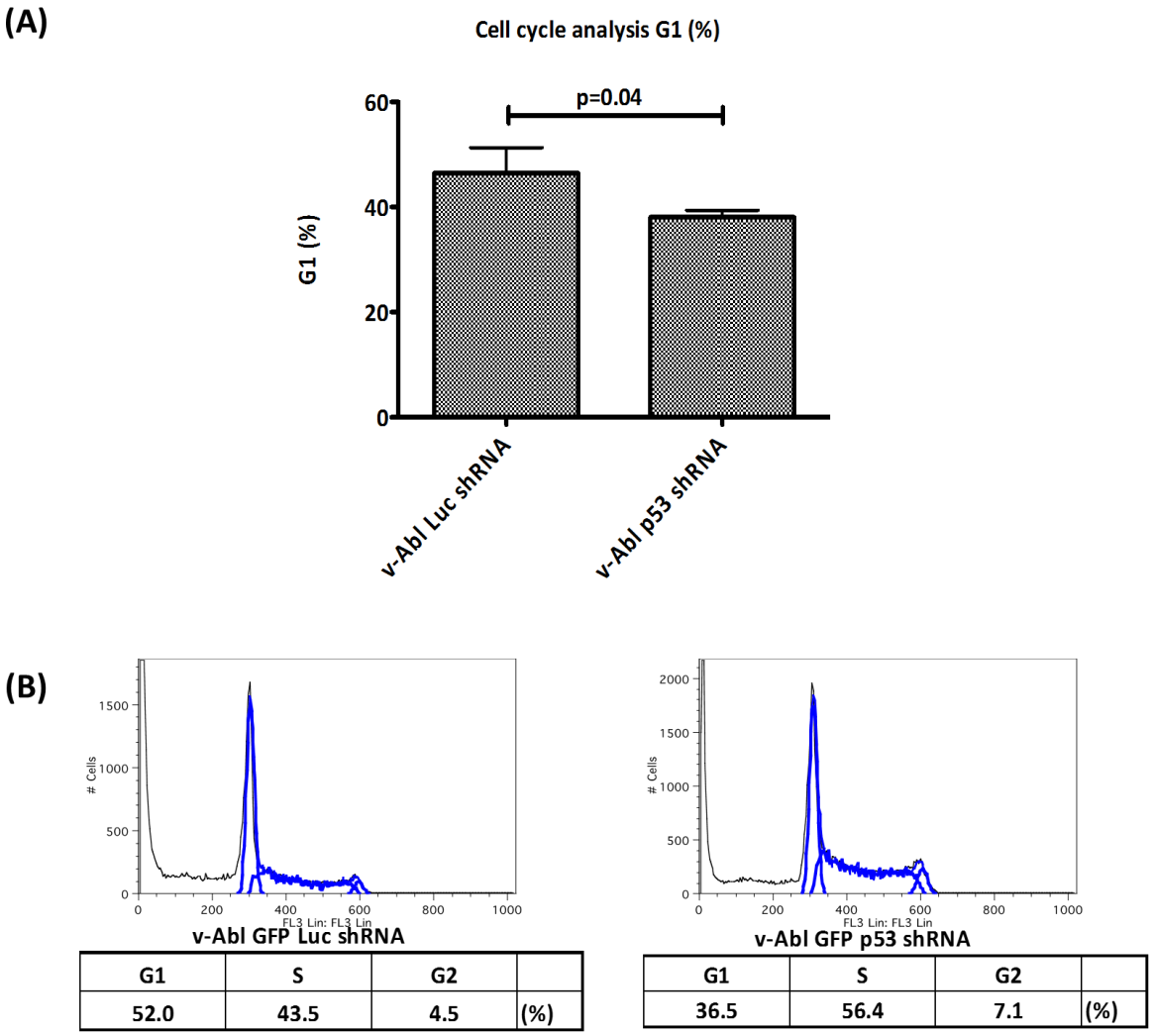


Figure 3.8. p53 knock-down decreased apoptosis levels in both early and late v-Abl transformation.

Wild-type bone marrow cells were infected with v-Abl GFP p53 shRNA or v-Abl GFP Luc shRNA. Apoptosis level was measured by staining with Annexin V-PE antibody every 3 to 4 days during the transformation. Two independent experiments were shown here.



G1	S	G2	
52.0	43.5	4.5	(%)

G1	S	G2	
36.5	56.4	7.1	(%)

Figure 3.9. p53 knock-down decreased G1 cell cycle arrest in early v-Abl transformation.

Wild-type bone marrow cells were infected with v-Abl GFP p53 shRNA or v-Abl GFP Luc shRNA. Two weeks after virus infection, cells were subjected to cell cycle analysis by PI staining. (A) Percentage of G1 phase cells in both samples is shown. (B) One representative data of cell cycle analysis is shown. This experiment has been repeated three times.

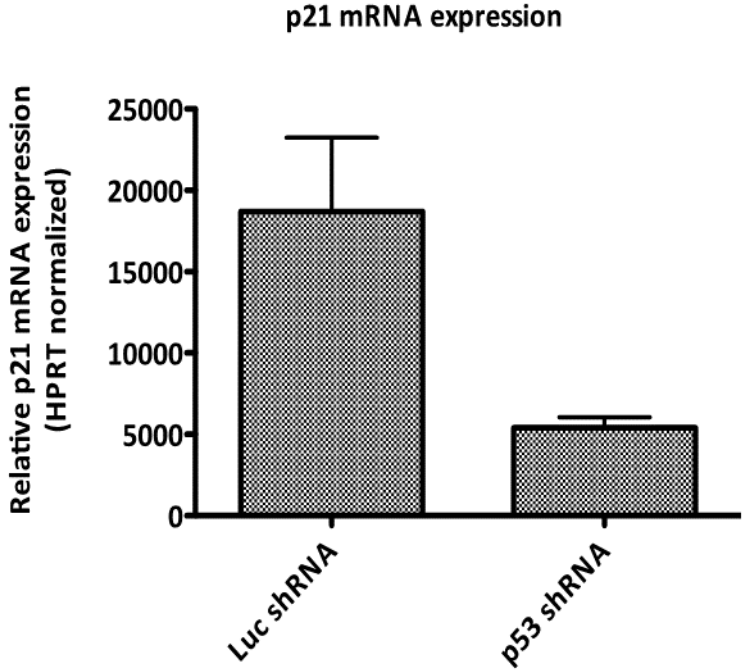


Figure 3.10. p53 knock-down decreased p21 expression in early v-Abl transformation.

Bone marrow cells were infected with v-Abl GFP p53 shRNA or v-Abl GFP Luc shRNA virus. Two weeks after virus infection, total RNA were isolated from transforming cells. p21 RNA expression levels were measured by qPCR with p21 primers. This data is the representative of three experiments.

Discussion:

Cellular factors are important in v-Abl transformation process, and we have analyzed role of p53 in v-Abl tumorigenesis. Previous studies showed that p53 is important in the late stage of v-Abl tumorigenesis. Our Initial transformation frequency assay and mixed virus competition experiments with p53 shRNA demonstrated that p53 knock-down can enhance v-Abl transformation also in the early stage. And this enhancement is associated with inhibition of both apoptosis and cell cycle arrest. Consequently, v-Abl transformation with p53 knock-down can be used to make Abelson cell lines effectively.

While p53 is well known to be involved in the late stage of v-Abl transformation, the apoptotic crisis stage, there was a disagreement in whether p53 is affecting the early v-Abl transformation step (Zou et al. 2000; Unnikrishnan et al. 1999). However, our experiments clearly proved that p53 is also involved in the early stage of v-Abl transformation. We could confirm that p53 shRNA increased colony formation in our initial transformation frequency assay, and p53 shRNA infected transforming cells were outgrowing uninfected cells in the mixed virus competition assay. Unnikrishnan et al. also pointed out that different strains of p53 knock-out mice might cause different effects. Certainly, Balb/c mice are more susceptible to v-Abl transformation and yield more colonies in the initial transformation frequency assay compared to C57BL/6 mice (data not shown; Rosenberg et al 1976). However, initial transformation frequency assay with Balb/C bone marrow cells confirmed that the v-Abl p53 shRNA virus still generates more transformation colonies over the control virus, though the fold difference was weaker compared to the C57BL/6 (data not shown). Therefore, p53 is involved in early v-Abl transformation, regardless of the mice backgrounds.

Our findings focused on the early stage of v-Abl transformation, which is less explored, compared to the later stage. Besides, it is true that p53 effects we have observed in the early step is weaker than the late step of v-Abl transformation (Figure3.6.). This is probably because of the extremely high apoptosis levels and dramatic clonal selection at the later stage, which is well-known (Unnikrishnan et al. 1999) (Figure 3.7. and Figure 3.8.). So, this raises many questions about the early v-Abl transformation: What is the role of the early stage in v-Abl transformation? What cellular events are occurring during this step? How does the transition from proliferation to the next stage happen? Although it is hard to answer these questions, one possibility seems to be that early proliferation of the initial transformation is providing enough number of cells to the later stage. Particularly, initial proliferating cells upon v-Abl virus infection are extremely small numbers, literally less than hundred. Thus it is critical to expand this cell population that can be used for clonal selection of subsequent mutational events leading to full transformation. Likewise, we have detected more early proliferation in p53 knock-down samples, and this will allow more candidate cells that can become final transformants successfully. However, many aspects of v-Abl transformation is still unknown and vague, and it will be essential to study more on both early and late stage of the v-Abl transformation.

Since, p53 knock-down can facilitate both early and late v-Abl transformation process, v-Abl p53 shRNA virus can be a very efficient tools to make Abelson cell lines. Previous studies have reported that less than 10% of the initial transforming colonies can be established as a transformed cell line after 1 or 2 months (Unnikrishnan et al. 1999). Considering low initial transformation frequency, which could be less than 1% of the infected cells, this could be a big obstacle in performing v-Abl transformation (Table 2.2.). Likewise, we frequently had experienced a failure in generating Abelson cell lines from bone marrows, because of the severe apoptotic crisis (data not shown). Thus, p53 knock-down can be used to accelerate producing v-Abl transformed cell lines, just as p53 null cells establish v-Abl transformation in higher efficiency and in short term (Unnikrishnan et al. 1999). Particularly, when Abelson pre-B cell lines are required from certain knock-out mice, this v-Abl p53 shRNA virus can be a very simple and certain method, without crossing the mice to the p53 null mice. In addition, p53 shRNA did not affect fully transformed Abelson cell lines (data not shown).

In consideration of the target cell population data and p53 knock-down experiments, we propose a better method to generate v-Abl transformed pre-B cell lines from mouse bone marrow cells: Infecting MACS sorted CD19+ cells with v-Abl p53 shRNA virus. The major v-Abl target population is Fraction B. However CD19+ MACS sorting is suggested, because FACS sorting has limitation in processing large quantity cells in a short time period. Actually, we have practiced this method to successfully produce several Abelson transformed pre-B cell lines from various knock-out mice (Garcia et al. 2012; Schulz et al. 2012; Amin et al. 2008).

The new initial transformation assay and the mixed virus competition experiment validated that p53 shRNA knock-down can enhance v-Abl transformation also in early v-Abl transformation, which is associated with decreasing both apoptosis and cell cycle arrest. v-Abl p53 shRNA virus can be an efficient way to make Abelson transformed pre-B cell lines. Now, we hope to investigate other cellular factors involved in v-Abl transformation. And this p53 shRNA can be a perfect positive control for later studies.

Chapter4. shRNA Library Screening to Identify novel cellular factors that suppress v-Abl Transformation

Backgrounds:

p53 and its pathway have been well studied in v-Abl transformation, and we have confirmed that p53 is also responsible for the initial v-Abl transformation stage. In addition, there have been some reports about other genes that might be involved in v-Abl transformation. For example, Pim1 and Pim2 kinases, and AID were reported to enhance v-Abl transformation, while NF-kappaB1 was found to be a negative regulator of the process (Chen et al. 2008; Gruber et al. 2010; Nakamura et al. 2002). Obviously, cellular factors, in addition to the v-Abl oncogene, are important during the v-Abl transformation process. However, many questions remain unanswered: Are there still important unknown factors involved in the multi-step tumorigenesis of v-Abl transformation? How are these factors modulated and regulated during the process? To tackle down these questions, we decided to perform library screening experiments.

I think it is the beauty of biological science that you can do functional genomic screening experiments. You can screen myriads of genes and factors in vector-based libraries, recue and amplify the outcomes by polymerase chain reaction (PCR), and re-introduce them back into the vectors for further round of screening. This large-scale screening method has been a very powerful tool to tease out unknown factors in many discovery studies of biology. Previously, our lab has exploited cDNA library screening to successfully identify a novel factor that up-regulates RAG expression in B cells (Amin et al. 2008). But in the v-Abl transformation system, unbiased library screening experiments to find unknown cellular factors have never been performed, so far.

Recently, Elledge and Hannon generated a second-generation genome-wide shRNA library (Silva et al. 2005). These shRNAs were designed after the endogenous micro-RNA, mir30, so they can utilize the natural micro-RNA processing pathway (Figure3.1.). Target sequence selection was also improved by better understanding of small RNA thermodynamics. This library is composed of more than 140,000 shRNAs covering all human and mouse predicted genes with maximized efficiency. Thus, highly efficient large-scale RNAi screening is possible with this library. One subset of this genome-wide shRNA library is called ‘cancer1000’. It has shRNAs against the cancer related genes, which were implicated in breast cancer, selected by using bioinformatics and data mining (Witt et al. 2006). This ‘cancer1000’ library contains about 2300 shRNAs targeting about 850 cancer relevant genes. In a recent study, Lowe lab used this shRNA library to perform an *in vivo* shRNA screen, and identified novel tumor suppressors involved in hepatocarcinogenesis (Zender et al. 2008). Similarly, we decided to employ this ‘cancer1000’

shRNA library to identify a novel cellular factors in v-Abl transformation, by screening experiments.

Observed very low initial transformation frequency, and involvement of p53 in v-Abl transformation led us to search for other unknown cellular factors that suppress v-Abl transformation. Here, we have tried loss-of-function screening experiments using the shRNA library. In order to identify candidate shRNAs that can facilitate v-Abl transformation, we developed a sequential infection methods for the screening. After examining several candidate shRNAs, Dpf2, a zinc-finger transcription factor, was validated as a positive hit, as Dpf2 shRNA can enhance v-Abl transformation. Further studies found some hints about how this Dpf2 shRNA might function during v-Abl transformation. However, we still do not understand much about a role of Dpf2 in v-Abl transformation, which remains to be elucidated.

Results:

Initial library screening failed to obtain positive hits.

In an attempt to identify novel cellular factors involved in early v-Abl transformation, we performed a shRNA library screen. This screening utilized a vector system in which v-Abl and an shRNA library are expressed from separate promoters in a dual-promoter vector. First, we transferred shRNAs from the cancer1000 shRNA library into the recipient vector, v-Abl GFP shRNA vector (Silva et al. 2005) (Figure 4.1.(A)). The original cancer1000 shRNA library was divided into 50 tubes, and each tube had about 50 different individual shRNAs, making a total of about 2500 shRNAs. Since p53 is expected to be a strong positive hit based on a previous 100 to 1 mixed virus experiment (Figure 3.6.), we segregated 2 tubes that had p53 shRNAs into a one group, the positive control group. The rest of the shRNAs were combined into 7 groups for testing. Construction of v-Abl GFP shRNA vectors was carefully performed, to maintain the library complexity and integrity by monitoring colony formation numbers and sequencing several individual colonies during bacterial transformation.

In order to screen important cellular factors in early v-Abl transformation, wild-type bone marrow cells were infected with each pool of v-Abl GFP shRNA library virus (Figure 4.1.(B)). Transforming cells were harvested at 14 days after infection, a time point before the apoptotic crisis begins (Green et al. 1989). Enriched shRNA cassettes, during the 14 days of transformation culture, were amplified from purified genomic DNA by PCR and re-introduced into the recipient v-Abl GFP shRNA vector for further rounds of screening. If any shRNA is enhancing the v-Abl transformation process, this shRNA should be enriched against other shRNAs during the screening. After three rounds of screening, many groups became oligo-clonal or mono-clonal, when checked by individual sequencing of rescued clones. The known positive control, p53 shRNA, was successfully enriched in its positive control group (data not shown). Screening from the library resulted in 5 candidate shRNAs. However, initial transformation frequency assay and mixed virus competition experiment with these individual shRNA did not reproduce any enhanced transformation ability compared to control virus (data not shown; see the discussion). Therefore, initial screening experiments failed to pick up any positive shRNAs that can facilitate the v-Abl transformation.

Library screening using sequential infection methods

Although many cells were infected with v-Abl virus, we have observed that only very small numbers of cells undergo initial transformation (Figure 2.3). Perhaps, this low number of transformation initiating cells might be the serious limiting factors in high complexity library screening. Still, p53 shRNA nicely enriched in initial screening as a positive control, but shRNAs with weak phenotype might not survive and become enriched in this experiment. In addition,

the level of shRNA knock-down in the two promoter system may also be problematic, since PGK promoter is weaker than the LTR promoter.

To circumvent these problems, we developed a sequential infection method (Figure 4.2.). This method utilized two vectors and two infection system, that v-Abl virus vector and shRNA library vector are used to perform separate infections. First, mouse bone marrow cells were infected with a normal hCD4-marked v-Abl virus, v-Abl hCD4 (Figure 4.2.(A)). Then, transforming cells were cultured for 7 days, to get enough proliferating cells. This transforming population was secondly infected with shRNA library virus, shRNA GFP. Finally, transforming cells were cultured for 12 more days, before the apoptosis level increases too high. Survived and enriched shRNAs were rescued by genomic DNA PCR and re-introduced into a recipient vector for further rounds of screening. This method is also a very good competition assay to monitor the enrichment of the shRNA by just measuring the GFP marker in the culture. If any shRNA is advantageous to v-Abl transformation, the GFP+ population will outcompete uninfected cells and increase in the culture. After 4 rounds of screening, many groups showed enrichment of the GFP+ population during transformation. Using this approach, we identified 6 candidate shRNAs, while p53 shRNA nicely enriched in the positive group.

To validate the result, individual candidate shRNAs were tested using the sequential infection method (Figure 4.3.). Among 6 candidates, 5 shRNAs recapitulated the enhancement of v-Abl transformation. These shRNA infected cells outcompete uninfected cells and expanded about 3-4 fold compared to control Luc shRNA, but not as much as p53 shRNA, which reached almost 100%. These candidate shRNAs are CYP19A1 (Aromatase), CTSH (Cathepsin H), Dpf2 (D4, zinc and double PHD fingers family 2 / Requiem), Klk1b11 (Kallikrein 1-related peptidase b11), and HLTF (Smarca3 / Helicase-like transcription factor) (This and later studies summarized in Table 4.1.).

Confirmation of knock-down by candidate shRNAs

To prove whether these candidate shRNAs really down-regulate their targets, we tested mRNA expression levels and protein levels in Abelson cells transduced with each shRNA knock-down (Only Dpf2 data shown in Figure 4.4.). First, Abelson cells (E47+/+) transduced with candidate shRNAs or control Luc shRNA were subjected to qPCR analysis to measure candidate gene mRNAs. For protein analysis, Abelson cells over-expressing flag-tagged version proteins were tested for its knock-down by shRNA. Briefly, Abelson cells (E47+/+) over-expressing flag-tagged each candidate gene's cDNA were infected with each candidate shRNAs or control Luc shRNA. Isolated protein samples were analyzed with Western blots using anti-flag antibody to compare the flag-tagged protein levels. We verified that 4 out of 5 candidate shRNAs (CYP19a1, Dpf2, Klk1b11, and HLTF shRNA) down-regulated target gene expression in both mRNA and protein levels.

Multiple Dpf2 shRNAs can enhance v-Abl transformation.

shRNAs like other RNAi reagents, have an inherent off-target effects potential (Echeverri et al. 2006). RNAi has relatively high tolerance of mismatches between RNAi and target mRNA sequences (Jackson et al. 2003). Therefore, it is impossible to rule out that experimental results might come from unwanted effects of non-targeted genes. Generally, it is recommended to generate multiple distinct shRNAs against the same target gene, since the probability will be very low that multiple shRNAs with different sequence will display the same off-target effects (Echeverri et al. 2006).

In order to preclude the possibility of candidate shRNA's off-target effects, 3 more shRNAs against each candidate genes were generated and examined in the sequential infection method. One additional Dpf2 shRNA (Dpf2 shRNA#4) exhibited enrichment phenotype more than control Luc shRNA sample in the assay, though less than original Dpf2 shRNA (Dpf2 shRNA#1) (Figure 4.5.(A)). This Dpf2 shRNA#4 is targeting 3' region of Dpf2 gene, while original Dpf2 shRNA (Dpf2 shRNA#1) is against 5' region of Dpf2.

To verify whether the additional Dpf2 shRNAs also down-regulate Dpf2 levels, Dpf2 mRNA expression levels in cells transduced with each of four Dpf2 shRNAs were measured by qPCR. Abelson cell lines (E47+/+) transduced with each Dpf2 shRNAs construct clearly demonstrated that Dpf2 shRNA#4 decreased Dpf2 mRNA level close to the original Dpf2 shRNA, more than other two additional Dpf2 shRNAs (Dpf2 shRNA#2 and #3) (Figure 4.5.(B)).

This multiple shRNA experiments confirmed that Dpf2 is involved in v-Abl transformation, and Knock-down of Dpf2 can enhance v-Abl transformation process. For other candidate hits, we could not find any other shRNAs against the same targets that could recapitulate the enhancement phenotype. So, we mainly focused on Dpf2 for further studies (see discussion).

Other phenotypes of Dpf2 in the Abelson transformation process

After confirming Dpf2 knock-down facilitates v-Abl transformation, we tried to search whether there are any other effects of Dpf2 during the transformation process. First, we tested whether Dpf2 cDNA over-expression affects v-Abl transformation. Since Dpf2 knock-down enhanced the transformation, Dpf2 cDNA over-expression might be expected to inhibit the transformation. We used a cDNA over-expression vector, MIG vector, which can express cDNA with a GFP marker (Figure 4.6.(A)). Then, Dpf2 MIG vector and control empty MIG vector were tested in sequential infection method during v-Abl transformation. The results showed that Dpf2 cDNA over-expression does not significantly influence v-Abl transformation compared to the control (Figure 4.6.(B)).

As p53 and its pathway is sometimes inhibited during v-Abl transformation, we asked whether Dpf2 is modulated during normal v-Abl transformation. Wild-type bone marrow cells

were infected with v-Abl GFP retrovirus, and transforming cultures were collected during the transformation and after transformation for qPCR assay with Dpf2 qPCR primers. Dpf2 mRNA expression levels did not change significantly during the transformation, and did not differ from levels found in IL-7 cultured bone marrow cells and fully established Abelson cell lines (Figure 4.7.). This indicates that Dpf2 expression level is not regulated during v-Abl transformation and Dpf2 down-regulation is not required for v-Abl transformation.

Dpf2 did not show any phenotype in established Abelson cells.

Since inhibiting Dpf2 facilitate v-Abl transformation, Dpf2 might be detrimental to v-Abl transformation, and can function as a tumor suppressor gene in transformed Abelson cell lines. To evaluate whether Dpf2 is important in transformed Abelson cell lines, Dpf2 was over-expressed or down-regulated in Abelson cells using retrovirus from Dpf2 MIG vector or Dpf2 GFP shRNA vector. Then, GFP positive population in the culture was measured by FACS over time. If Dpf2 over-expression or knock-down is disadvantageous or advantageous to the Abelson cells, then retrovirus infected cell population should be decreased or increased respectively. Over-expression and knock-down of Dpf2 in Abelson cells did not displayed significant effects compared to control Luc over-expression and knock-down sample (Figure 4.8.). Thus, Dpf2 is not playing an important role in fully transformed Abelson cells.

Dpf2 shRNA enhancement of v-Abl transformation is p53 dependent.

Since, p53 is well known factor involved in v-Abl transformation, we tried to test whether Dpf2 phenotype in v-Abl transformation is dependent on p53. Bone marrows from p53 -/- mice were assayed with Dpf2 GFP shRNA vector in the sequential infection method (Figure 4.9.). Interestingly, the results showed that Dpf2 shRNA enrichment disappeared in p53 null cells, while CYP19a1 shRNA, another candidate shRNA found in the screening, still enriches as in wild-type cells (Figure 4.9. and Figure 4.3.). This result demonstrated that Dpf2 shRNA phenotype requires p53.

After we observed this p53 dependent Dpf2 phenotype, we again examined whether Dpf2 over-expression affects v-Abl transformation of p53 null cells. So, Dpf2 MIG vector was tested with p53 -/- bone marrow cells in the sequential infection method (Figure 4.10). However, Dpf2 MIG samples did not exhibit noticeable difference compared to control empty MIG. Thus, we concluded that Dpf2 over-expression does not affect in v-Abl transformation both in wild-type and p53 null backgrounds.

This p53 dependency of Dpf2 shRNA enhancement, led to idea that Dpf2 knock-down might negatively affect p53 in v-Abl transformation. To test this, we first measured p53 mRNA expression levels upon Dpf2 knock-down during v-Abl transformation (Figure 4.11.(A)). However, Dpf2 knock-down did not influence p53 mRNA levels. Similarly we have examined

p53 protein levels, and found they were not affected either (Figure 4.11.(B)). Although, Dpf2 is known as a transcription factor, it could interact with p53 through protein modification rather than expression levels. p53 could undergo as many as 50 posttranslational modifications such as phosphorylation, acetylation and ubiquitylation (Meek et al. 2009). We chose to test the phosphorylation site of Ser15 in p53, since it is one of the well-studied and is induced by DNA damage signals (Shieh et al. 1997). But, Dpf2 knock-down transforming cells exhibited similar levels of p53 activation to control (Figure 4.11.(B)). Therefore, Dpf2 shRNA does not affect p53 expression levels or activation levels.

In order to solve this Dpf2 puzzle, we have tried microarray experiments. Transcription profiles from v-Abl transforming cells with Dpf2, Luc, or p53 shRNA were analyzed. However, Dpf2 knock-down samples were very similar to control samples, as shown in the dendrogram (Figure 4.12.(A)). Not many genes were up-regulated or down-regulated significantly (Figure 4.12.(B) and (C)). Gene ontology analysis with this list of genes did not give meaningful results, probably because of small number of genes. We have tested some genes individually, such as Pim2, but could not verify any involvement in v-Abl transformation. And we are still looking for the possible downstream players of Dpf2 phenotype in this list of the genes.

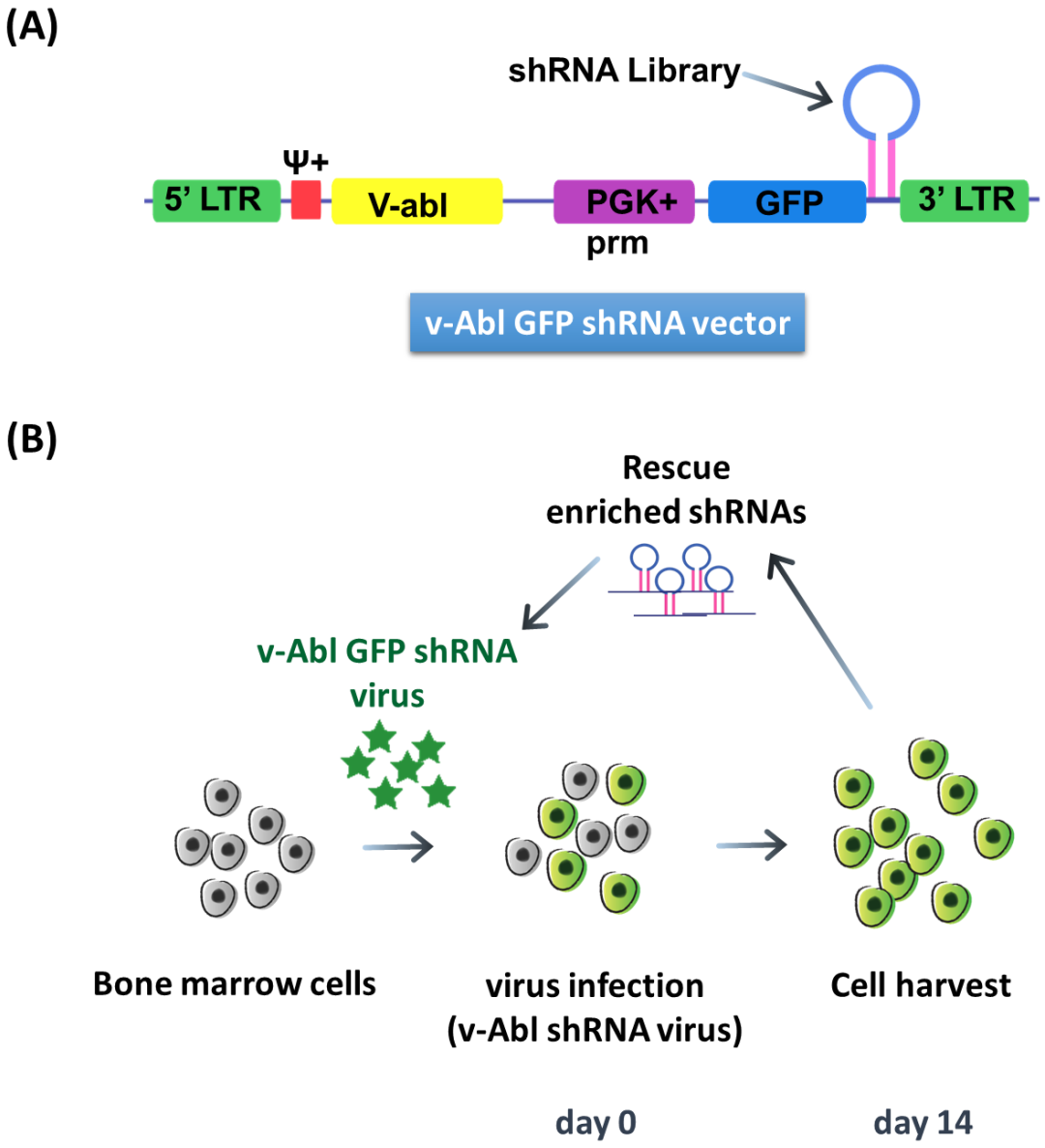


Figure 4.1. Diagram of v-Abl GFP shRNA vector and initial shRNA library screening method

(A) v-Abl GFP shRNA vector is based on two-promoter system. v-Abl is under LTR promoter and shRNA is located at the tail of GFP marker under the PGK promoter. "Cancer1000" shRNA library is transferred to the shRNA region of this vector.
(B) Mouse bone marrow cells were infected with retrovirus from v-Abl GFP shRNA library vector. Cells were harvested at 14 days after virus infection. Enriched shRNAs in the culture were rescued by PCR amplification and re-introduced into the recipient vector for further rounds of screening.

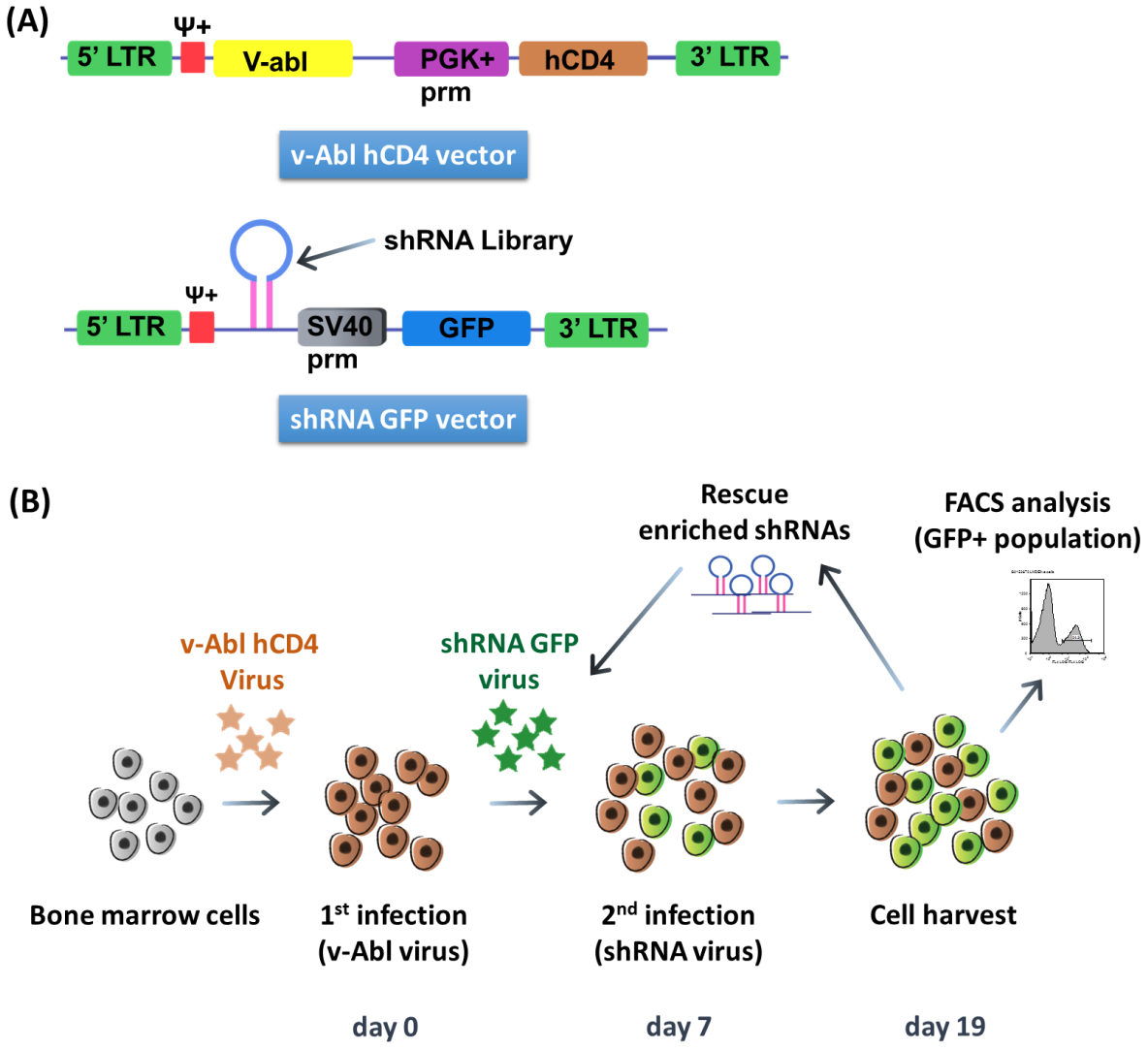


Figure 4.2. Diagram of v-Abl hCD4 and shRNA GFP vector, and sequential infection method for screening shRNA library

(A) Diagram of v-Abl hCD4 vector and shRNA GFP vector. These vectors were used for sequential infection method. Both v-Abl and shRNA are driven by LTR promoter. "Cancer1000" shRNA library was transferred to the shRNA region of the vector.
(B) Mouse bone marrow cells were infected with retrovirus from v-Abl hCD4 vector to initiate v-Abl transformation. At day 7, these transforming cells were secondly infected with shRNA GFP vector, the shRNA library vector. Transforming cells were cultured for 12 more days. Then, cells were harvested and enriched shRNAs in the culture were rescued by PCR amplification and re-introduced into the recipient vector for further rounds of screening. After second virus infection, shRNA virus infected cells among transforming population can be measured by FACS analysis of GFP marker in shRNA virus.

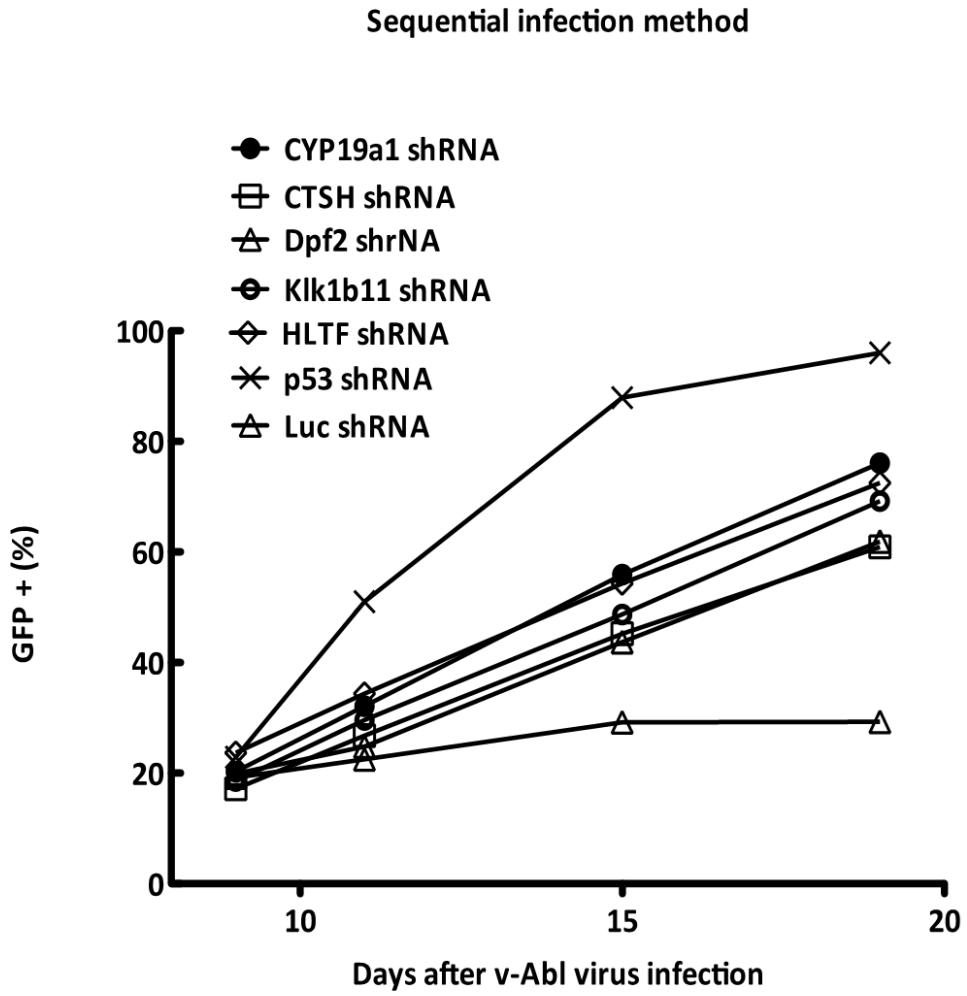


Figure 4.3. Candidate shRNAs from library screening enhance v-Abl transformation in sequential infection method assay

Sequential infection method is performed as described in Figure 4.2. Briefly, day 7 v-Abl transforming cells were infected with retrovirus from candidate shRNAs, p53 shRNA and control Luc shRNA GFP vector. shRNA virus marker GFP positive cells were monitored by FACS during the 12 days of culture. This data is the representative of three experiments.

	transforming cells	Knock-down confirmation	transforming cells	p53 null backgrounds transforming cells	p53 null backgrounds transforming cells	Abelson cells	Abelson cells	multiple shRNA confirmation
	Individual shRNA test		cDNA over expression test	individual shRNA test	cDNA over expression test	individual shRNA test	cDNA over expression test	
CYP19a1	O (Enhancement)	O (Confirmed)	X (No effect)	O (Enhancement)	X (No effect)	X (No effect)	X (No effect)	X
CTSH	O	X						
Bmp6	X							
Dpf2	O	O	X	X	X	X	X	O (Confirmed)
Klk1b11	O	O	X	O	X	X	X	X
HLTF	O	O	X	O	X	X	X	X

Table 4.1. Summary of experiment results with candidate shRNAs in this chapter

Candidate shRNAs from screening with sequential infection method were tested in various experiments. All experiments are described in the following sections.

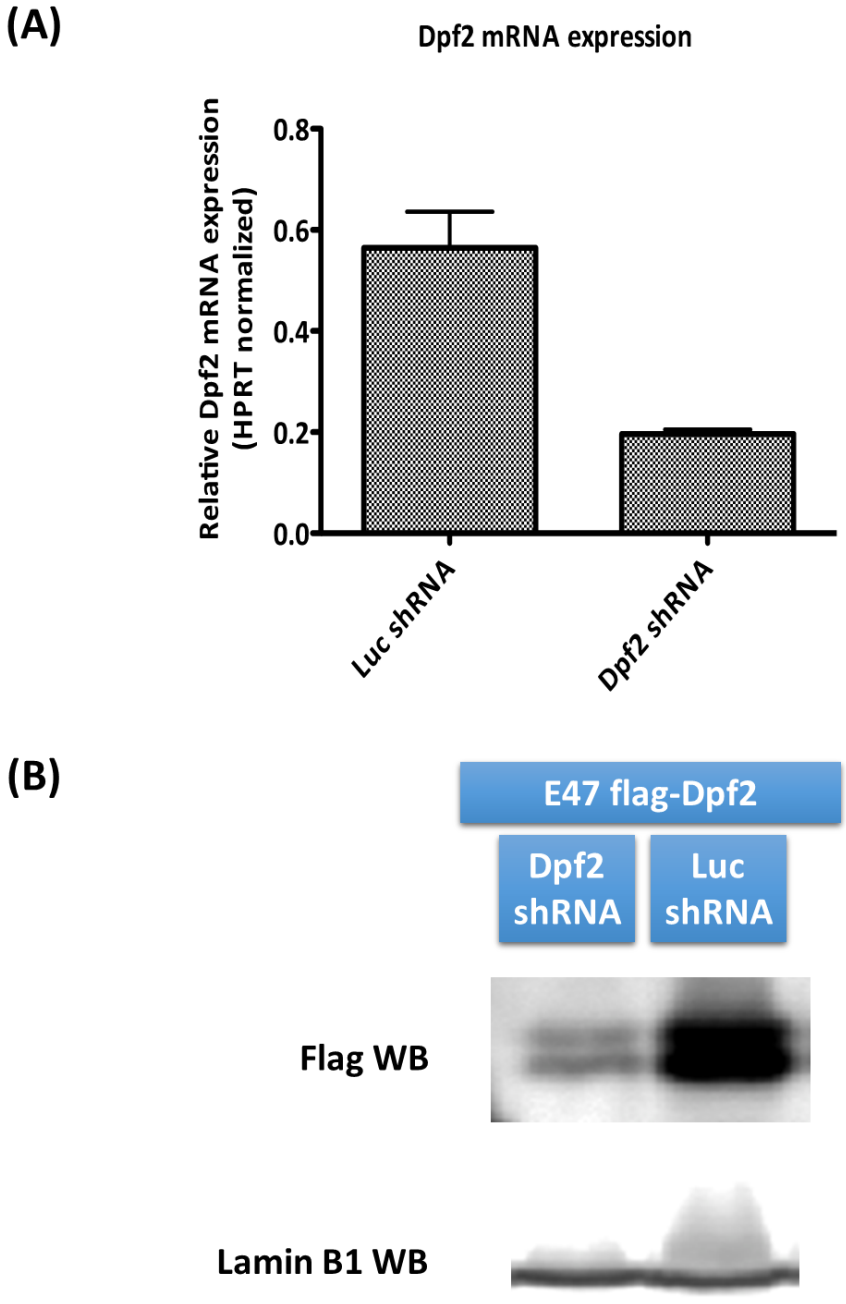


Figure 4.4. Dpf2 shRNAs down-regulates Dpf2 expression in Abelson cell lines.
(A) Abelson cell line E47+/+ was transduced with Dpf2 GFP shRNA or control Luc GFP shRNA vector. Virus infected cells were sorted and isolated total RNA is subjected to qPCR assay with Dpf2 qPCR primers.
(B) Dpf2 flag IRES hCD4 vector transduced E47 +/+ Abelson cell line was infected with Dpf2 GFP shRNA or control Luc GFP shRNA retrovirus. FACS sorted GFP marker positive cells were harvested and extracted proteins were subjected to Western blot assay with anti-flag antibody and control Lamin B1 antibody. These data are the representative of three experiments.

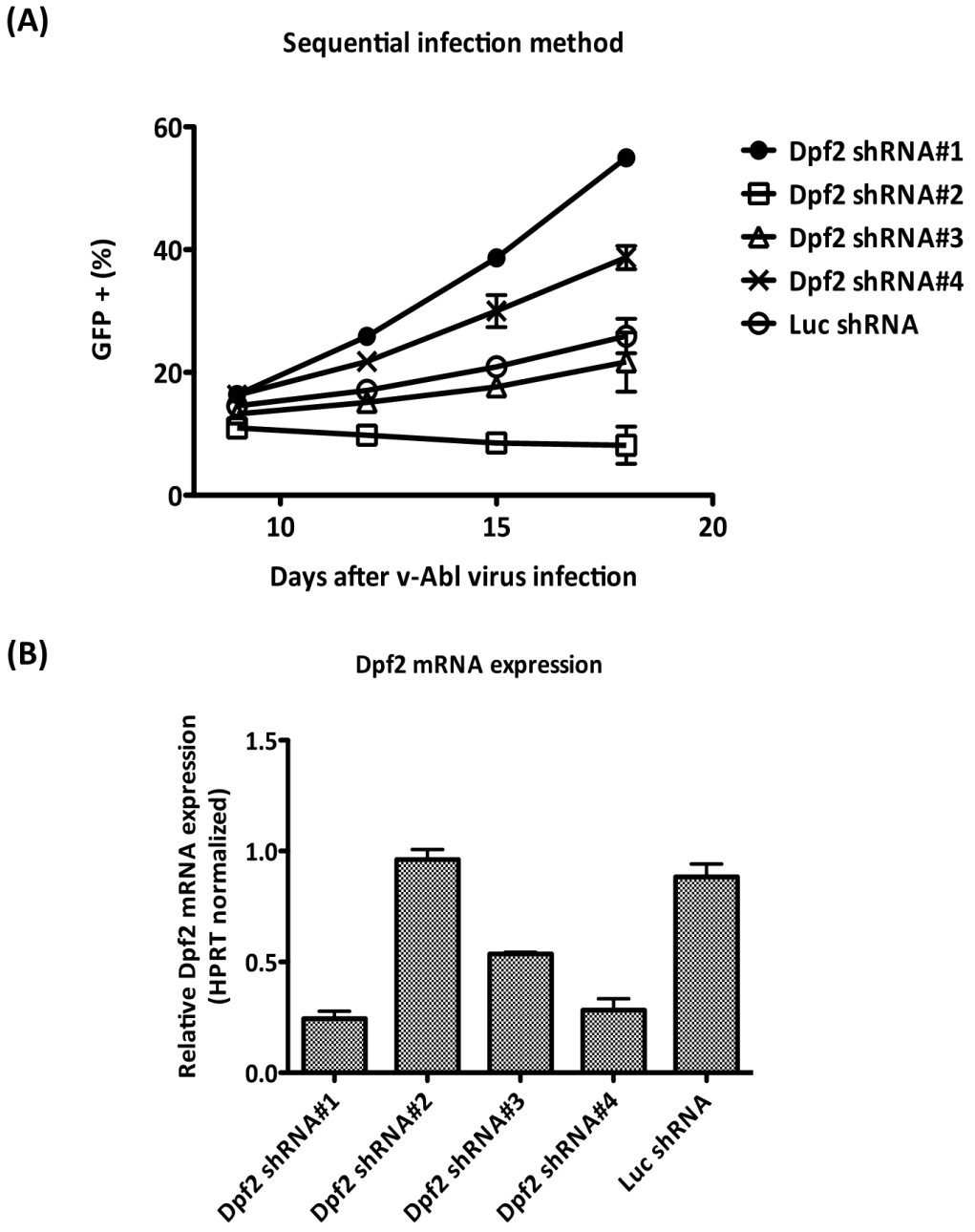
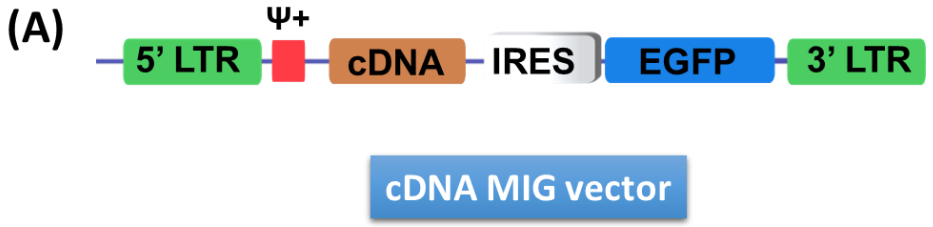


Figure 4.5. Multiple Dpf2 shRNAs facilitated v-Abl transformation.

(A) In addition to the original Dpf2 shRNA (Dpf2 shRNA#1), three more Dpf2 shRNAs (Dpf2 shRNA#2, 3, 4) were generated and assayed in the sequential infection method. Dpf2 shRNA#4 displayed enhancement of v-Abl transformation compared to control Luc shRNA.
(B) Knock-down abilities of these Dpf2 shRNAs were tested. Abelson cell line E47 +/+ was infected with retrovirus from these four Dpf2 shRNA GFP vector and control Luc shRNA GFP vector. Virus infected, GFP marker positive cells were sorted and isolated total RNA is subjected to qPCR assay with Dpf2 qPCR primers. These data are the representative of three experiments.



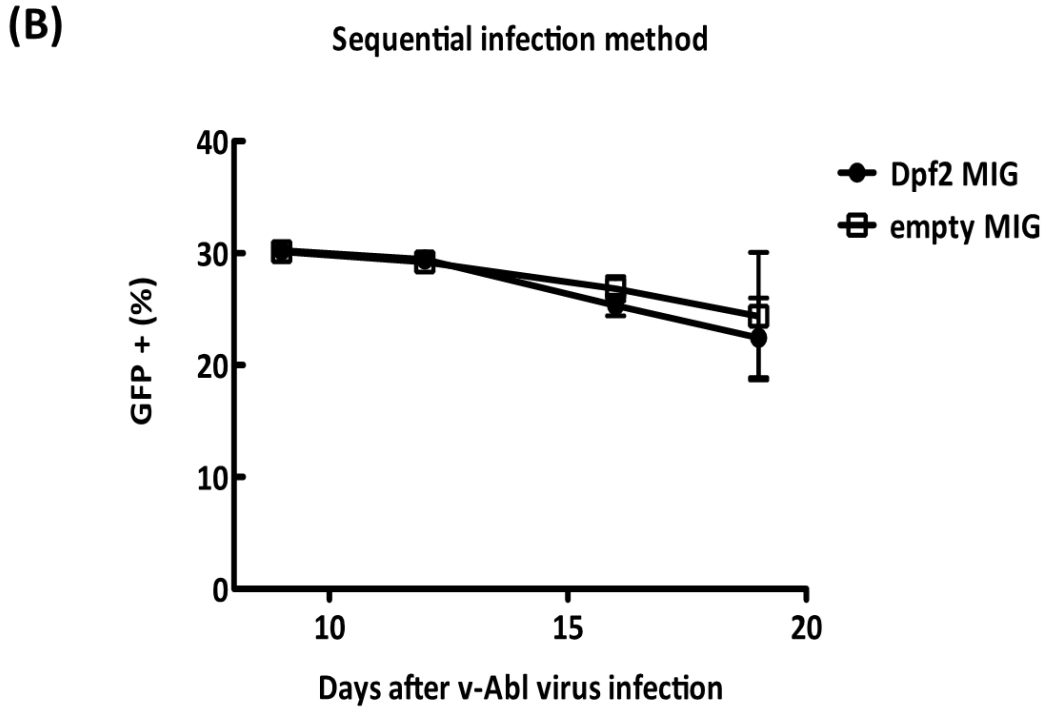


Figure 4.6. Dpf2 over-expression does not affect v-Abl transformation process.

(A) Diagram of cDNA over-expression vector, cDNA MIG vector. cDNA is under LTR promoter, followed by IRES-GFP cassette as a marker. Over-expression of Dpf2 and all other cDNAs used in this study are based on this vector.
(B) Over-expression of Dpf2 was tested in v-Abl transformation with sequential infection method. Dpf2 MIG vector infected cells showed no effect during v-Abl transformation, just like control empty MIG vector infected cells. This data is the representative of three experiments.

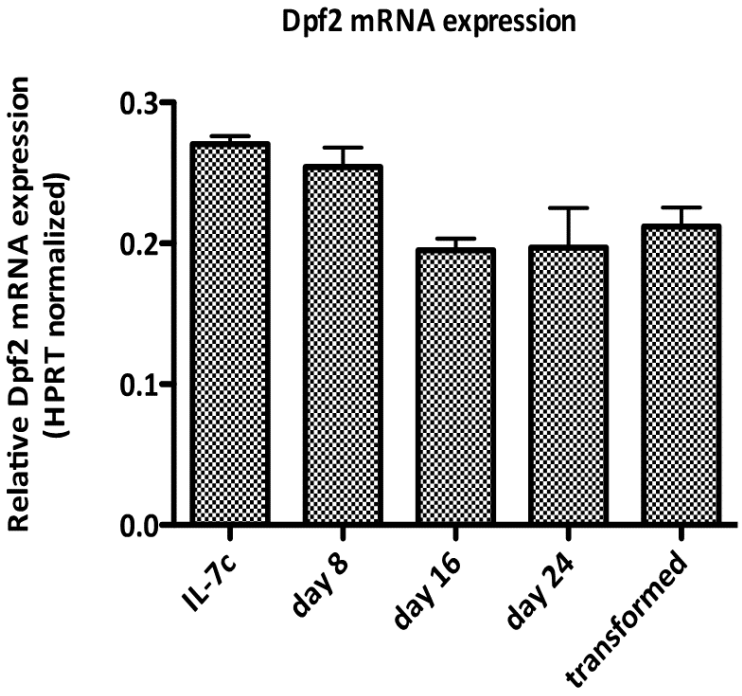


Figure 4.7. Dpf2 is constantly expressed before, during, and after the v-Abl transformation.

Wild-type bone marrow cells were infected with normal v-Abl GFP virus to initiate v-Abl transformation. Transforming cells were cultured until fully established as a cell line, which was after three months of transformation. At various times during the transformation process, part of cell culture was harvested and total RNA was isolated. Later, these RNA and control RNA from control IL-7 bone marrow cultures were subjected to qPCR assay with DPf2 qPCR primers. In all samples, Dpf2 mRNA expression was in similar levels. This data is representative of two experiments.

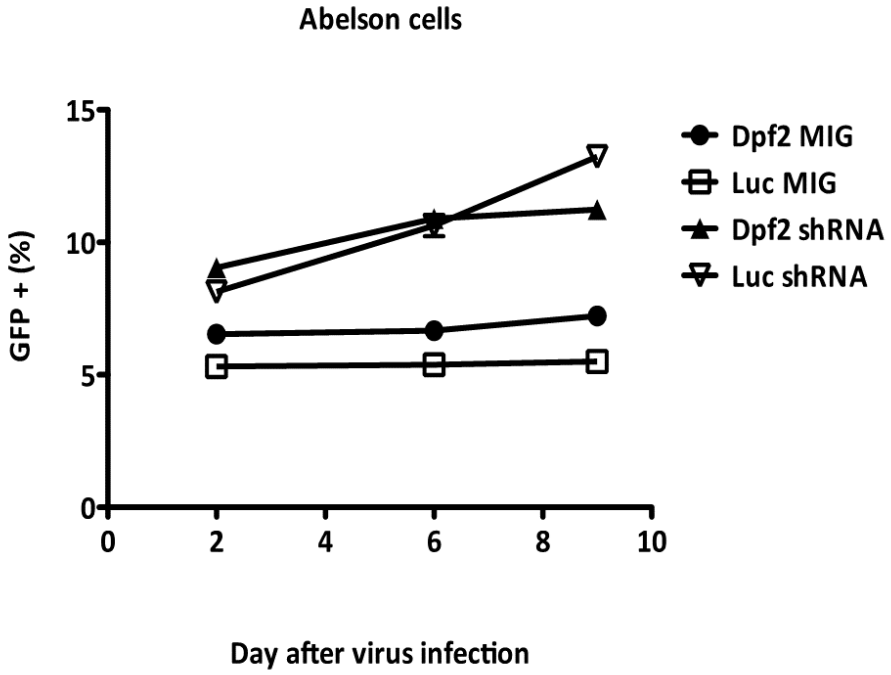


Figure 4.8. Dpf2 does not affect fully transformed Abelson cell lines.

Abelson cell line (112112#1) was infected with over-expression construct Dpf2 MIG or control Luc MIG vector, or knock-down construct Dpf2 GFP shRNA or control Luc GFP shRNA vector. While infected Abelson cells were cultured, retrovirus infected marker GFP positive cells were monitored by FACS analysis. For 9 days of culture, both Dpf2 over-expression and knock-down samples were not significantly different from control Luc samples. This data is the representative of three experiments.

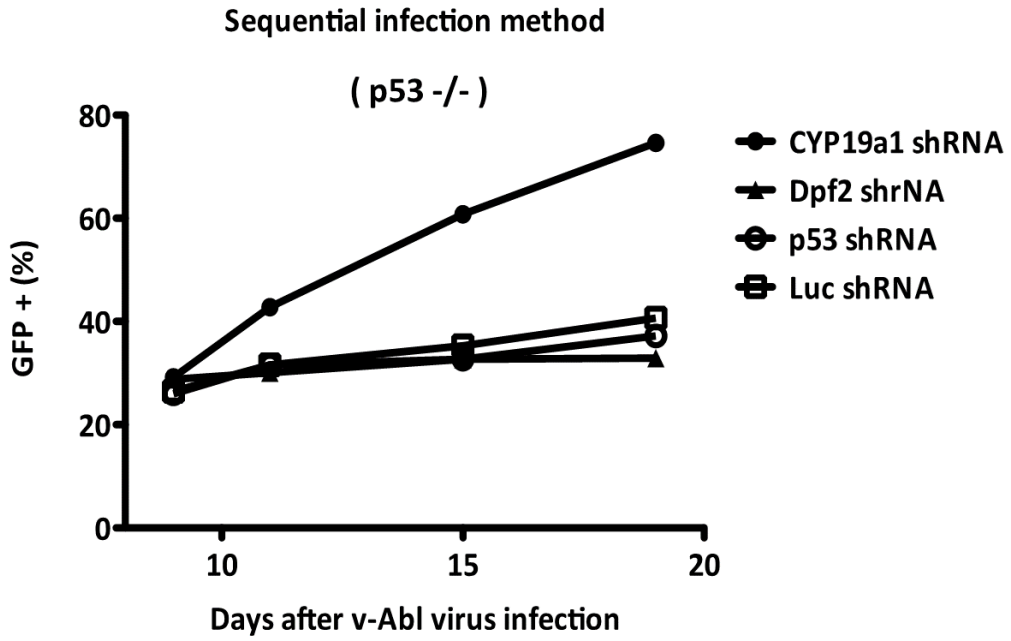


Figure 4.9. v-Abl transformation enhancement of Dpf2 knock-down requires p53.

Same experiment as in Figure 4.3. was performed in p53 -/- backgrounds. Briefly, p53 null bone marrow cells were infected with v-Abl hCD4 retrovirus to start v-Abl transformation. Day 7 v-Abl transforming cells were infected with retrovirus from Dpf2, CYP19a1, p53 and control Luc shRNAs GFP vectors. shRNA virus marker GFP positive cells were monitored by FACS during the 12 days of culture. This data is the representative of three experiments.

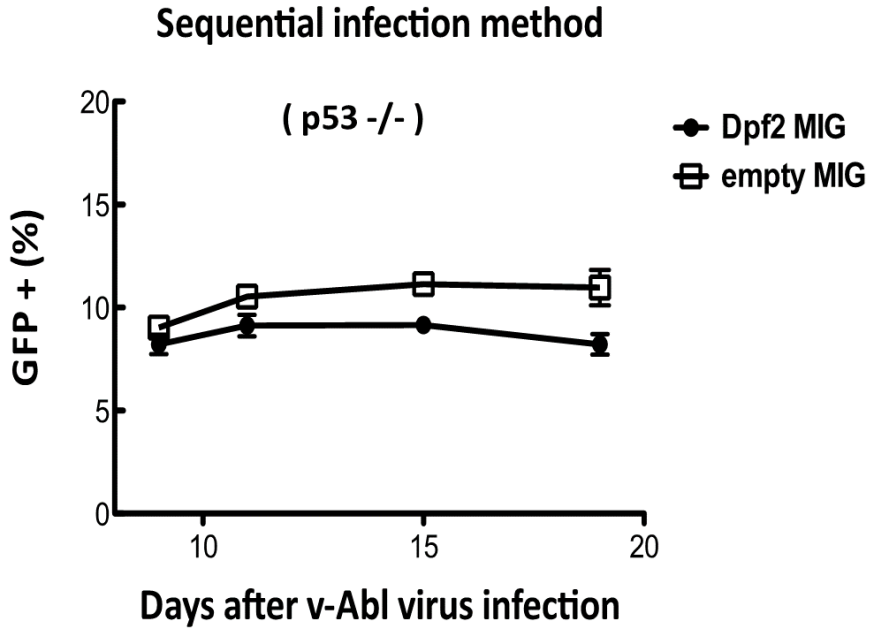


Figure 4.10. Dpf2 over-expression does not affect v-Abl transformation process in p53 null backgrounds.

Over-expression of Dpf2 were tested in v-Abl transformation with sequential infection method in p53 null backgrounds. p53 -/- bone marrow cells were infected with v-Abl hCD4 virus. Day 7 transforming cells were infected with Dpf2 MIG or control empty MIG vector. Dpf2 MIG vector infected cells showed no effect during v-Abl transformation, just as in wild-type backgrounds. This data is the representative of three experiments.

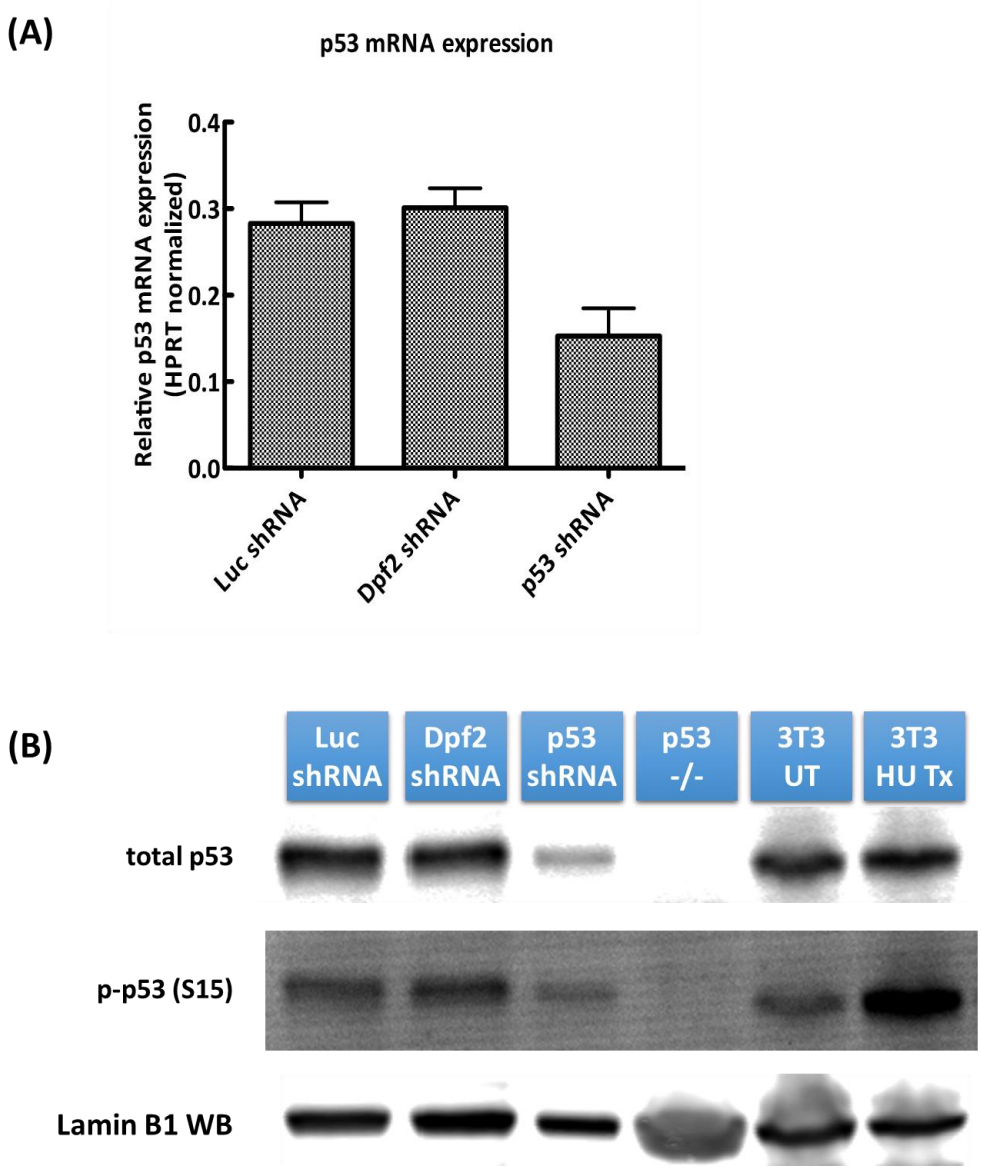
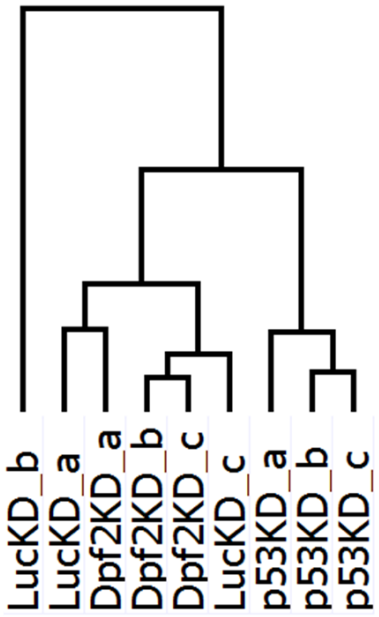


Figure 4.11. Dpf2 knock-down does not affect p53 expression levels or p53 activation levels.

During the sequential infection method assay, v-Abl transforming cells with Dpf2, p53, or Luc shRNA GFP virus infected cells were FACS sorted. Then, cells were harvested for total RNA and protein isolation.
(A) Total RNA was subjected to qPCR assay with p53 qPCR primer.
(B) Protein was subjected to Western blot assay with p53 , phosphorylated p53 (Ser15), and control Lamin B1 antibody. For Western blot assay, control proteins from p53 knock-out transforming cells, wild-type 3T3 fibroblast, and 3T3 with hydroxyurea treatment (20mM, 12 hours) were added. These data are the representative of three experiments.

(A)



(B)

	Dpf2 KD/Luc KD	p53 KD/Luc KD
S100a6	3.33	4.54
Hs3st2	2.30	0.84
Csf1	2.02	2.19
Rag1	1.83	1.58
Pfn2	1.83	1.38
Zscan4a	1.82	2.30
Scd1	1.80	3.06
P2rx3	1.70	1.56
Plod2	1.69	2.11
Trpc4	1.68	1.13
Il10ra	1.63	0.95
Trem1	1.62	1.62
Gfra2	1.62	0.80
Gab1	1.57	1.20
Lphn2	1.55	1.40
Rbp1	1.54	1.42
1700034H14Rik	1.53	1.38
Gbp2	1.53	1.05
Arhgap25	1.53	1.25
Il2ra	1.52	0.96
Lphn2	1.51	1.32
Rpl17	1.50	1.12
Car2	1.50	1.37
Pim2	1.50	2.18

Figure 4.12. Microarray analysis of transforming cells with Dpf2, p53, and Luc knock-down

(A) Dendrogram of hierarchical clustering of transcription profiles. Triplicate samples from Dpf2, p53, and Luc knock-down v-Abl transforming cells were used. While p53 knock-down samples were clustered with each other closely, Dpf2 knock-down and control Luc knock-down samples were intermingled.
(B) List of genes that were up-regulated in Dpf2 knock-down samples. mRNA level fold change of Dpf2 knock-down vs. Luc knock-down samples and p53 knock-down vs. Luc knock-down samples were indicated.
(C) (See next page) List of genes that were down-regulated in Dpf2 knock-down samples.

(C)

	Dpf2 KD/Luc KD	p53 KD/Luc KD
Ly6a	0.33	1.30
Cxcr5	0.34	0.54
Scarna13	0.37	0.33
Ly6d	0.38	0.23
Gldc	0.39	0.74
Snora44	0.40	0.52
Tubb3	0.41	0.93
Ubash3a	0.44	0.78
Dpf2	0.44	1.09
H19	0.46	1.58
Cd9	0.49	0.41
Slc17a8	0.49	0.42
Ppic	0.49	0.80
Gm2799	0.51	0.35
Lat	0.53	0.35
Snora20	0.53	0.43
Ly6c1	0.53	1.22
Actn1	0.53	0.41
Gm10708	0.54	0.29
Gm13926	0.54	0.44
Hist1h3h	0.55	0.67
Npm3	0.55	0.71
Ramp3	0.55	0.37
Fcrl6	0.55	0.93
Uaca	0.55	1.29
Mgat4a	0.55	1.24
Sertad4	0.56	0.66
Rpl15	0.57	0.88
Cacnb2	0.58	0.58
Arpp21	0.59	0.64
Alox5ap	0.60	0.67

Figure 4.12. (See before page)

Discussion:

After finding a role for p53 in early v-Abl transformation, we wanted to ask what other cellular factors are involved in the transformation process. So, we have performed library screening experiments with a state-of-the-art shRNA library. In order to fish out the candidate shRNAs, we implemented a new screening strategy, the sequential infection method, resulting in finding some candidate shRNAs that can enhance the v-Abl transformation. Multiple shRNA experiments confirmed that Dpf2 is involved in v-Abl transformation. However, we could not find other phenotypes of Dpf2 in previously established transformed Abelson cells. Studies with p53 null mice demonstrated that Dpf2 shRNA enhancement requires p53, and p53 null cells blunted v-Abl transformation enhancement of Dpf2 knock-down. Nevertheless we could not find other hints of Dpf2, and the mechanism how Dpf2 knock-down in v-Abl transformation is still largely vague.

Initial screening using a single vector system failed to yield candidate shRNAs. First, initial transformation frequency of the v-Abl transformation is extremely low (Figure 2.3) (Rosenberg et al. 1976). Although the v-Abl GFP shRNA library vector infects many cells, only few cells actually can survive and start transformation, which is literally less than 100 colonies out of a million cells. In addition, many of these colonies are very small sized, which could not proliferate very well. Although our library complexity in one group was only about 300 individual shRNAs, there could be not enough cells initiating v-Abl transformation. Secondly, in a single vector system, the shRNA library is under the PGK promoter, which is weaker than LTR promoter (Figure 4.1.(A) and Figure 4.2.(A)). We have seen that GFP markers under PGK promoter are dimmer than GFP under LTR promoter in FACS analysis (data not shown). Similarly, others also reported weak shRNA can be sensitive to the promoter strength, compared to strong shRNA (Silva et al. 2005). So, some weak shRNAs might not be able to exhibit its ability in this one vector system. However, p53 shRNA, the strong positive control, worked well and enriched in this screening method. Therefore, this one vector system screening was not sensitive enough to screen weak candidate shRNAs.

To save the shRNA library screening, we implemented the sequential infection method. This method separates v-Abl virus infection from shRNA library virus infection (Figure 4.2.). So, it provides more uniform and constant cell populations for library screening. In spite of limited transformation initiating cells, the early proliferation burst generates sufficient amount of cells, which can be easily more than several million cells at 7 days after v-Abl virus infection. These cells are all v-Abl virus infected, initial transformation initiated cells with high proliferation rate. Furthermore, experiment using cells from one population, eliminates problems such as unequal v-Abl expression from the positional effects of retrovirus insertion. Thus, this sequential infection method provides a safe experimental platform for studying v-Abl transformation. Though, this method cannot test the very beginning of v-Abl transformation before 7 days post infection.

On the other hand, we have to collect the data at least on day 19 after virus infection in the experiments. Around the later time point, severe apoptotic crisis begins, and transforming cultures become very unstable and inconsistent. Even in the control virus infected cells, the marker positive population among these cells begins fluctuating at this late time point. We divided control virus infected transforming cells from the same pool into 48 well plates, and found all the wells are with different marker positive percentage from 0% to 100% at the later stage of transformation. In addition, we have seen many marker positive populations became mono- or oligo-clonal in the FACS analysis. These support that high apoptosis and clonal selection at the late stage confer transforming cells more randomness and unstableness, which makes it hard to perform experiment. To study this late apoptotic stage, you would have to design the experiment to collect data from large population in short-period of time.

Dpf2 is a zinc-finger transcription factor, a member of zinc and double PHD fingers family. Initially, one screening experiment discovered this Dpf2 as a factor that is required for IL-3 withdrawn programmed cell death in myeloid cells, where it had its first name, Requiem (Gabig et al. 1994). Thus it is closely associated with apoptosis. However, Dpf2 has been barely studied, and the role of DPf2 in apoptosis is still under on-going research. And here, we have found that Dpf2 phenotype is p53-dependent in the v-Abl transformation, which brings a new clue to the Dpf2 studies. In addition, v-Abl transformation system can be another way to investigate more about Dpf2. So far, Dpf2 knock-out mice has not been generated. Once, it is available, we could examine it with v-Abl transformation.

Our screening assay resulted in a total of four candidate shRNAs. In addition to Dpf2 shRNA, CYP19a1, Kl1kb11 and HLTF shRNAs also enhanced v-Abl transformation. Among these, CYP19a1 is also called Aromatase, a member of the cytochrome P450 superfamily that catalyzes the last step of Estrogen synthesis. Estrogen has been long studied as a potent activator in autoimmune disease (Grimaldi et al. 2002). Also, some studies have reported that Estrogen and Estrogen pathway are involved in immune cell apoptosis and development (Mor et al. 2003;Kincade et al. 2000). Thus it is intriguing that CYP19a1 knock-down can enhance v-Abl transformation. Regarding this, we have tried treatment of v-Abl infected bone marrow cultures with Estrogen, CYP19a1 inhibitors, or Estrogen receptor inhibitors, but none of these recapitulated CYP19a1 shRNA phenotype. However, we have observed that CYP19a1 mRNA expression level was transiently increased, then came down during v-Abl transformation. In addition, overexpression of Estrogen receptor inhibited v-Abl transformation process. Thus, it is still not clear whether CYP19a1 is involved in v-Abl transformation, and testing CYP19a1 knock-out mice would be essential.

In order to find unknown cellular factors involved in v-Abl transformation, we performed shRNA library screening in sequential infection method. After rounds of screening, we found Dpf2 knock-down can enhance v-Abl transformation. This Dpf2 is not required for v-Abl transformation, and is not affecting transformed cell lines. However, we found p53 is required for enhancement of v-Abl transformation by Dpf2. v-Abl transforming cells become unstable and unpredictable at the later apoptotic stages, which makes it hard to study. But this is what

happens in cancer development. Spontaneous and stochastic mutants are picked up and selected continuously (Hanahan et al. 2011). In this way, I think v-Abl transformation system is a very attractive tool to directly test transformation in a relatively short period of time. In these days, a very powerful technique, next-generation sequencing (NGS), comes in handy (Zeng et al. 2012). It would be encouraging to use this high-throughput sequencing, and ask how cellular factors are modulated and regulated during the v-Abl transformation process.

Chapter5. Materials and Methods

Mice and genotyping

C57BL/6 mice were used as a wild-type mice in all experiments, unless other mouse strain is specified. p53 knock-out mice ($Trp53^{tm1Tyj}$) in C57BL/6 backgrounds were genotyped using the published protocol (Jacks et al. 1994). Mice used in all experiments were 3-8 weeks old. C57BL/6, Balb/c, and p53 knock-out mice were purchased from Jackson Laboratories. All mouse experimentation was approved by the Animal Care and Use Committee of the University of California at Berkeley.

Cell lines and cell culture

Abelson cell line 112112#1 was generated by v-Abl transformation of wild-type bone marrow cells. Briefly, cells were infected with v-Abl hCD4 retrovirus, and cultured in 20% RPMI media for three months. Another Abelson cell line E47+/+ is described in Muljo et al. 2003.

The established Abelson cell lines including E47+/+ and 112112#1 were cultured in the 5% RPMI media: RPMI (Invitrogen) supplemented with 5% fetal calf serum (FCS), 50uM 2-mercaptoethanol, 2mM $_{L}$-Glutamine, and antibiotics. All the cell cultures in the v-Abl transformation were grown in the 20% RPMI media: RPMI (Invitrogen) supplemented with 20% FCS, 50uM 2-mercaptoethanol, 2mM $_{L}$-Glutamine, and antibiotics. Fresh bone marrow cells were grown in 20% RPMI with 5 ng/ml IL-7.

Ecopack2-293 and 3T3 fibroblast for retrovirus production and titration test were grown in 10% DMEM media: DMEM (Invitrogen) media supplemented with 10% FCS and 800uM sodium pyruvate without antibiotics.

Plasmids

All retroviral vectors used in the experiments were based on the Murine stem cell virus (MSCV) vector. All vectors used are outlined below.

v-Abl GFP vector: LTR-v-Abl-PGK promoter- GFP

v-Abl puro vector: LTR-v-Abl-PGK promoter-puro

v-Abl hCD4 vector: LTR-v-Abl-PGK promoter-hCD4

> Truncated hCD4 marker substituted GFP in the vector.

v-Abl GFP shRNA vector: LTF- v-Abl-PGK promoter – GFP –shRNA

> mir30 based p53 shRNA (p53.1224) and Luc shRNA (Luc.1309) was incorporated at the downstream of GFP

shRNA GFP vector: LTF – shRNA – SV40 – GFP

> Original shRNA library vector from Scott Lowe lab (Silva et al. 2005).

cDNA MIG vector: LTR – cDNA – IRES GFP
> From MSCV PIG vector (OpenBiosystems), puro was substituted with cDNAs.
cDNA flag IRES hCD4 vector: LTF-cDNA-flag-IRES-hCD4
> described in (Amin et al. 2008), surface marker truncated hCD4 marker is expressed.

All the shRNAs used were mir30 based shRNAs (Figure 3.1.) (Stegmeier et al. 2005). Many of the shRNA sequences were derived from RNAi codex (Olson at al. 2005).

Dpf2 shRNA#1 (the Original screened shRNA): HP_290321 (Olson at al. 2005)
Dpf2 shRNA#2: HP_294301 (Olson at al. 2005)
Dpf2 shRNA#3: HP_302790 (Olson at al. 2005)
Dpf2 shRNA#4: HP_297753 (Olson at al. 2005)
CYP19a1 shRNA: HP_235282 (Olson at al. 2005)
Klk1b11 shRNA: HP_290185 (Olson at al. 2005)
HLTF shRNA: HP_249251 (Olson at al. 2005)
p53 shRNA: p53.1224 from Bric et al. 2009.
Luc shRNA: Luciferase.1309 from Silva et al. 2005.
Dpf2 cDNA was amplified from total RNA of bone marrow cells
Luc cDNA was transferred from firefly Luciferase gene of Psicheck2 vector (Promega).

shRNA library

Cancer1000 shRNA library was kindly provided by Scott Lowe lab from Cold Spring Harbor Laboratory (now in Memorial Sloan-Kettering Cancer Center) (Silva et al. 2005). The library consists of 2500 individual mir30 based shRNAs targeting about 800 cancer related genes. It came as 50 tubes, and each tube has about 50 individual shRNAs. We divided the library into 8 groups. Group#1, positive control group, had only 2 tubes that contain p53 shRNAs. The other groups, Group#2 to Group#8, had about 6 to 7 tubes each, corresponding to 300 to 350 individual shRNAs. These 8 groups were used both in initial library screening and sequential infection method screening.

Isolation of bone marrow cells from mice

Mouse bone marrow cells were isolated from tibia, humerus, and femur of mice by flushing with 5% RPMI media. Then, Histopaque-1083 (Sigma) was used as manufacturer's protocol to deplete red blood cells and non-lymphocytes. Then, the cells were washed with 5% RPMI media for further studies.

Retrovirus production and infection

Retrovirus packaging cell line Ecopack2-293 (Clonetech) was prepared as manufacturer's instruction. Briefly, Ecopack cell line grown in 10% DMEM media, was plated in 3 cm plates containing OPTI-MEM (Invitrogen) media with 10% FCS. Next day, plated cells were transfected with retroviral vector and vesicular stomatitis virus G (VSV-G) expressing vector (pseudotyping vector), by Lipofectamine 2000 (Invitrogen), according to manufacturer's protocol. After two days, viral supernatant was filtered through 0.45um syringe filter, and virus aliquots were frozen at -80'C.

Some of the retroviruses were titrated by infecting 3T3 cell lines. Briefly, 3T3 cells were plated on 6 well plates a day before infection. 10 times diluted retroviruses were added with 4ug/ml polybrene. After 24 hours incubation, virus infection rate of each virus was measured by FACS analysis or qPCR analysis from isolated genomic DNA.

For strong retrovirus infection, we used spin infection protocol. Briefly, 5 million cells in 200ul of 20% RPMI media were plated in 12 well plates. 1ml retrovirus soup with 4ug/ml polybrene was added to the cells. Then, the plate was spun at 840g, 2 hours, 32'C. Finally, samples were diluted out with 20% RPMI media.

Total RNA preparation and qRT-PCR

Total RNA was isolated using Trizol Reagent (Invitrogen), following the manufacturer's protocol. Then, RNA was subjected to reverse transcription for cDNA synthesis using Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV-RT) Kit (Invitrogen) with random hexamers.

Quantitative reverse transcription PCR (qPCR) was performed with appropriate qPCR primers using Jumpstart Taq (Sigma) and EvaGreen (Biotium) on an ABI 7300 Thermocycler (Applied Biosystems). PCR amplification was as follows: 95'C 5 min, and 40 cycles of 95'C 30sec 60'C 30sec and 72'C 30sec (data collection), followed by 70'C 3min.

qPCR primer sequences were obtained from PrimerBank (Wang et al. 2012).

p53 qPCR primers
F: CTC TCC CCC GCA AAA GAA AAA
R: CGG AAC ATC TCG AAG CGT TTA
p21 qPCR primers
F: ACG GTG GAA CTT TGA CTT CG
R: CAG GGC AGA GGA AGT ACT GG
Dpf2 qPCR primers
F: AGC TCC TTG GCG AGC AAT AC
R: CTC TGG GCT ACT CCA GTC TGT
HPRT qPCR primers
F: CTG GTG AAA AGG ACC TCT CG
R: TGA AGT ACT CAT TAT AGT CAA GGG CA

Microarray experiments

During the sequential infection methods (see below) Dpf2, p53, and Luc shRNA GFP virus infected v-Abl transforming cells were FACS sorted using GFP marker. At day 14 after first v-Abl virus infection, these cells were harvested, then total RNA was prepared as described above. RNAs from triplicate experiments of Dpf2, p53, and Luc knock-down, total 9 samples, were cleaned up by RNeasy Kit (Qiagen) as manufacturer's protocol. Then, microarray experiment was performed with Affymetrix GeneChip Mouse Gene 1.0 ST Array (Affymetrix), in Functional Genomics Laboratory at University of California, Berkeley. Finally, array data was analyzed with Genepattern program (Reich et al. 2006).

Western Blot analysis

Total protein was isolated by lysing harvested cells with RIPA buffer (1% NP-40, 0.1% SDS, 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.5% Sodium Deoxycholate, 1 mM EDTA, 1mM DTT, Protease Inhibitors, Phosphatase Inhibitors). Protein samples were separated on 10% SDS-PAGE, and transferred to PVDF membrane (Bio-rad). After blocking, the membrane was stained with primary antibody, and appropriate secondary antibody. Finally it was analyzed with Odyssey Infrared Imaging system (LI-COR).

Primary antibodies: total p53 (Cell Signaling) / p-p53(Ser15) (Cell Signaling) / Lamin B1 (Abcam) / GAPDH (Cell Signaling) / anti-flag M2 antibody (Sigma)
Secondary antibodies: anti rabbit IgG IRD 680 (Invitrogen) / anti mice IgG IRD 800 (Invitrogen)

Flow cytometry and FACS sorting experiments

Cells were harvested and prepared in FACS wash buffer: PBS with 5% BSA and10mM HEPES. Then, cells were stained with primary antibodies, and then with secondary antibodies if required. FACS analysis was done using FC-500 (Beckman-Coulter) or LSRFortessa (BD Biosciences). FACS sorting was performed for other experiments on Influx (Cytopeia, now BD Biosciences) or Moflo (Cytomation, now Beckman-Coulter). For sorting experiments, pre-sort and post-sort samples were FACS analyzed. All the flow cytometry work was done in Flow Cytometry Facility in University of California, Berkeley. Data analysis used FlowJo program (FlowJo).

Antibodies: CD19-PEcy7 (1D3, BD Pharmingen), c-kit-PE (ACK45, BD Pharmingen) / BP-1– biotin (6C3, BD Pharmingen) with SA-APCcy7 (BD Pharmingen) / B220–pacific blue (RA3-6B2, BD Pharmingen) / IgM-FITC(II/41, eBioscience) / CD43-PE (S7, BD Pharmingen)/ HSA-PEcy5 (M1/69, eBioscience)

MACS sorting and depletion

Cells were harvested and prepared in MACS wash buffer: PBS with 0.5% BSA and 2mM EDTA. In CD19+ MACS sorting, cells were stained with anti-CD19 antibody conjugated with magnetic beads, the CD19 MicroBeads (Miltenyi Biotec). Then, CD19+ cells were enriched using a MS column (Miltenyi Biotec), following the manufacture's instruction.

For MACS depletion, cells were stained with surface marker antibodies conjugated with PE. Then, secondary antibody, anti-PE MicroBeads (Miltenyi Biotec), labeled the sample. Finally, antibody stained cells were depleted using a LD column (Miltenyi Biotec). To confirm depletion process, pre-sort and post-sort samples were FACS analyzed.

Antibodies: CD19-PE (1D3, BD Pharmingen) / Bp-1-biotin (6C3, BD Pharmingen) with SA-PE (eBioscience) / CD25-PE (PC61, BD Pharmingen) / IgM-PE (Southern Biotech)

Round-bottom 96 well plate assay: Initial transformation frequency assay

Cells were prepared from Histopaque depletion of bone marrows, FACS sorting, or MACS depletion. Then, they were infected with v-Abl GFP retrovirus. After 36 hours, cells were harvested and GFP positive cells were sorted by FACS machine into round-bottom 96 well plate with 200ul RPMI 20% media. Unless indicated, 20 cells/well sorting was used in the experiments. Day 14 after sorting, colonies in the plates were counted by low magnification (X40) microscopy. Colony size was also arbitrarily determined comparing with the field of view in microscopy (X40): Colonies larger than 50% of the field of view as "Large" / Colonies with 25% to 50% size of the field of view as "Small". For each sample, more than 4 plates were sorted.

Sort agar assay and 96 well plate culture assay were previously described (Rosenberg et al. 1976; Chen et al. 2008).

Mixed virus competition assay

v-Abl puro virus and v-Abl GFP p53 shRNA or v-Abl GFP Luc shRNA virus were mixed in 100 to 1 ratio. All three viruses were titrated to assure appropriate amount of virus could be used. Wild-type bone marrow cells were infected with these mixed virus, and cultured in 20% RPMI media. From day 2 to day 20 after the virus infection, GFP positive cell population was measured by FACS analysis. v-Abl puro virus and v-Abl GFP Luc shRNA virus were mixed in 10 to 1 ratio, and used for additional control experiment.

Library screening – initial one vector system

shRNA library was transferred into v-Abl GFP shRNA vector. Retrovirus from this vector infected wild-type bone marrow cells. Day 14 after virus infection, transforming cells were harvested and genomic DNA was isolated. Enriched shRNAs were rescued by PCR amplification with mir30XhoI and mir30EcoRI primers. Then, shRNA amplicons were re-introduced into the recipient vector, v-Abl GFP shRNA vector, using XhoI and EcoRI sites. After three rounds of screening, individual bacterial transformation colonies during cloning were sequenced to identify enriched shRNAs. 10 colonies from each group were sequenced.

mir30XhoI primer: CAG AAG GCT CGA GAA GGT ATA TTG CTG TTG ACA GTG AGC G
mir30EcoRI primer: CTA AAG TAG CCC CTT GAA TTC CGA GGC AGT AGG CA

Library screening – sequential infection method

Wild-type bone marrow cells were infected with v-Abl hCD4 virus. At day 7, 5 million transforming cells were infected with 1ml of original shRNA library GFP virus and 4ug/ml polybrene. Then the cells were cultured in 20% RPMI media for 12 more days (day 19 after initial v-Abl virus infection), and harvested to rescue enriched shRNAs. From isolated genomic DNA, enriched shRNAs were PCR amplified, using mir30XhoI and mir30EcoRI primers. shRNA amplicons were re-introduced into the recipient vector, shRNA GFP vector, using XhoI and EcoRI sites. After four rounds of screening, individual bacterial transformation colonies during cloning were sequenced to identify enriched shRNAs. 10 colonies from each group were sequenced.

Sequential infection method assay

Experimental procedure was exactly same as library screening with sequential infection method. Briefly, initial transforming cells were infected with shRNA GFP virus or cDNA MIG virus. After the second virus infection, GFP positive population in the culture was monitored by FACS analysis, every three to four days, until day 19 after the first v-Abl virus infection.

Proliferation assay / apoptosis assay / cell cycle analysis

Trypan blue excluded viable cells were counted on hemacytometer to measure total cell numbers in v-Abl transformation culture with v-Abl GFP p53 shRNA and Luc shRNA. Apoptosis level was assayed by Annexin V-PE Apoptosis Detection Kit (BD biosciences), following the manufacturer's instruction. Then, Annexin V positive cells were analyzed by FACS. Cell cycle was assayed by Propidium Iodide (PI) staining, followed by FACS analysis. Apoptosis assay, and cell cycle analysis data were processed with FlowJo program (FlowJo).

Chapter6. References

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