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P27 deficiency accelerates the development of PTEN-deficiency-induced myeloproliferative disease

Master thesis in Biomedical Engineering

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ABSTRACT

PTEN acts as a phosphatase for PIP3 and negatively regulates the PI3K/AKT pathway, and CDKN1B ($P27^{KIP1}$) is a cyclin-dependent kinase inhibitor that regulates G0 to S phase transitions by binding to and regulating the activity of cyclin-dependent kinases. Genetic alternations of *Pten* or *Cdkn1b* are common in hematological malignancies. Combined loss of PTEN and $P27^{KIP1}$ expression is associated with tumor cell proliferation and poor prognosis in prostate cancer. However, it is not so clear how two mutations would cooperate in leukemogenesis. Here, we show that combined inactivation of PTEN or $P27^{KIP1}$ in the hematopoietic compartment in mice results in a more severe myeloproliferative disease phenotype with shorter lifespan, lower hemoglobin and more enlarged spleen, lever comparted inaction of Pten or p27KIP1 alone.

Key words

PTEN; $P27^{KIP1}$; myeloproliferative disease; leukemogenesis;

CONTENTS

1. INTRODUCTION

Leukemia characterized by high mortality and lack of special treatment have become a serious threat to human health. Most of the signs and symptoms of leukemia are caused by the over-proliferation of some abnormal blood cells (blasts) [1]. Though the mechanism of these abnormalities were still unknown, more and more evidences suggest that they are developed from a combination of genetic and environment factors [2]. Targeting the mutated DNA, genetic therapy was introduced into the treatment of the leukemia. Not only correcting the genetic mutation, this therapy can also help to regulate the cell signaling pathway [3]. It is well-known that the cell signaling pathway plays important roles in the domain of adjusting the cell cycle and cell proliferation [4]. By effecting the expression of specific genes, this therapy is turned to be a more selective treatment strategy for leukemia with minimal residual disease [5]. On the other hand, the treatment advances also place greater demands on the knowledge of genes, including oncogenes (such as *Stat3*) and antioncogenes (such as *P53*).

Phosphatase and tensin homolog (PTEN), which is a protein encoded by *Pten* gene, was introduced into this study. *Pten* acts as a tumor suppressor gene and its mutation is detected in many kinds of human cancer [6] [7]. Some previous studies proved that PTEN is able to negatively regulate the PI3K/Akt/mTOR pathway. For this reason, it can regulate the cell cycle and introduce acute leukemia in mouse model [8].

In the domain of cell cycle regulation, a group of protein called Cyclin-dependent kinases (CDKs) also plays important roles in driving cell cycle phases. CDK inhibitors (CKI), including INK4, p57, and p21, can inactivate CDKs and are well-known for their tumor suppressive property. P27 which is another CKI under the Cip/Kip family, was reported strongly correlate anaplastic large-cell lymphoma (ALCL), in which loss of PTEN is frequently detected [9] [10]. Accumulating evidences show the potential connection between the “leukemia induced by PTEN deficiency” and “cell cycling regulated by P27”.

In this research, Floxed *P27*^{kip1} gene were crossed into the *Pten* deficiency leukemia mouse model to study the role of P27^{kip1} in PI3K/Akt/mTOR pathway and how PTEN can regulate cell cycling through P27.

2. THEORETICAL FOUNDATIONS

1.1 MOUSE

1.1.1 EXPERIMENTAL MICE

Mice and rats play critical roles in developing new drugs and therapies for human diseases nowadays. According to a survey of Foundation for Biomedical Research (FBR), over 95 percent of lab animals are mice and rats [11]. It is not only because these animals' small size and short generation time, but also for the reason that their genetic, biological and behavior characteristics closely resemble those of human [12]. These similarities make mice and rats become a desirable resource to create disease models by modifying their genes (e.g. transgenic mice). Besides the genetic similarity between human and rodent, the use of mice (rats) also brings several other advantages to a study. First, researchers are able to monitor how a disease develops under their control, since they can terminate the experiment and harvest samples anytime they want; secondly, the mechanism of the disease can be understood easier, because the anatomy, physiology and genetics of rodents have been fully-studied already; most importantly, since a large number of animals can be involved in a single project, the experiment result is possible to be statistically significant [13].

1.1.2 TRANSGENIC MICE

"Transgenic" is the technique producing organism with modified gene of interest, including knock-in, knock-out or reverse the target gene. In order to have a successful transgenic mouse line, at least five steps are compulsory: purification of transgenic construct, harvesting donor zygotes, microinjection, implantation and genotyping of transgene expression [14]. With these processes, researchers combine target DNA segments into plasmids which are known as 'vectors', and then microinject these prepared transgene DNA construct into mouse zygote to have a "transgenic embryo". At last, transgenic mice were gained after the delivery of those implanted mice [15] [16].

1.1.3 MOUSE LINE

C57BL/6 is one of the inbred strains and is widely used in the laboratory experiments [17]. After over 20 generations of brother-sister mating, individuals from this mouse line share similar genetic background. C57 family of strains including C57BL, C57BR and C57L are mostly inbred in large scale after World War I. The laboratorial performances of C57 family mice are quite stable in the past several years, especially in the field of genic studies. C57BL/6 mice are covered in black and show higher sensitivities to noise, smell, light and drugs. Normal C57BL/6 mice have low tumor incidence with the lifespan of 1.5 to 2 years [18].

1.2 PATHOLOGY

1.2.1 ENLARGEMENT OF SPLEEN

Spleen is an important tissue with the main function of filtration of blood, reserve of blood cells and response immune reaction [19]. The enlargement of it can be caused by many different reasons. For most of time, it is a sign of overreaction or overload. Once spleen outgrows its own blood supply, some part of it may be damaged which lead to bleeding and lose of function. Disorders such as infections, cancer (leukemia/lymphoma) and inflammatory diseases share the enlarged spleen as one of their common complications [21].

1.2.2 LEUKEMIA

Leukemia is also known as a cancer of the blood cells or bone marrow. This disorder starts from the blood-forming cells in the middle of the bone which are also known as the stem cells or progenitor cells. In the case of leukemia, the uncontrolled immature white blood cells (blast cells) were produced in the bone marrow. These over-produced blast cells not only fail to function in the immune system, but also crowd out normal blood cells in the circulatory system [5]. For instance, the loss of platelets may result poor blood clotting ability and loss of red blood cells can lead to low oxygen carrying ability (anemia).

1.2.3 LYMPHOMA

In the case of lymphoma, the cancer originates from the lymphatic organs including bone marrow, spleen, thymus and lymph nodes [21]. As an important part of the immune system, these lymphatic organs produce, store, and carry white blood cells which fight against infections and the spread of tumors [22]. A lymphoma develops when some of lymphocytes divide faster than normal cells or fail to carry out the apoptosis process. The swelling of the lymph nodes or lymphatic organs are the most common symptoms of lymphoma, and other discomforts include fever, weight loss and fatigue [21].

1.2.4 MYELOPROLIFERATIVE NEOPLASM (MPN)

MPNs are a collection of several diseases with the similar cause - the over proliferation of bone marrow cells or blood cells. The over-produced cells may function normally and the increase in number may not cause any symptoms. However, with its progressing, some of the MPNs can turn to acute leukemia. Comparing with acute leukemia, MPNs develop much slower and get worse gradually [23].

1.3 GENE

1.3.1 GENETIC RECOMBINATION

During the pairing of homologous chromosomes during meiosis, DNA strands undergo breaking or rejoining to generate new combination of alleles [24]. This process, as known as genetic recombination, gives novel sequences and phenotypes to the next generation. In this way, mutations and new phenotypes can be introduced, therefore adapting the new generation to the variable environment. Most of the recombination occurs naturally, while in the field of genetic engineering, geneticists used the idea of genetic recombination developed a technology called site-specific recombination (SSR). In this method, recombination only takes place between the gene segments of interest. They

can be deleted, inserted and inverted with high fidelity [25]. SSR has become one of the most important means to study the mechanism and the expression of genes nowadays [26].

1.3.2 CRE-LOX TECHNOLOGY

Cre-lox technology is based on a tyrosine recombinase (cyclization recombinase/Cre) derived from P1 bacteriophage [27]. This enzyme is a 38 kD protein controlled by the recombinase gene called cre, and it can catalyzes the SSR event between two DNA recognition sites called loxP. LoxP is a 34 base pair (bp) sequence consists of an 8 bp core spacer sequence and two 13 bp palindromic sequences [27]. Since the core sequence in the middle is asymmetric, its order defines the orientation of the whole loxP. DNA fragment targeted with one loxP site at either end of the sequences are said to be "floxed". With different orientation of the loxP sites, floxed DNA sequence can either be deleted or inversed [28]. Cre-loxP recombination can be processed without any additional co-factors or extra energy. One of the most straightforward and widely-used method to process cre recombination is simply crossing a general Cre expresser transgenic line into the mice line with floxed gene [29]. This method is most commonly seen in the animal model of conditional knockout technology.

Mx1-cre

In order to control the expression of Cre recombinase, inducible systems was combined into the Cre-lox technology. In this strategy, mice containing a modified gene are crossed with Cre gene driven by a specific promoter which is only expressed in the desired target tissue, therefore resulting tissue-specific Cre expression and tissue-specific gene ablation [30].

Mx1-cre is one of such tissue-specific inducible system used in animal studies. Mx1 protein is the major protein inhibiting the replication of influenza virus in mice immune system. Previous researches showed the Mx1 promoter can be induced by either interferon (IFN) or IFN inducer [31]. Mx1-Cre system which use Mx1 promoter to control the expression of Cre recombinase transgene has been built. In this regard, Mx1 promoter can be activated by the application of IFN-alpha, IFN-beta or pI-pC, thereby inducing the expression of Cre recombinase only in immune organ [32 33].

1.3.3 GENE BACKGROUND

Signaling pathway

As the smallest functional unit of all living organisms in this world, cells achieve their functions of migration, proliferation and differentiation through a complex system of communication known as cell signaling. In general, a standard signal transduction process involves detection of stimulus on the cell membrane; receptors help to pass information through membrane; transmission of intracellular signals (second messenger); and the final affects which can adjust the cell's behavior [34].

PTEN and PI3K/AKT/mTOR Pathway

PTEN becomes well-known since it was realized to be a tumor suppressor gene [35]. Accumulating evidences indicated the important function of PTEN in dephosphorylating reaction which involved not only in cell division but also in cell migration [36]. However,

PTEN alone cannot achieve its complete function. More downstream proteins get involved and formed the signaling network called PI3K/Akt/mTOR pathway. This is an intracellular pathway which plays essential roles in the cell process of apoptosis and was regarded as a potential therapeutic target in many types of cancers. mTOR (mammalian target of rapamycin) is the key protein kinase in this pathway. It directly and indirectly targets to several components of ribosome recruitment machinery, thereby modulating the protein synthesis, transcription and cell cycling [37]. While, for the other two important elements - Akt and PI3K, both of them showed their positive regulating function on mTOR [38 - 41].

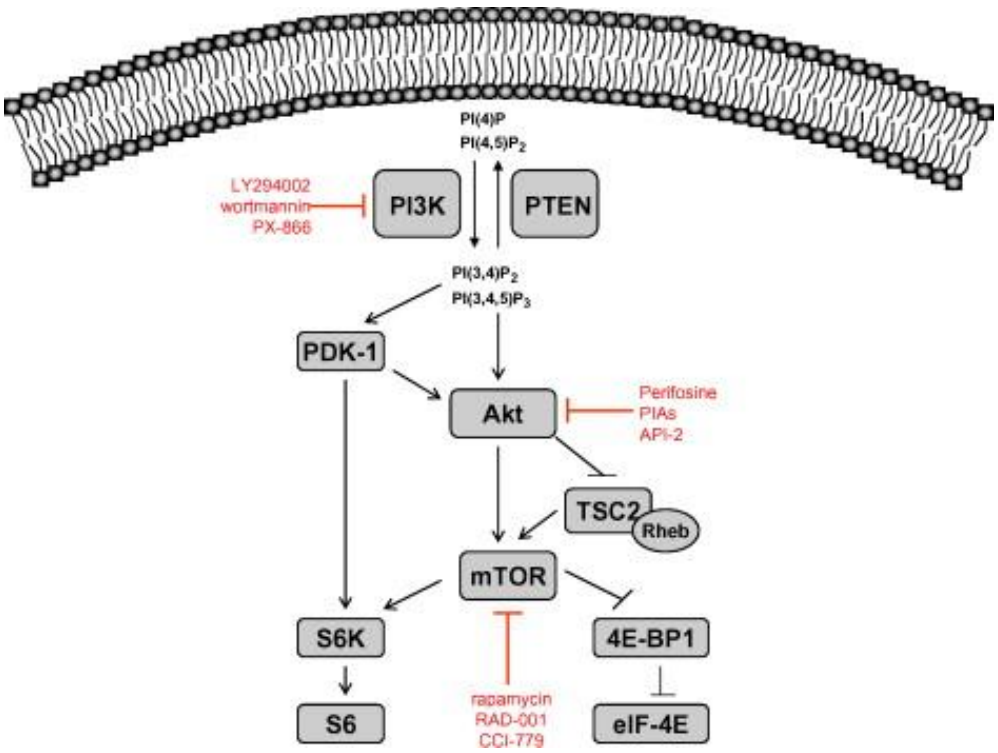

Figure 1. PI3K/AKT/mTOR pathway, loss of PTEN accelerates s synthesis of Ptdlns(3,4,5)P(3) thus activating Akt and mTOR (picture cited from [15]).

In order to initiate the PI3K/Akt/mTOR chain, the accumulation of a second messenger of PI3K called Phosphatidylinositol 3,4,5-trisphosphate (Ptdlns(3,4,5)P(3)) is necessary. However, PTEN (phosphatase and tensin homolog on chromosome 10) which is a phosphatidylinositol-3 phosphatase has the ability to dephosphorylate Ptdlns(3,4,5)P(3) and convert it into Ptdlns(4,5)P(2), therefore inhibiting the Ptdlns(3,4,5)P(3)/Akt signaling [42]. That is to say, the normal expression of PTEN gene can negative regulates the PI3K/Akt/mTOR pathway. A lot of studies showed the loss of Pten can lead to myeloproliferative disorders and acute leukemia in the end [43 - 45]. Mediated by different Cre-loxP system (VEC-Cre [43], LysM-Cre [44] and Mx1-Cre[45]), the progressing of leukemogenesis maybe at different paces, but the leukemogenesis of losing Pten is confirmed.

CDKs

A complete cell cycle can be divided into 4 directional phases: G1 (Gap1) phase, S (Synthesis) phase, G2 (Gap2) phase and M (Mitosis) phase [24]. Cyclin-dependent kinases

(CDKs) work at the checkpoints between phases to decide if the cell has already prepared for entering to the next stage [24]. Among all of the Cdk family (from Cdk1 to Cdk20) , Cdk1, Cdk2 and Cdk4 have more outstanding performance in regulating cells' status from quiescent to proliferation, while other Cdks are believed to function mainly in the transcription process [46]. Recent studies showed that the tissue regeneration and tumor-associated cell cycle is often medicated by the Cdks. [47] And the disorder of the Cdk expression leads to the cell proliferative disease [48 49]. It indicates that Cdk genes may also be considered as a drug target in gene therapy for cancer or even leukemia. In this research, the role of Cdk1 and Cdk2 in the development of leukemia and hematopoiesis were studied in vivo.

CDK1

As the original member of the Cdk family, Cdk1 was reported to have dominant performance in mammalian cell division and DNA replication [47 50]. Previous researches pointed out that the over-expression of Cdk1-CyclinB can lead to the premature entering to M phase and disorder of cell division, which indicated Cdk1-CyclinB complexes are highly required for proper procedure of mitosis [51]. Another important cyclin associate with Cdk1 is the A type cyclin. The complex formed between them mainly regulates the DNA replication during G2-M transition [52].

CDK2

As another important member of the interphase Cdks (iCdks), CDK2 was reported to play essential roles in completing G1 phase and starting S phase [53]. Specifically, CDK2 can bind to E-type cyclins (Cyclin E1 and E2) to start the replication assembly and also interacts with cyclin A to initiate the DNA synthesis [54]. A direct correlation between CDK2 and human cancer haven't been found yet. However, a lot of experiments confirmed the absence of CDK2 may lead to efficient proliferation in some cell lines, and the inhibition of the CDK2 inhibitor can also cause the development of tumors [55 - 57]. These evidences indicated the potential involvement of CDK2 in controlling cell proliferation and tumorigenesis.

$p27^{KIP1}$

$p27^{KIP1}$, encoded by the CDKN1B gene, is one of the Cip/Kip family proteins. They can inhibit the activity of specific CDKs and negatively regulate the proliferation process of the cell [48]. For $p27^{KIP1}$, as shown in the Figure 2A, it mainly inhibits the Cdk2-CyclinE complex, thus block the cell cycling progress in the G1 phase [24]. Moreover, not only with Cdk2, P27 also play roles in regulation of CDK6-CyclinD and CDK4-CyclinD [48]. Although the mutant of P27 is rarely detected in the case of human cancer, the decrease of $p27^{KIP1}$ content in several tumor cell lines was confirmed by some previous studies [58]. These linkages between the protein and tumor cells make $p27^{KIP1}$ an interesting target in the research of cell proliferation and human cancer.

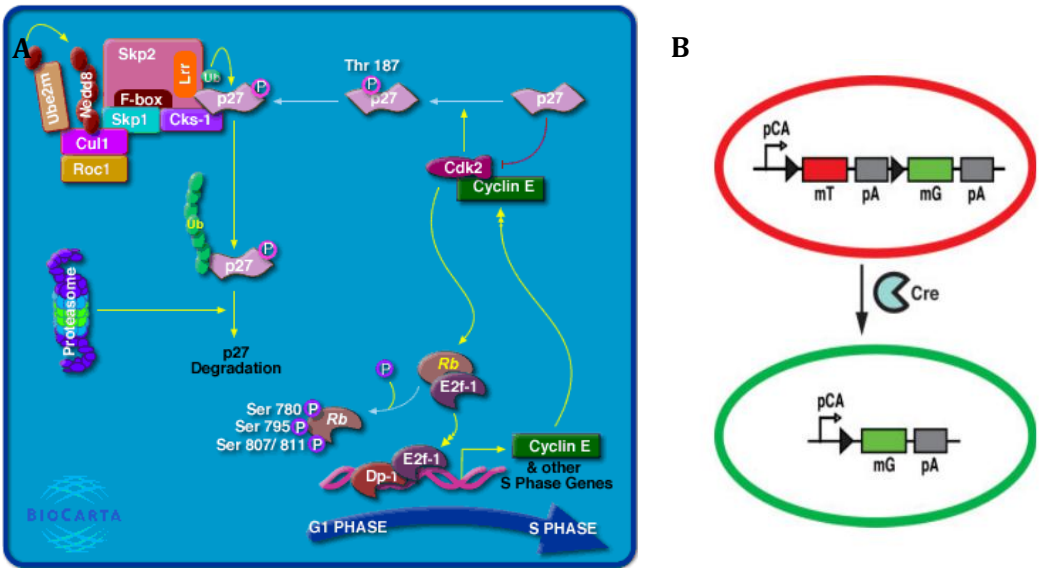

Figure 2 A. Signaling Pathway involving P27, P27 rests Cdk2/CyclinE complex thereby stopping cells entering S phase (picture cited from [66]); **B**. Without Cre, only mT get expressed and the DNA transcription stops at the first pA. However, when Cre is expressed, the first mT-pA sequence is knocked out and only mG gets expressed (picture cited from [59]).

mT/mG

Known as the global double-fluorescent Cre reporter gene, mT/mG gives its carrier an ability to show how the cre-loxp system works. As a double Cre-reporter, mT/mG expresses tandem dimer Tomato (tdTomato) which is a kind of red fluorescent protein (RFP) on the cell membrane before recombination and then expresses EGFP after cre's excision. The mT/mG construct consists of a chicken beta actin promoter with a CMV enhancer (pCA) and a loxP-flanked mT sequence following by an mG sequence as shown in Figure 2B [59] .

3. METHOD

3.1 ANIMAL PROCEDURES

3.1.1 BREEDING

Mice with conditional *Pten*^{fl} alleles [45 60] (designated *P*) with a mixed genomic background of 129S4/SvJae and C57BL/6J were bred with *P27*$^{fl/fl}$ mice (designated *P27*) to generate PP27 mice (*Pten*$^{fl/fl}$ *P27*$^{fl/fl}$). PP27 mice were bred with mice harboring the interferon (IFN)-inducible Mx1-Cre transgene [31 32] (designated *M*) to generate PP27M mice (*Pten*$^{fl/fl}$ *P27*$^{fl/fl}$ Mx1-Cre). PP27M mice were compared with littermate PM and P27M mice; P, P27 and PP27 mice were used as healthy controls (designated Ctr). Other floxed gene such as CDK1$^{fl/fl}$ and CDK2$^{fl/fl}$ are also crossed into the *Pten*$^{fl/fl}$ Mx1-Cre mice line by the same means. The mice were housed by MEB (Department of Medical Epidemiology and Biostatistics), under controlled environmental conditions with free

access to water and food. Illumination was on between 0600 and 1800 h. All mice were monitored daily and experimental protocols were approved by the regional ethical committee of the University of Gothenburg, Sweden.

3.1.2 MICE INJECTION

In order to active the Cre-Lox system, thereby conditionally knocking out the target gene. Intraperitoneal (IP) injection of 400ug polyriboinosinic /polyribocytidylic acid (pIpC; Sigma, St Louis, MO) was performed for both the experimental and control mice. In this study, 4-week-old mice in each group received 3 injections every 48 hours for the complete gene deletion. (Figure 3)

Figure 3. Injection of pIpC.

pIpC is a kind of immunostimulant which has a similar structure with double-stand RNA. It can interact and activate the B-Cells, dendritic cells and macrophages thereby inducing immune responses. The solid pIpC is prepared into 1mg/ml solution with water and froze in -80 for storage. When injection needed, pIpC solution is taken out and slowly melted on ice.

3.1.3 EUTHANASIA

Mice were euthanized with isoflurane inhalation followed by cervical dislocation. For the mice kept for survival studies, they will be euthanized when the mice get ruffled fur and become listless or lose more than 10% of body weight.

3.2 GENOTYPING

Genotyping was performed by PCR amplification of genomic DNA extracted from mouse tails. The *Pten*^{fl} allele was detected with forward primer 5'-CAA GCA CTC TGC GAA CTG AG-3' and reverse primer 5'-AAG TTT TTG AAG GCA AGA TGC-3', yielding a 328-bp fragment from the *Pten*^{fl} allele and a 156-bp fragment from the *Pten*$^{+}$ allele. The *P27*^{fl} allele was detected with forward primer 5'-TAG GGG AAA TGG ATA GTA GAT GTT AGG ACC-3' and reverse primer 5'-GGT ATA ATA TGG AAA GTG ACT CTA ATG GCC-3', yielding a 400-bp fragment from *P27*^{fl} allele and a 370-bp fragment from the *P27*$^{+}$ allele. For *CDK1*^{fl} allele, forward primer 5'-GGA GCT TGC TTA GCT CCT ACT TT-3' (PKO 651) and reverse primer 5'-CAG GCC AAC CTG GGT GAG CTA GAG-3' (PKO 652) were applied to yield a 390-bp fragment from *CDK1*^{fl} allele and a 260-bp fragment from the *CDK1*$^{+}$ allele.

For *CDK2*^{fl} allele, forward primer 5'- CAA GCA CGA ACC AAC ACA CAT AAG T -3' (PKO 1131) and reverse primer 5'- GCG CCA CCA CTG CTT GGC TCA GAT A -3' (PKO 1101) were used to yield a 441-bp fragment from *CDK2*^{fl} allele and a 342-bp fragment from the *CDK2*$^{+}$ allele. The Mx1-Cre transgene was detected with forward primer 5'-GCG GTC TGG CAG TAA AAA CTA TC-3' (oIMR 1084) and reverse primer 5'- GTG AAA CAG CAT TGC TGT CAC TT-3' (oIMR 1085), and the amplified fragment was 100-bp.

Electrophoresis

The phosphate groups give negative charges to DNA and make it move toward anode during the electrophoresis. The gel matrix limits the speed of DNA migration and relates the moving rate to the DNA size. DNA with larger molecules meets greater resistance and moves slow, while the smaller DNA moves faster.

Staining

DNA can be visualized under UV light, after stained with the ethidium bromide. Ethidium bromid will fluoresce with an orange color and this fluorescent signal will be amplified 20-fold after binding to DNA. By comparing with the DNA ladder, the size of the tested DNA sequence can be approximated.

3.3 TISSUE AND CELL SAMPLES

Three weeks after injection, groups of mice were sacrificed and their tissues were harvested for further analysis. Newly harvested tissues were soaked in ice-cold Dulbecco's Modified Eagle Medium (Gibco® DMEM) for temporary keep and transportation. For femur and tibia, after being cut off, they would be placed into the 5ml Eppendorf tube and kept on ice.

3.3.1 CELL SUSPENSION

Cell suspension was made and stored for cell culturing, flow cytometry analysis and PCR. 50mg of target tissue was placed in a petri dish on ice and minced into slurry by scalpel blades. Dissolve the chopped tissue into 1ml ice-cold PBS, and pipette the sample up and down for 10 times by using a 3ml syringe with an 18 gauge needle. Then pipette the sample with a 21 gauge needle to have a complete homogenization. Transfer the sample to a 1.5ml Eppendorf tube and centrifuge at 300g for 5 min at 4 degree. Decant supernatant liquid and resuspend the cell pellet in 1ml PBS. Centrifuge the cell suspension again with above settings. After repeating the washing process for one more time, the cell suspension is ready.

For long-term preservation, cells need to be preserved in the frozen state to retain their viability. In this case, cell pellet need to be resuspended into the freezing media and store in Freezing Containers to -80^{o}C immediately. And transfer to -180^{o}C after an overnight cooling down process.

3.3.2 HISTOLOGY

In order to monitor pathological changes caused by diseases, the intact structure of the tissue need to be observed and analyzed. Sectioning was performed and slices were observed by microscope for this purpose.

Fixation

To prevent the tissue from both intrinsic and extrinsic damage, fixatives such as formalin and paraformaldehyde (PFA) were applied. In this study, harvested organs were fixed in 4% PFA which is 20 times the volume of the tissue for at least 24 hours. In the case of bone, 15 ml PFA is needed for each tibia/femur, and the fixation time should be no less than 72 hours.

Decalcification

Unlike other organs, an additional procedure of decalcification is necessary for softening the bone samples. EDTA is a commonly used chelating agent in biology and was applied in this study. A tibia/femur sample needs to be soaked in 10ml EDTA reagent for ten days. And the EDTA with bone sample should be refreshed every other day.

Dehydration, clearing, infiltration and Embedding

These processes are automatically done by the dehydration machine in this project (Figure 4A).

Sectioning

And the produced wax blocks are ready for sectioning. Vertical sectioning perpendicular to the surface plane is applied for all of the tissues. 8-micrometer-thick tissue sections were made on the microtome (Figure 4B). For bone samples, since the high cell density of the bone marrow, thinner sections (4.5 micrometer) are made to get a satisfying microscopic image.

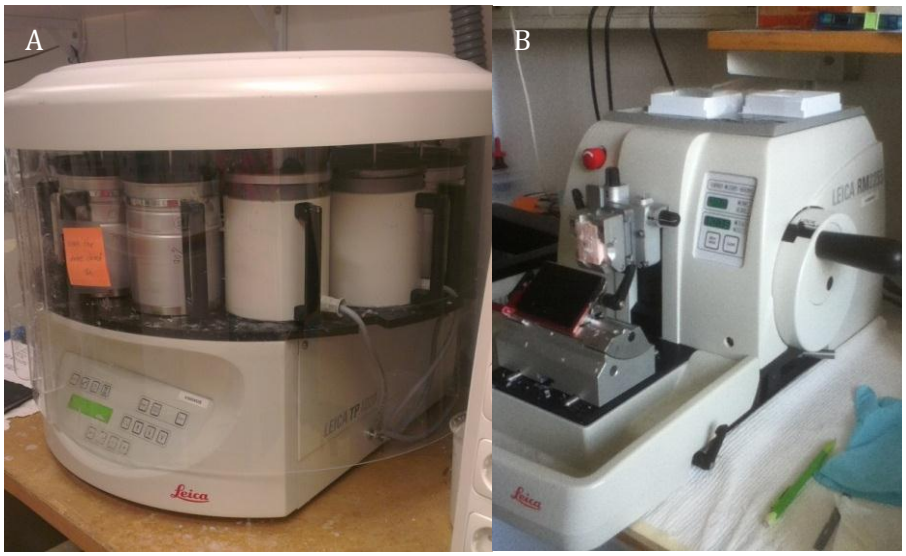

Figure 4 A. Dehydration machine; **B**. Sectioning machine.

3.4 Western Blotting

Harvest tissue pieces can also be used in immunoblot analysis. Proteins in the cells were extracted by lysing the target tissue with the ice-cold lysis buffer(50 mM Tris-HCl, 120 mM NaCl, 5 mM MgCl2, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 1% NP-40, 20 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 2mM orthovanadate, and the Complete Mini protease inhibitor cocktail). Lysates were homogenized, and centrifuged at 20000g

for 20 minutes, and equal amounts of total protein of the supernatant were size-fractionated on 10% to 15% sodium dodecylsulfate polyacrylamide gels. The proteins were transferred onto nitrocellulose(NC) membranes and incubated with antibodies recognizing phosphorylated ERK1/2 (#9106), total ERK (#9102), phosphorylated AKT (#9271), PTEN (#9559), p27KIP1 (2552; Cell Signaling, Danvers, MA), Cyclin D1(#sc-718), Cyclin D3 (#sc-182) and Beta-actin (#sc-47778; Santa Cruz Biotechnology, Inc., Dallas, TX). Protein bands were visualized with a horseradish peroxidase–conjugated secondary antibody (#170-5046 and #170-5047; Bio-Rad Laboratories, Inc., Hercules, California) and the Enhanced Chemiluminescence kit (Amersham, Little Chalfont, Buckinghamshire, United Kingdom).

3.5 FLUORESCENCE-ACTIVATED CELL SORTING (FACS)

Splenocytes and bone marrow cells were incubated with antibodies and analyzed with FACS Diva software (BD Biosciences, San Jose, CA, USA) in this study.

3.5.1 ANTIBODIES

Antibodies which are labeled with different fluorescent groups, can bind specific surface proteins (Cluster of Differentiation or CD), thereby targeting certain cell population.

A lot of antibodies were used in this project. CD4 (#342447) was used to identify T helper cells, monocytes and so on. CD8 (#342447) is widely expressed on cytotoxic T cells. CD3 (#342447) expresses on both T helper cells and cytotoxic T cells, and can therefore be used to separate T cells' population from B cells in the blood or tissue samples. CD45 (#342447) is used to distinguish hematopoietic stem cells from the bone marrow. CD45R/B220 (#550286) is a B-cell marker, and is expressed on B lymphocytes at all stages from early pro-B cells to mature B cell. CD11b/Mac1 (#552850) targets many leukocytes in the innate immune system. Ly-6G&ly-6C (#552985) helps to find out the immature cell from the bone marrow, but not for erythroid cell lineage. CD34 (#553733) is normally found on the hematopoietic progenitor/stem cells in the bone marrow. Sca-1 (#561228) expresses on many hematopoietic stem cells (HSC). CD117/c-kit (#553356) is used to identify HSC, multipotent progenitors (MPP), common myeloid progenitors (CMP) from bone marrow cells. lineage cocktail/Lin (#340546, BD Biosciences, Becton,Dickinson and Company.), – is a mixture with CD3, CD14, CD16, CD19, CD20 and CD56, which can bind from monocyte to macrophage and almost all kinds of lymphocytes, this cocktail helps to mark all the mature cells.

3.5.2 CELL TYPES AND LABELLING

Hematopoietic stem cells (HSCs) in the BM generate all types of blood cells. The antigens expressed on the cell membrane provide information about the cell type and differentiation stage. Monitoring the expression of these antigens helps to estimate the progression of hematopoietic diseases. (Figure 5)

Common myeloid progenitor (CMP): lin-, SCA-1-, c-kit+, CD34+, CD16/32mid

Granulocyte-macrophage progenitor (GMP): lin-, SCA-1-, c-kit+, CD34+, CD16/32hi

Megakaryocyte-erythroid progenitor (MEP): lin-, SCA-1-, c-kit+, CD34-, CD16/32low

Long-term hematopoietic stem cell (LT-HSC) : lin-, C-kit+, SCA-1+, Mac-1 (CD11b)lo, CD135-, CD34-

Short-term hematopoietic stem cell (ST-HSC) : lin-, C-kit+, SCA-1+, Mac-1 (CD11b)lo, CD135-, CD34+

Multipotent progenitor cells (MPP) : lin-, C-kit+, SCA-1+, Mac-1 (CD11b)lo, CD135+, CD34+

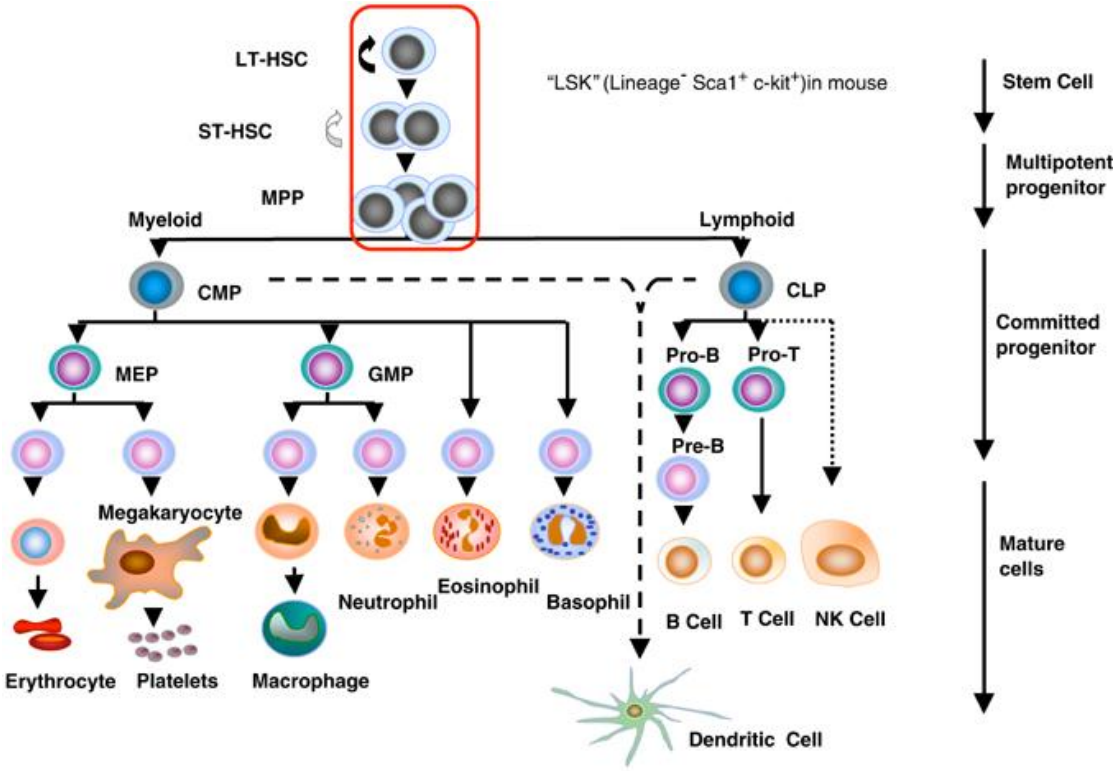

Figure 5. Haematopoietic lineage tree (picture cited from [67])

3.5.3 GATING

Since the cell suspension is a mixture of living cells, dead cells and cellular debris. Gating process helps to identify specific cell types according to their morphology and fluorescent markers. Although this is a subjective work, some routine steps need to be followed.

For cells

To get rid of cellular debris and other impurities, the first gating is based on the size and transparency (FSC v.s. SSC). Small particles might be cellular fragments, while large ones could be cell cluster. It also seems impossible for a cell to be completely transparent or opaque. Given the above consideration, the gate was put in the middle of the dot plot.

For singlets

In the setting of the machine, FSC-H and FSC-A should be linearly correlated [68]. By gating the diagonal cell population, the singlets (round objects) can be separated from the plot of FSC-A v.s. FSC-H.

For live cells

The change of permeability on the cell membrane is the most discernible differences between live and dead cells. Some fluorescent indicator can permeate into the dead cells and bind to intracellular substances. However, for the live cells, their semipermeable membrane can prevent the migration to the interior of the cells.

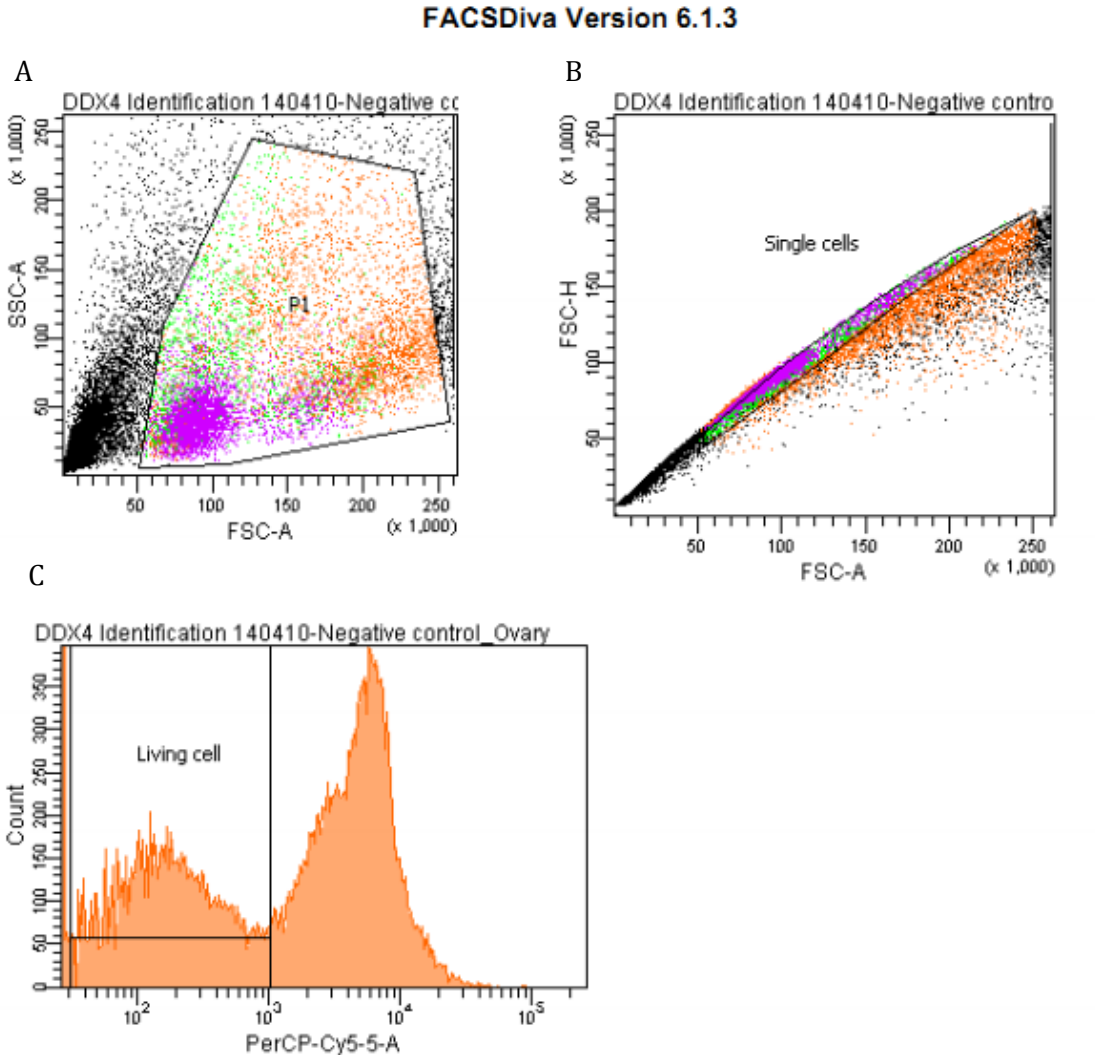

Figure 6 A. Gating in the FSC direction gets rid of the very small and very large particles, and the gating in SSC direction keep completely transparent or opaque particles out of consideration; **B**. FSC-A v.s. FSC-H helps to assess the cell clumping; **C**. Chromosome dye is used to target the dead cells.

3.6 BLOOD TESTING AND SMEAR

Blood was taken weekly from tail veins and temporarily stored in an EDTA-coated tube (Microvette, CB 300; Sarstedt, Nümbrecht, Germany). Around 100 ul blood was collected from each mouse and analyzed with a hematology analyzer KX-21 (Sysmex Europe, Norderstedt, Hamburg, Germany).

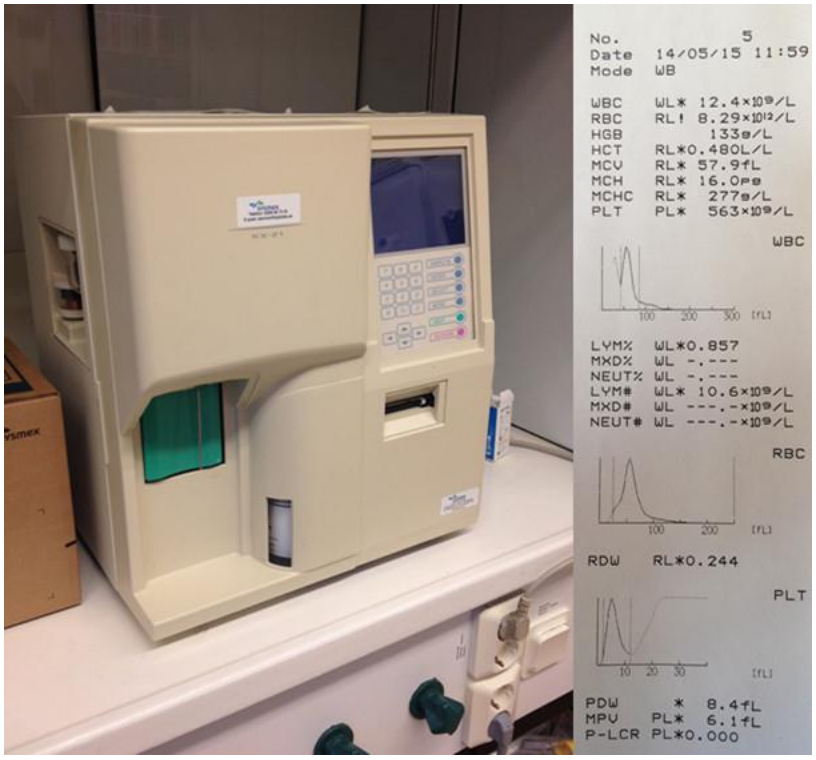

Figure 7. Blood testing machine and analysis report.

5 to 10 ul blood samples were spread on the microscope slides followed by air-dry and Wright-Giemsa staining. (Figure 8)

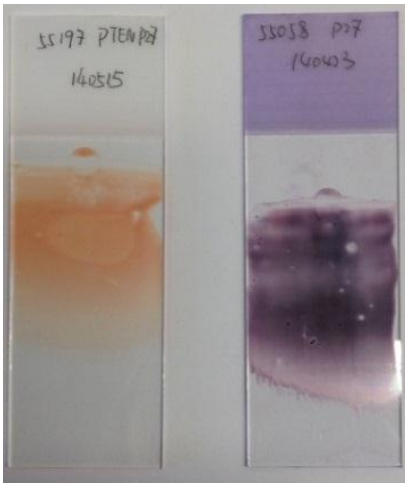

Figure 8. Blood smear before (left) and after (right) Wright-Giemsa staining.

3.7 Colony-Forming Cell (CFC) assay

splenocytes (1×10^5) and bone marrow cells (2×10^4) harvested from experimental mice were seeded in duplicate wells in methylcellulose medium (MethoCult M3434; StemCell Technologies, Vancouver, BC, Canada). Cells were cultured in the environment of 37C, 5% CO2 and >95% humidity for six days, then the numbers of colonies were scored.

LEUKEMIC CFC ASSAY

For bone marrow samples, when leukemic cells involved, these immortal cells are able to form clusters infinitely, while normal stem cells have limited replating ability. The number of cell clusters after replating is an important assessment of leukemia [61]. In this project, after counting the cell clusters on the 6th day, the cultured BM cells were washed, collected, and replated for the next round of incubation.

4. RESULTS

4.1 THE EXAMINATION OF KNOCKOUT EFFECTIVITY

By introducing the mT/mG gene into Mx1-Cre mice line (designated *TGM*), the performance of Mx1-Cre system can be evaluated by detecting the fluorescent signal from the cell membrane. TGM mice group were injected 200ug pIpC at the age of 28 days, and were sacrificed at the age of 48 days. Harvested tissue and BM were made as sections and BM smears, respectively. Without any stain, these slices were observed under fluorescent microscopy. As shown in Figure 9, bone marrow cells form the control group express red fluorescent signal on the membrane. However, in the sample of *TGM*, most of the cells turn into green color, which means the loxp-cre system has been activated successfully.

Some cells were still expressing red fluorescence signal in figure 9B, which demonstrate that the efficiency of the Mx1-Cre activation cannot reach 100%. Based on some previous studies, 250ug/mouse pIpC injection gives 100% of floxed gene deletion in liver, 94% in spleen [32] and only 50% in bone marrow [62].

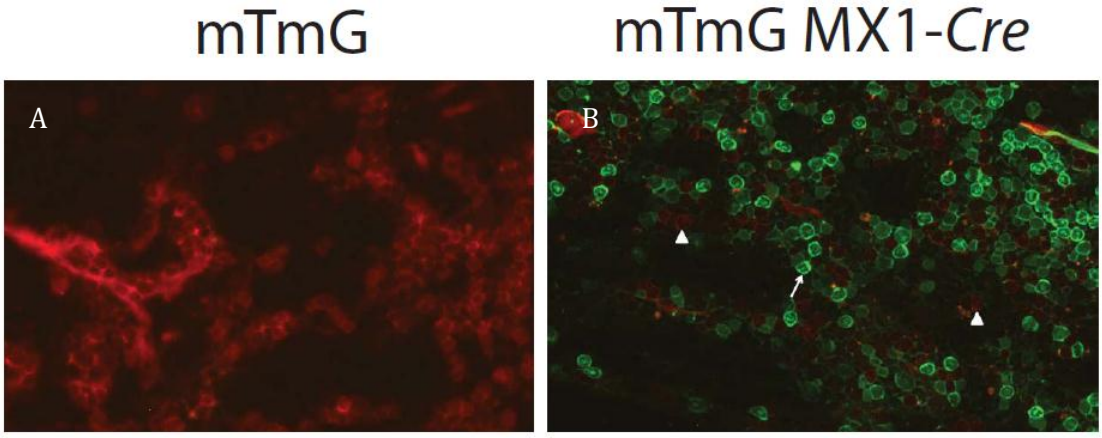

Figure 9 A. Bone marrow smear from mouse injected pIpC at age of 4 weeks without Mx1-Cre; **B.** Bone marrow smear from mouse injected pIpC at 4 weeks age with Mx1-Cre, the activation of loxp-Cre system is comfirmed.

In order to achieve higher efficiency, larger volume and multiple times of pIpC injection were applied (3 times for every 2 days, 400ug/time). The knockout of gene was also monitored by PCR testing as shown in Figure 10.

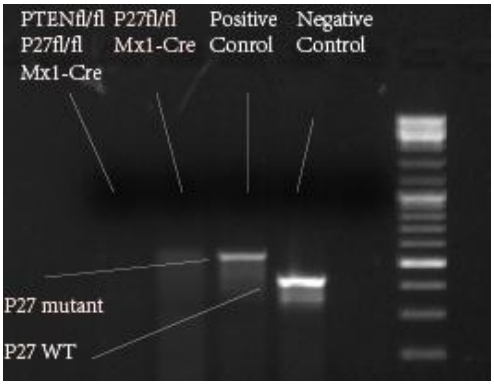

Figure 10. Four spleen lysates from each group were examined, the loss of *P27*^{kip1} is detectable by PCR.

Western blotting was applied to check the expression of the target protein. This method can also be used to verify the knockout of genes from the protein level. Clearly shown in the Figure 11, *PP27M* showed very weak signal in both PTEN and P27 cases. However, for the *PM* group, the P27 were still normally expressing, but PTEN protein was highly suppressed. On the contrary, samples from P27M group showed highly expressed PTEN while loss of P27.

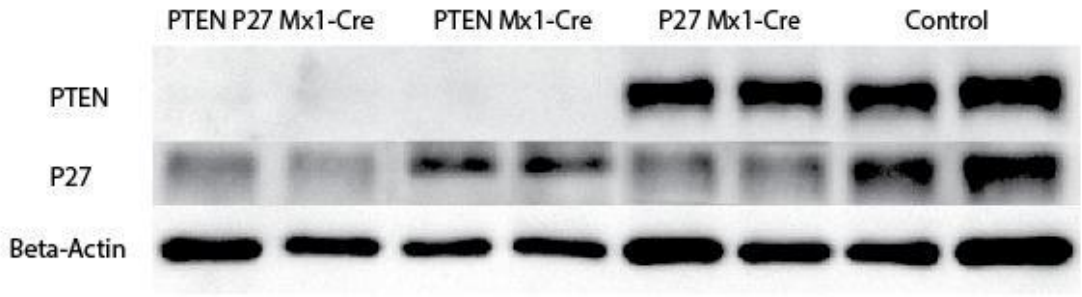

Figure 11. Western blot results confirm the knockout of Pten and P27kip1; from the case of PTEN Mx1-Cre group, the loss of Pten also clearly decreases the expression of P27kip1. Beta-Actin works as the control.

4.2 P27 IN THE NORMAL HEMATOPOIETIC SYSTEM

The health state of *P27* mice was monitored following the rules from Laboratory Animals [63]. Body weight and blood information were recorded every two weeks. In both female and male P27 KO group, a remarkable increase of overall weight can be observed.

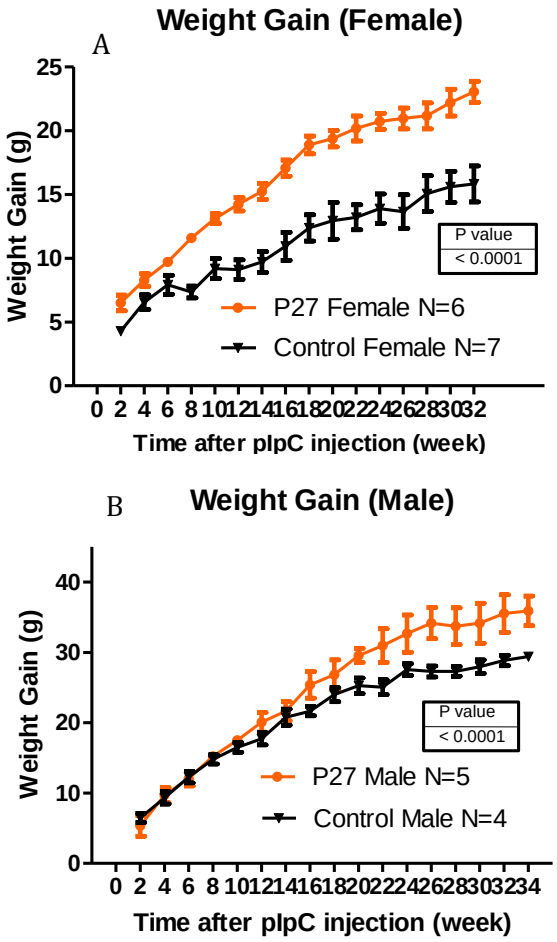

Figure 12. Loss of P27 gain weight to mice in both female (A) and male (B) cases.

However, the ratio of organ weight and body weight doesn't change a lot. A slight increase of spleen-body rate can be detected in P27M group as shown in Figure 13, but the difference was not significant (P>0.05). The same pattern can also be found in the case of thymus, liver and kidney (data not shown). All the organs functions well and no visible pathological changes can be found by microscopy. The loss of *P27^{kip1}* in hematopoietic system cannot cause any lethal side effects for the adult mice (Figure 14).

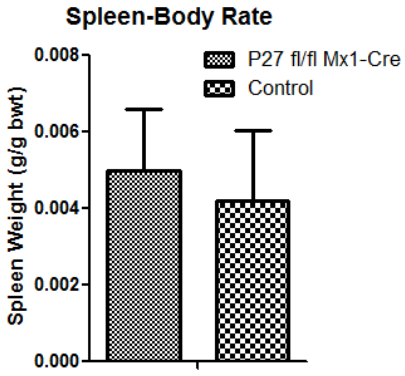

Figure 13. Spleen-body rate from P27M group and control mice at the same age were compared, no significant difference (P>0.05).

4.3 LOSE OF P27 IN LEUKEMIA MICE MODEL

To determine the impact of combined deficiency of PTEN and p27KIP1 in the hematopoietic compartment, pI-pC was injected into PP27M, PM, P27M, and Ctrl mice.

4.3.1 SURVIVAL CURVE

Consistent with previous studies [64], all PM mice died from MPN by 98 days after pI-pC injections (median survival 62 days), whereas P27M and Ctr mice lived much longer and no MPN phenotype was observed in P27M mice. However, the maximum survival of PP27M mice was 30 days (median 24 days; $p < .001$ versus PM; Figure 14).

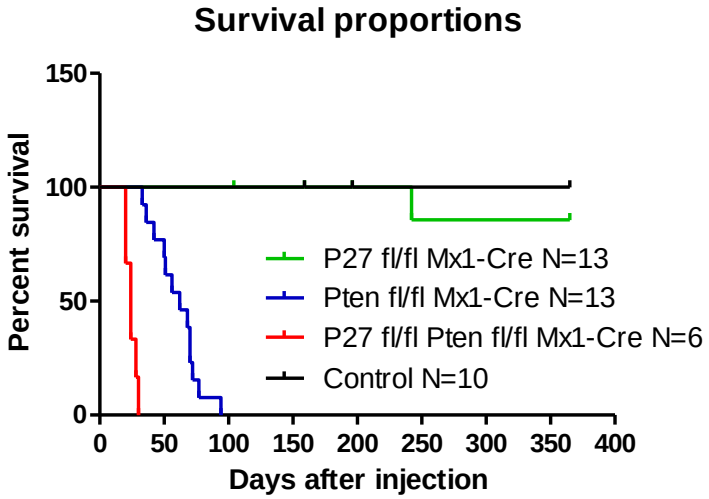

Figure 14. Survival cure for all experimental groups after pIpC injection at week 4, PP27M group have the shortest average lifespan (24 days), followed by PM group (62 days).

4.3.2 ENLARGEMENT OF ORGANS

2 weeks after the first pIpC injection, mice from all 4 groups were sacrificed. Tissues were taken pictures and weighed. The swelling of spleen was clearly shown in figure 15.

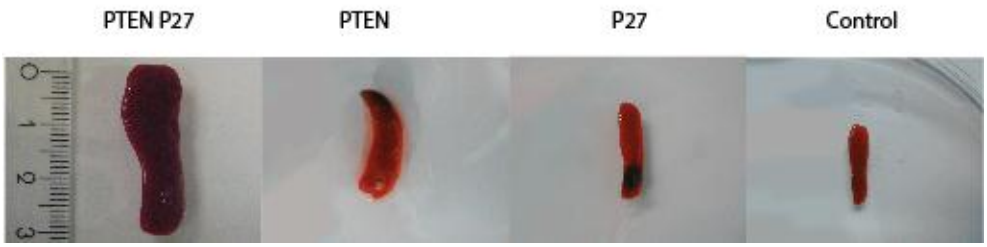

Figure 15. Spleens from different groups of mice were taken pictures and compared. The swelling of the tissue in PP27M group is obvious. Comparing with the Ctrl group, spleen samples from PM group also witness a mild size increase.

The similar result can also be found in the case of liver and thymus (pictures not shown). In Figure 16, the differences of organ-body rate were compared. Spleen and liver weights in PCM mice increased by 2.3–5.6 fold and 1.2–2.4 folds, respectively, compared with PM, CM, and Ctrl mice. Student's t-tests were applied for all of these data, and the differences were significant ($P<0.05$).

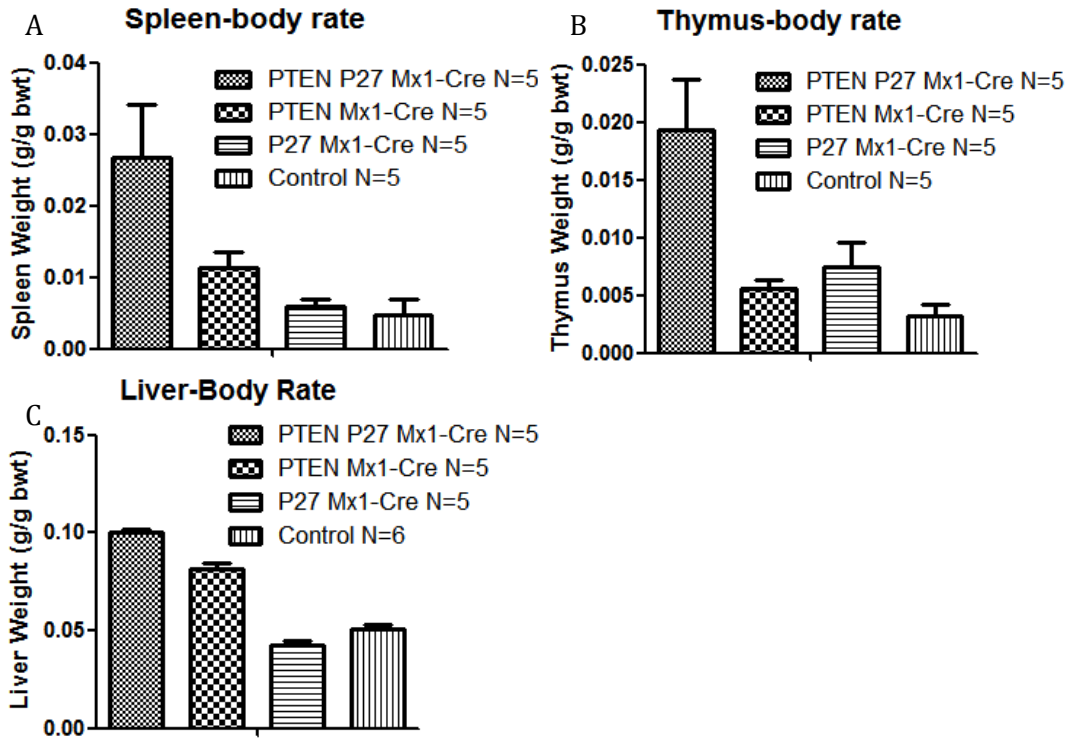

Figure 16 A. Average weight of spleen from PP27M group increased by 5.6 fold comparing with the Ctrl group; **B.** Weight of thymus from PP27M group increased by 6.1 fold comparing with the Ctrl group; **C.** Weight of liver from PP27M group increased by 2.1 fold comparing with the Ctrl group.

4.3.3 BLOOD TESTING

Two weeks after pI-pC injections, white blood cell counts were 20.8 × 10^9 cells/L in *PP27M* mice compared with mean counts of 18.3 × 10^9, 13.9 × 10^9, and 13.6 × 10^9 cells/L for *PM*, *P27M*, and *Ctrl* mice, respectively (Figure 17A). The content of hemoglobin in *PP27M* group (96 g/L) was decreased by around 2/3rd comparing with the *Ctrl* group (148 g/L) (Figure 17B).

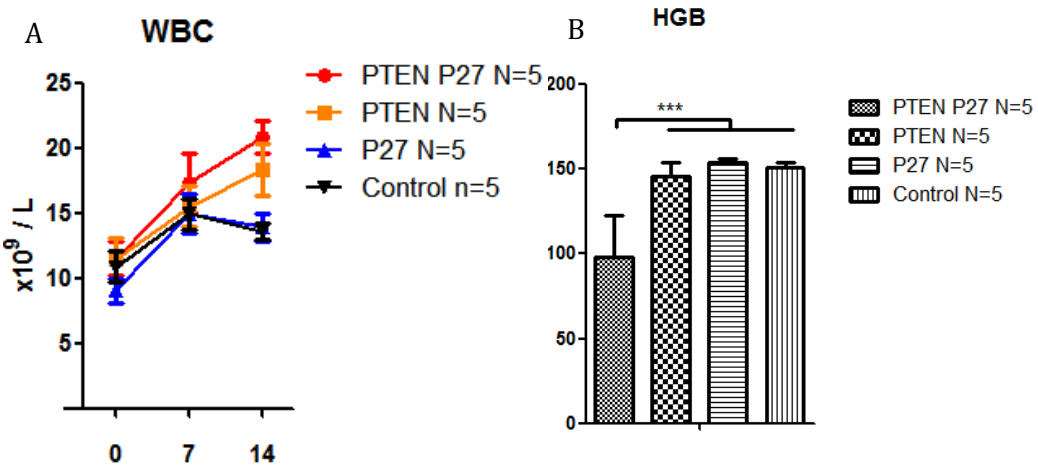

Figure 17 A. White blood cell count increased sharply in the PP27M and PM group but not in P27M group; **B.** Rapid decrease of hemoglobin content was only observed in the PP27M group.

4.3.4 *BLOOD SMEAR AND TISSUE SECTION*

The increase of the nucleated cells could be detected by blood smear (Figure 18A). However the differences in cell count are not statistically significant. A high level of myeloid blast cell infiltration can be found in the liver section from *PP27M* mice (Figure 18B), while in the other groups of samples, the symptoms were imperceptible.

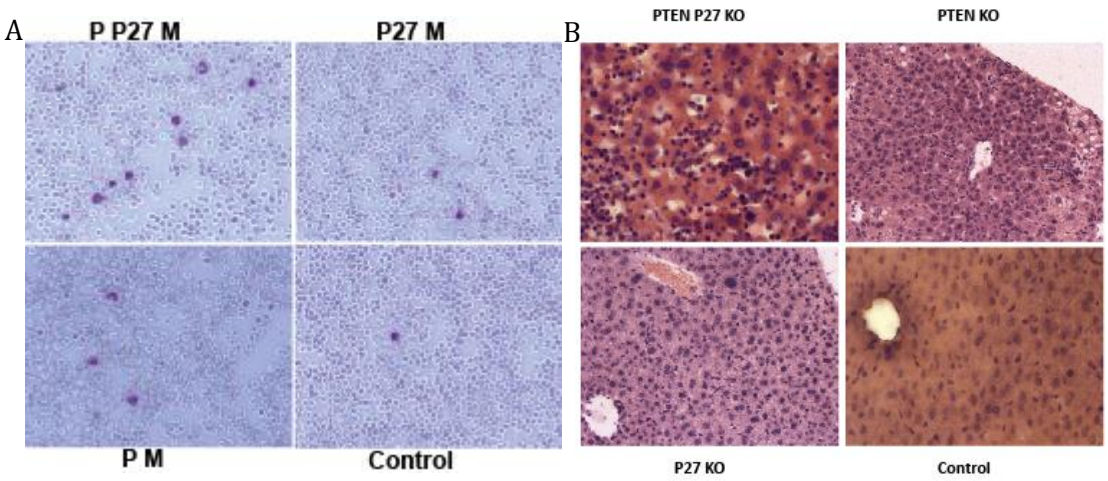

Figure 18 A. A mild increase of nucleated cells can be observed in PP27M group from the blood smear;
B. Myeloid blast cell infiltration can be found in the liver section in the case of PP27M mice

More tissues from Immuno system were examined. However, no morphological changes and no increase in the amounts of immature cells, including myeloblasts, could be detected in the blood and bone marrow in PCM mice compared with the other three groups. Even in the extremely enlarged spleen sample, we only detected the loss of red pup and internal bleeding (Figure 19).

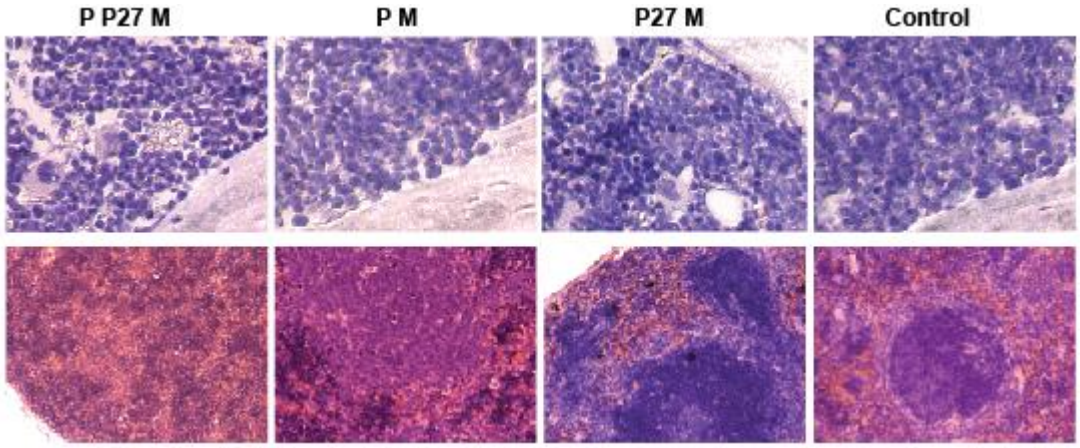

Figure 19. Spleen and bone sections were compared among all groups

4.3.5 *COLONY GROWTH*

To confirm the cell type in the harvested tissue (BM and spleen), CFU analysis and leukemic CFU analysis were applied.

BM

Figure 20 shows the percentage of the progenitor cells among the BM samples after 1 week of culturing and another week of replating. After replating(Figure 20B), cell

number in the control group remain the same comparing with the result of first week, while other groups (PP27M, PM and P27M) witness an explosive increase in cell number.

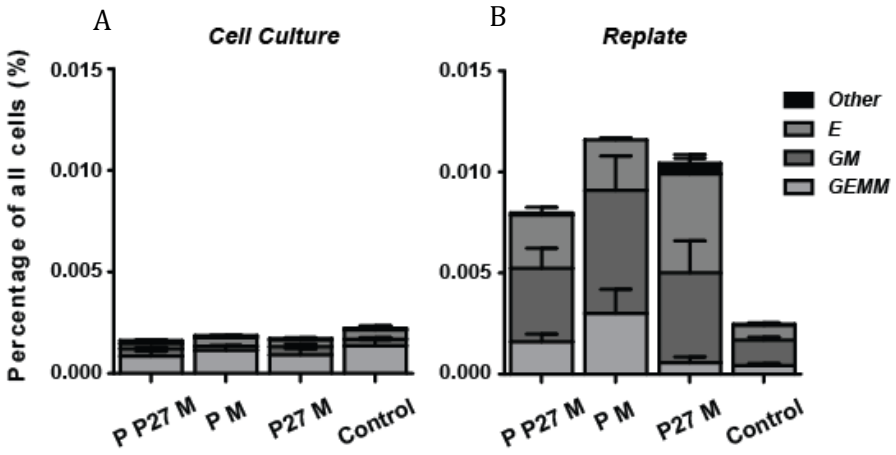

Figure 20 A. After one week of incubation, the count of cell clusters was recorded. By dividing the original plated cell number, the percentage of stem cells or progenitors was calculated. **B.** After replating, cell number in PP27M, PM and P27M groups witness an explosive increase, while Ctrl group maintains the same cell count.

Spleenocyte

Culturing for the splenocyte always gives much less cell clusters comparing with the BM cells. However, in the *PP27M* group, the amount of splenocyte clusters shared the same level with its BM outcome (around 0.0015%). None of groups returned positive results in leukemic CFU analysis (no cluster formed after replating).

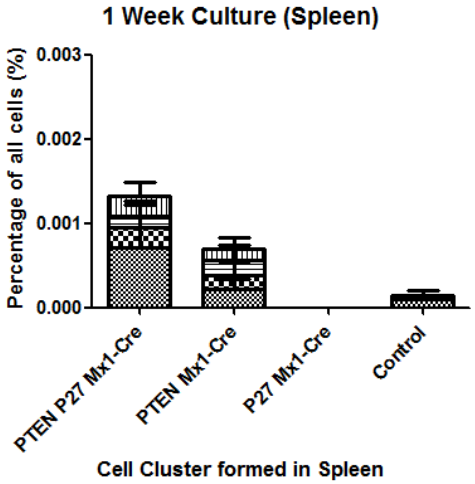

Figure 21. Splenocytes form fewer cell clusters comparing with the BM sample, and no cell cluster can be observed in all of the experimental groups after replating.

4.3.6 FACS ANALYSIS

FACS analysis was performed to have some detailed information about cell components in blood samples, BM samples and spleen samples. In this study, the antibody CD45,

together with CD34, are used to target the hematopoietic stem cells. Mac-1 and Gr1 are working together to identify the myeloid-derived suppressor cells (MDSC) which are strongly immunosuppressive and thought to be a subset for tumor-infiltrating lymphocytes. Cells expressing lin-, sca-1+ and c-kit-1+ are known as LSK cells (or KSL cells), which is regarded as the most primitive hematopoietic stem cells. At last, CD4, CD8 and CD3 were used to identify the T cells and B cells.

Peripheral blood (PB)

For PB samples, B cells, T cells and infiltrating lymphocytes were marked and compared among different groups. Both PM and PP27M group have an obvious decrease in the content of (mostly T helper cells) and distinct increase of the Mac-1+ Gr-1+ cells (MDSC). A slight decrease of B cells can also be detected in both PP27M and PM group.

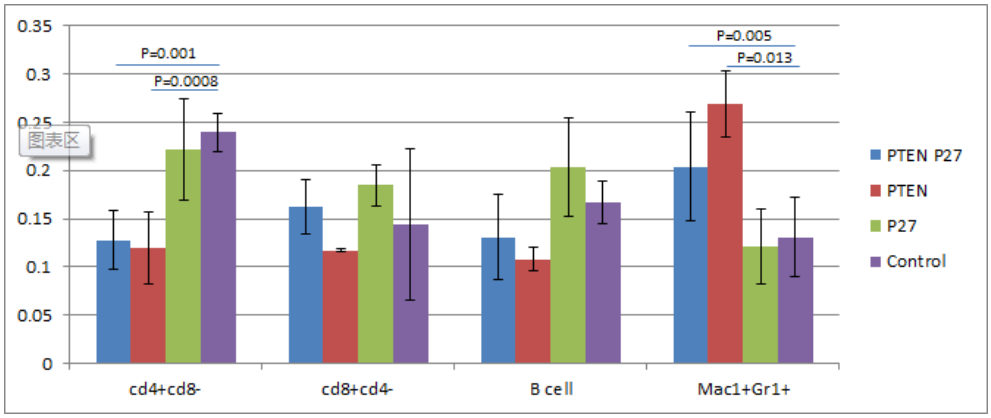

Figure 22. Y-axis shows the percentage of cells in each sub-populations. Comparing with the Control, CD4+ CD8- cells drop rapidly and Mac-1+ Gr-1+ cells increase sharply in the blood samples of PP27M and PM.

Bone Marrow (BM)

For the cells extracted from BM, the infiltrating cells and hematopoietic stem cells were monitored. In PP27M and PM group, the number of B cells drops a little, while the amount of Mac-1+ Gr-1+ cells increased slightly. However, since the high variance in the case of Ctrl group, the statistics did not reveal the significance (Figure 23).

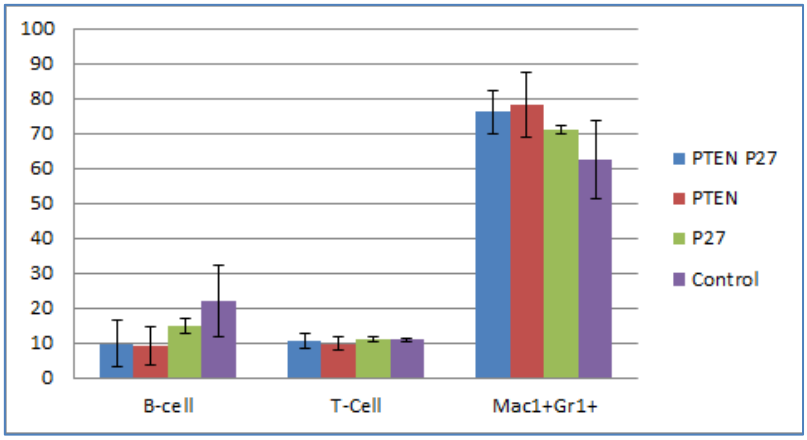

Figure 23. Y-axis shows the percentage of cells in each sub-populations. Amount of T cell, B cell and MDSCs were compared. No statistically significant difference was found.

For the content of Lin- Sca1+ c-Kit+ cells (LSK) and CD45+ CD34+ cells, the expected growth of cell amount didn't appear in the *PP27M* group. However, consistent with previous studies [64], the increase of LSK cells and loss of hematopoietic stem cells in *PM* group were observable (Figure 24).

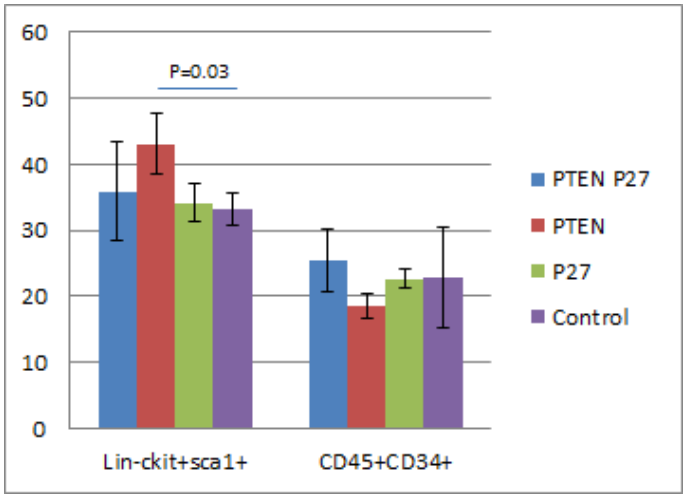

Figure 24. Y-axis shows the percentage of cells in each sub-populations. The increase of LSK cells and loss of hematopoietic stem cells in PM group were confirmed.

Spleen

Splenocytes were also examined by FACS analysis. A rapid drop of B cell content and a dramatic growth of Mac1+ Gr1+ cells (MDSC) were detected in the *PP27M* group as shown in the Figure 25.

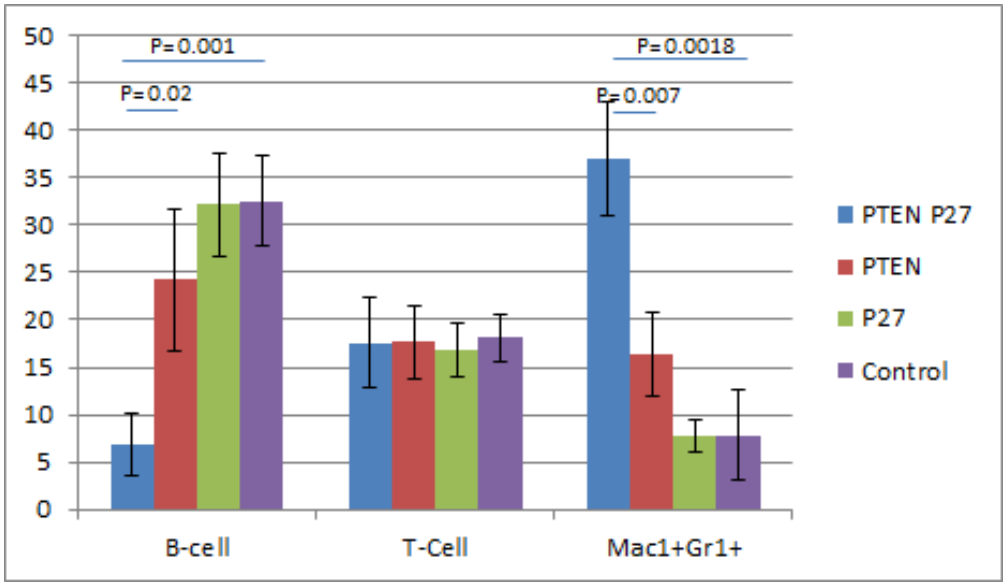

Figure 25. Y-axis shows the percentage of cells in each sub-populations. **B-Cell.** Rapid drop of B cell count was observed in PP27M group followed by PM group. **T-Cell**. Neither loss of PTEN nor P27 can affect the amount of T cells in spleen samples. **Mac1+Gr1+**. MDSCs witness a sharp increase in the PP27M group followed by PM group.

The increase of LSK cells and CD45+ CD34+ cells are also remarkable in the PP27M group (Figure 26).

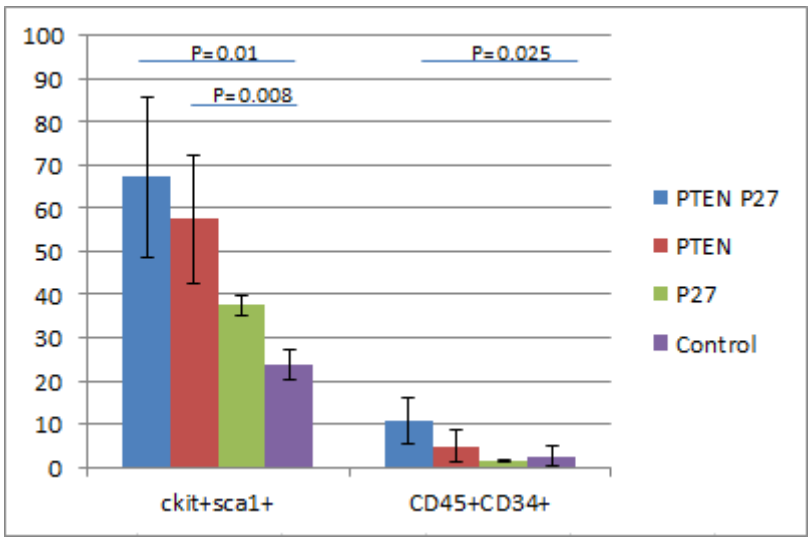

Figure 26. Y-axis shows the percentage of cells in each sub-populations. Comparing with the Control, the number of LSK cells and immature cells in PP27M group increase rapidly followed by PM and P27M group.

More additional antibodies were applied to subdivide the increased cell population (lin- Sca1+ c-Kit+ cells and lin- Sca1- c-Kit+ cells) into different categories and stages. As shown in figure 27, LT-HSC and ST-HSC witness a remarkable drop in the cell count. However, MPP undergoes a rapid growth, and it contributes to the main part of all the LSK cells' bulge. The rapid growth of MPP also leads to the distinct increase of CLP. In the case of CMP, MEP declined rapidly while the GMP increases slightly. Since the hematopoietic stem cells and progenitors are pretty rare in the sample, individual variances always make the gating process very hard. Almost all the data have a very wide error bar.

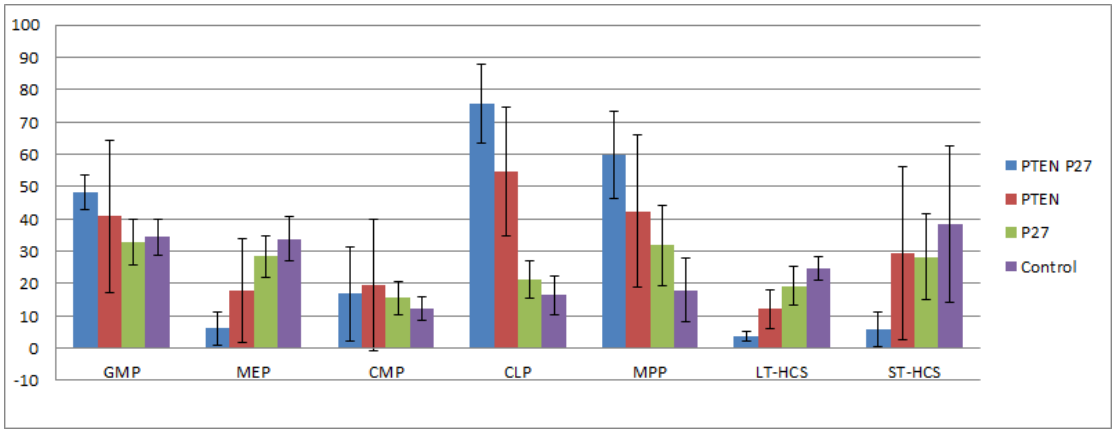

Figure 27. Y-axis shows the percentage of cells in each sub-populations. Further divisions for the LSK cells and CMP were examined. Comparing with Control, the rapid increase of CLP and MPP were detected in PP27M group. The MEP, LT-HCS and ST-HCS drop sharply in PP27M case.

5. Discussion

5.1 P27 IS NOT ESSENTIAL IN ADULT MICE

The absence of P27 did not cause any lethal harmless to the mice. Although The P27 KO mice witness a distinct enlargement on their body size, organ-body ratios were not significantly different from those of wild type mice. The FACS analysis also showed the loss of P27 cannot affect the cell differentiation and development in hematopoietic system.

For another, the female sterility in P27 deficient mice [65] was not confirmed in this study. Even though a rapid drop of fertility rate can be observed, some of the P27 KO female mice still give birth to the new pups. However, since the Mx1-Cre is not specifically targeting reproductive system, the gene knock-out rate of reproductive cells might be not complete.

5.2 P27 ACCELERATE THE DEVELOPMENT OF MPD

P27 accelerate the development of MPD, but not develop acute leukemia. From the FACS analysis, infiltrating cells can be found in PB, BM and spleen samples in PP27M and PM group, but not in P27 M and control group. Immature cells were slightly increased in both of the BM sample from PP27M and PM group. For the hematopoietic stem cells and progenitors in BM, the increase of LSK cells in the PM group is evident, but not in the PP27M group. That is to say, there is no evidence showing the absence of P27 can develop acute leukemia in the PTEN deficient mice model.

In the spleen samples, PP27M group has the largest amount of LSK cells and immature cells, followed by PM group, P27M group and control. Further division for the LSK cells provides more detail information about cell types in spleen cell samples. Mentioned in 3.3.2, LSK cells can be divided into three groups: MPP, LT-HSC and ST-HSC. MPP differentiated into CMP and CLP, in which CMP results MEP and GMP, while CLP gives all the B, T, NK cells (Figure 8). Based on Figure 30, we can find that the rapid increase of MPP make a great contribution to the growth of LSK cells, since the LT-HSC and ST-HSC tend to decrease in the meantime. That means stem cells and progenitors in the spleen are losing their capability of self-renew and start to differentiate faster than usual. The increase of MPP leads the acceleration of CLP. However, the amount of downstream cell line (B-cell, T-cell and NK cell) didn't increase. On the contrary, B cells witness a dramatic drop in the spleen. It indicates that CLP may fail to differentiate Pro-B cell or Pre-B cell and that maybe the cause of immature cell accumulation. On the other hand, the differentiation of CMP also doesn't work very well. GMP rises while MEP drops, which may be the reason of losing hemoglobin in the blood test.

5.3 AKT MIGHT BE THE LINK BETWEEN PTEN AND P27

It's well-known that PTEN deficiency can introduce MPD and develop to leukemia. One widely accepted explanation is that PTEN works as a suppressor in the PI3K/AKT/mTOR pathway. The absence of PTEN improve the expression of PI3K and AKT, thus raise the content of mTOR for controlling protein synthesis, cell cycling et cetera. However, more evidences showed the AKT can also cause the phosphorylation of

P27 and inactive P27. That is to say, the loss of PTEN can somehow deregulate the normal function of P27. And in this study, except the disease becomes more serious, there are not so many differences between PTEN KO mice and double KO mice. That means maybe P27 is one the most important factors in the PTEN deficiency induced MPD. In order to examine this suspicion, more work need to be done, such as western blotting for AKT and other relative proteins, cell proliferation and cell cycle analysis.

6. REFERENCE

1. What You Need To Know About Leukemia. (n.d.). *National Cancer Institute*. September 2013.

2. Leukemia. (n.d.). *The Columbia Encyclopedia, 6th ed.*. The Columbia University Press. 2015.

3. **Fredric PM, David CB, Ronald JM, et al.** Regulated protein expression for in vivo gene therapy for neurological disorders: Progress, strategies, and issues. *Neurobiology of Disease*. 2012;48(2): 212–221.

4. **Nicholas R** and **Paul R**. Signaling Pathways that Regulate Cell Division. *Spring Harbor Laboratory Press*. 2012.

5. **Zeijlemaker W, Schuurhuis GJ.** Chapter 6: Minimal Residual Disease and Leukemic Stem Cells in Acute Myeloid Leukemia. In: **Margarita G, Gueorgui B.** Leukemia, *InTech*, 2013

6. **Bangyan S, Matthias G, Shunyou W, et al.** PTENless means more. *Developmental Biology*. 2004(273): 175-184.

7. **Cully M, You H, Levine AJ, et al.** Beyond PTEN mutations: the PI3K pathway as an integrator of multiple inputs during tumorigenesis. *Nature Rev. Cancer*. 2006;6: 184-192.

8. **Jiwang Z, Justin CG, Tong Y, et al.** PTEN maintains haematopoietic stem cells and acts in lineage choice and leukaemia prevention. *Nature*. 2006;441.

9. **Rassidakis GZ, Feretzaki M, Atwell C**. Inhibition of Akt increases p27Kip1 levels and induces cell cycle arrest in anaplastic large cell lymphoma. *Blood*. 2005.

10. **Uner AH, Sağlam A, Han U.** PTEN and p27 expression in mature T-cell and NK-cell neoplasms. *Leuk Lymphoma*. 2005.

11. Education - About Animal Research. (n.d.). *Foundation for Biomedical Research*. July , 2013.

12. **Remy Melina,** Why do medical researchers use mice? *Livescince*. November 2010.

13. **Simmons, D.** The use of animal models in studying genetic disease: transgenesis and induced mutation. *Nature Education*. 2008;1(1)

14. **Andrew Cho, Naoto Haruyama, Ashok B. Kulkarni,** Generation of Transgenic Mice*. Curr Proroc Cell Biol*. Mary 2009.

15. Transgenic Mouse and Transgenic Rat Outline. (n.d.). *Transgenic Animal Web*. October 2012.

16. Transgenic Mice. (n.d.). *Access Excellence @ the national health museum*. 1999.

17. The Trouble With Black-6: A tiny alcoholic takes over the lab. **Engber Daniel**. *Slate*. November 2011.

18. Aurora's Guide to Mouse Colony Management. **Connor AB**. *Cell Migration Gateway*. December 2013.

19. **Mark FC. Normal S**, Function, and Histology of the Spleen. *Toxicologic Pathology*. 2006;34:455–465.

20. Mayo Clinic Family Health Book. **Scott CL**. *Oxmoor House*. May 2003.

21. Lymphoma. **Bethesda, Maryland**, *US: MedlinePlus, National Institutes of Health*. May 2015.

22. **Lauralee S**. Human Physiology: From Cells to Systems. *Cengage Learning*. 2012.

23. Chronic Myeloproliferative Neoplasms Treatment. (n.d.). *National Cancer Institute*. August, 2015.

24. **Bruce A, Alexander J.** DNA Replication, Repair, and Recombination. In: **Bruce A, Alexander J, Julian L. et al.** Molecular Biology of the Cell, 4th edition. *Garland Science*. 2002.

25. **Bode J, Schlake T, Schuebeler D, et al.** The transgeneticist's toolbox: novel methods for the targeted modification of eukaryotic genomes. *Biol. Chem*. 2000;381(9–10) 801–813.

26. **Catherine S. Branda, Susan M. Dymecki,** Talking about a Revolution: The Impact of Site-Specific Recombinases on Genetic Analyses in Mice. *Developmental Cell* . 2004; 6(1): 7 - 287.

27. **Sauer B, Henderson N.** Site-specific DNA recombination in mammalian cells by the Cre recombinase of bacteriophage P1. *Proc. Natl. Acad. Sci. USA.* 1988; 85: 5166–5170.

28. **Nagy A.** Cre recombinase: the universal reagent for genome tailoring. *Genesis*. 2000 Feb; 26(2):99-109.

29. **M Anton, F L Graham**. Site-specific recombination mediated by an adenovirus vector expressing the Cre recombinase protein: a molecular switch for control of gene expression. *J. Virol.* 1995 August; 69(8)4600-4606.

30. **Brian S.** Inducible Gene Targeting in Mice Using the Cre/loxSystem. *Methods*. 1998 April; 14(4): 381-392.

31. **Hug H, Costas M, Staeheli P, et al**. Organization of the murine Mx gene and characterization of its interferon- and virus-inducible promoter. *Mol. Cell. Biol.* 1988 August; 8(8): 3065-3079.

32. **Ralf K, Frieder S, Michel A, et al**. Inducible Gene Targeting in Mice. *Science*. 1995 Sep. 8; 269(5229): 1427-1429.

33. **Heinz A, Susan S, Mathieu N, et al**. Transgenic mice with intracellular immunity to influenza virus. *Cell*. 1990 July 13; 62(1): 51-61.

34. (n.d.). Chapter 20, Cell-to-Cell Signaling: Hormones and Receptors. In: **Lodish H, Berk A, Zipursky SL, et al**. *Molecular Cell Biology. 4th edition*. New York: W. H. Freeman; 2000.

35. **Chen Z, Trotman LC, Shaffer D, et al**. Crucial role of p53-dependent cellular senescence in suppression of Pten-deficient tumorigenesis. Nature. 2005Aug; 436(7051): 725–730.

36. **Min SS, Leonardo S, Pier P P.** The functions and regulation of the PTEN tumor suppressor. *Nature Reviews Molecular Cell Biology*. 2012 May; 13: 283-296.

37. **Nissim H, Nahum Sonenberg**. Upstream and downstream of mTOR. *Genes & Dev*. 2004; 18:1926-1945.

38. **Gingras AC, Kennedy SG, O'Leary MA, et al**. 4E-BP1, a repressor of mRNA translation, is phosphorylated and inactivated by the Akt(PKB) signaling pathway. *GENES & DEVELOPMENT*. 1998 Feb 15; 12(4): 502-513.

39. **Hay N, Sonenberg N.** Upstream and downstream of mTOR. *GENES & DEVELOPMENT*. 2004 Aug 15; 18(16):1926-1945.

40. **Chung J., Grammer, T.C., Lemon, K.P., Kazlauskas, A., and Blenis, J**. PDGF- and insulin-dependent pp70S6k activation mediated by phosphatidylinositol-3-OH kinase. *Nature*. 1994; 370: 71-75.

41. **Mendez, R ; Myers, MG ; White, MF ; et al**. Stimulation of protein synthesis, eukaryotic translation initiation factor 4E phosphorylation, and PHAS-I phosphorylation by insulin requires insulin receptor substrate 1 and phosphatidylinositol 3-kinase. *Molecular and cellular biology*. 1996 Jun; 16(6):2857-2864.

42. **Lewis C. Cantley**. The Phosphoinositide 3-Kinase Pathway. *Science*. 2002 May; 296(5573): 1655-1657.

43. **Guo W, Lasky JL, Wu H, et al.** Multi-genetic events collaboratively contribute to Pten-null leukaemia stem-cell formation. *Nature*. 2008 May 22; 453(7194):529-33.

44. **Yu H, Li Y, Luo HR, et al**. Relevant mouse model for human monocytic leukemia through Cre/lox-controlled myeloid-specific deletion of PTEN**.** *Leukemia*. 2010 May; 24(5):1077-80.

45. **Yilmaz ÖH, Valdez R, Theisen BK, et al**. Pten dependence distinguishes haematopoietic stem cells from leukaemia-initiating cells. *Nature*, 2006; 441(7092): 475-82.

46. **Anton M, Tomislav K, Sabine H, et al**. A structural perspective of CTD function. *Genes & Dev*. 2005; 19: 1401-1415.

47. **Kasim DM, Chandrahas KR, Philipp K,** Cyclin-dependent kinase 1 (Cdk1) is essential for cell division and suppression of DNA re-replication but not for liver regeneration. *PNAS*. 2012 February 21; 109 (10): 3826-3831.

48. **Marcos M, Mariano B**. Milestones in cell division : To cycle or not to cycle: a critical decision in cancer. *Nature Reviews Cancer*. 2001 December;1: 222-231.

49. **Marcos M, Mariano B**. Cell cycle, CDKs and cancer: a changing paradigm. *Nature Reviews Cancer*. 2009 March; 9: 153-66.

50. **Deepak A, Kui L, Yan S**. Cdk1 drives meiosis and mitosis through two different mechanisms. *Cell Cycle*. 2012 August 1; 11(15): 2763–2764.

51. **Ford HL, Pardee AB**. Cancer and the cell cycle. *J. Cell. Biochem. Suppl*. 1999; 32-33: 166–72.

52. **Jeffrey PD, Russo AA, Polyak K, Gibbs E, et al.** Mechanism of CDK activation revealed by the structure of a cyclinA-CDK2 complex. *Nature*. 1995 July; 376 (6538): 313–20.

53. **Heuvel S, Harlow E**. Distinct roles for cyclin-dependent kinases in cell cycle control. *Science*. 1993 December 24; 262 (5142): 2050-2054.

54. **Dawn C, Heike L, Ronald AL**. Distinct roles for cyclins E and A during DNA replication complex assembly and activation. *Nature Cell Biology*. 2002; 4: 523 – 528.

55. **Tetsu O, McCormick F**. Proliferation of cancer cells despite CDK2 inhibition. *Cancer Cell*. 2003 Mar;3(3):233-45.

56. **Akli S, Van Pelt CS, Bui T**. Cdk2 is required for breast cancer mediated by the low-molecular-weight isoform of cyclin E. *Cancer Res*. 2011 May 1;71(9):3377-86.

57. **Geoffrey IS**. Cyclin-Dependent Kinase Pathways As Targets for Cancer Treatment. *Journal of Clinical Oncology*. 2006 April 10; 24.

58. **Chu IM, Hengst L, Slingerland JM.** The Cdk inhibitor p27 in human cancer: prognostic potential and relevance to anticancer therapy. *Nat Rev Cancer*. 2008 Apr；8(4):253-67.

59. **Muzumdar MD, Tasic B, MiyamichiK, et al.** A global double-fluorescent Cre reporter mouse. *Genesis*, 2007; 45: 593–605.

60. **Zhang J, Grindley JC, Yin T, et al.** PTEN maintains hematopoietic stem cells and acts in lineage choice and leukemia prevention. *Nature*. 2006; 441(7092), 518-22.

61. **Buick RN, Minden MD, McCulloch EA**. Self-renewal in culture of proliferative blast progenitor cells in acute. *Blood*. 1979; 54: 95-104.

62. **Jiwang Z, Chao N, Yuji M, et al.** Identification of the haematopoietic stem cell niche and control of the niche size. *Nature*. 2003 October 23; 425: 836-841.

63. **Mahler M, Berard M, Feinstein R**. FELASA recommendations for the health monitoring of mouse, rat, hamster, guinea pig and rabbit colonies in breeding and experimental units. *Laboratory Animals*. 2014; 48(3): 178–192.

64. **Wey S, Luo B, Tseng CC, et al**. Inducible knockout of GRP78/BiP in the hematopoietic system suppresses Pten-null leukemogenesis and AKT oncogenic signaling. *Blood*. 2012; 119: 817-825.

65. **Fero ML, Rivkin M, Tasch MA**. syndrome of multiorgan hyperplasia with features of gigantism, tumorigenesis, and female sterility in p27(Kip1)-deficient mice. *Cell*. 1996 May 31; 85(5):733-44.

66. Regulation of p27 Phosphorylation during Cell Cycle Progression. *National Cancer Institute*. http://cgap.nci.nih.gov/Pathways/BioCarta/h_p27Pathway

67. **Jonas L, and Stefan K**, The role of Smad signaling in hematopoiesis, *Oncogene*. 2005; 24: 5676–5692.

68. A Guide to Absolute Counting Using the BD Accuri® C6 Flow Cytometer. *BD Biosciences*. 2011 Aug.