**An Induced Pluripotent Stem Cell-Based Platform for In Vitro Study of Chronic Myeloid Leukemia Stem Cell Development and Drug Resistance**

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**An Induced Pluripotent Stem Cell-Based Platform for In Vitro Study of Chronic Myeloid Leukemia Stem Cell Development and Drug Resistance**

**Abstract**

Chronic myeloid leukemia (CML), a myeloproliferative disorder characterized by unregulated growth of predominantly myeloid cells and their accumulation in the bone marrow and peripheral blood, originates in hematopoietic stem cells (HSCs) with t(9;22)(q34;q11.2) translocation which produces Bcr/Abl fusion transcript. A tyrosine kinase inhibitor (TKI), imatinib, employed as a first line of treatment for CML, is able to induce complete cytogenetic response in most of the patients. However, it does not cure CML due to persisting leukemic stem cells (LSCs). Therefore, an eradication of CML requires understanding the mechanisms of imatinib resistance and developing drugs targeting alternative cell survival pathways in CML LSCs. Due to a very low number of $Bcr/Abl^{+}$ cells within the most primitive hematopoietic cell compartments compared to dominance of leukemic cells in more mature and terminally differentiating compartments, study in LSCs is limited. Here we used induced pluripotent stem cells (iPSCs) to generate $lin^{-}CD34^{+}CD45^{+}$ in vitro and determine whether these iPSC-derived cells can be used to screen for new drug targets to eliminate CML LSCs. We found that $lin^{-}CD34^{+}CD45^{+}(iCD34^{+})$ primitive hematopoietic cells generated from CML iPSCs display LTC-IC potential, high ALDH activity, and efflux rhodamine. These cells express functional Bcr/Abl with kinase activity (pCrkl) compared to normal iPSCs. Besides hematopoietic stem cell properties, the CML iPSCs-derived cells display distinctive LSC features, including adhesion defect, imatinib resistance, and rapid limited cytokine independent proliferation. Following maturation/expansion with growth

factors, $lin^-CD34^+CD45^+$ cells lost CD34 expression and regained sensitivity to imatinib. All together, these data suggest that $lin^-CD34^+CD45^+$ cells obtained from CML iPSCs represent the functional equivalent of LSCs. Comparative analysis of gene expression in CML iPSCs-derived $lin^-CD34^+CD45^+$ cells treated and not treated with imatinib identified olfactomedin 4 (Olfm4) as the top-ranked gene among 127 genes that were induced by imatinib. Olfm4 knockdown using siRNA increased apoptosis in vitro of $lin^-CD34^+CD45^+$ cells from CML iPSCs. The effect was enhanced when cells were treated with imatinib, indicating that Olfm4 may represent a potential novel drug target for somatic CML-LSCs. To confirm our hypothesis, we performed Olfm4 knockdown in $lin^-CD34^+$ from a parental CML sample. We found that similar to iPSC-derived $iCD34^+$ cells, Olfm4 knockdown in somatic CML stem/progenitor cells significantly increased apoptosis and reduced colony-forming activity. Thus, identification of primitive hematopoietic cells with LSC properties in iPSC cultures provides an iPSC-based platform to study leukemia stem cell development and mechanisms of drug resistance in a patient-specific manner.

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**List of Figures and Tables**

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**List of Abbreviations**

| | |
|---|---|
| ALDH | Aldehyde dehydrogenase |
| BL-CFC | Hemangioblast colony-forming cell |
| BSA | Bovine serum albumin |
| CD | Cluster of differentiation |
| CFC-E | Colony forming cell-erythrocyte |
| CFC-G | Colony forming cell-granulocyte |
| CFC-GEMM | Colony forming cell-granulocyte/erythrocyte/ macrophage/megakaryocyte |
| CFC-GM | Colony forming cell-granulocyte-macrophage |
| CFC-M | Colony forming cell-macrophage |
| CFC(s) | Colony forming cell(s) |
| CFSE | Carboxy-fluorescein diacetate succinimidyl diester |
| CML | Chronic myeloid leukemia |
| DEAB | Diethylaminobenzaldehyde |
| DM | Differentiation media |
| ESC(s) | Embryonic stem cell(s) |
| FACs | Fluorescence activated cell sorting |
| FBS | Fetal bovine serum |
| FGF2 | Fibroblast growth factor 2 |
| Flt3L | Fms-like tyrosine kinase 3 ligand |
| G-CSF | Granulocyte colony-stimulating factor |
| GF(s) | Growth factor(s) |

| | |
|---|---|
| GM-CSF | Granulocyte/Macrophage colony-stimulating factor |
| HSC(s) | Hematopoietic stem cell(s) |
| IC50 | Inhibition concentration 50 |
| IL-3 | Interleukin 3 |
| IL-6 | Interleukin 6 |
| IMDM | Iscove's modified Dulbecco's medium |
| iPSC(s) | Induced pluripotent stem cell(s) |
| LSC(s) | Leukemia stem cell(s) |
| LTC-IC | Long term culture initiating cell |
| MACs | Magnetic activated cell sorting |
| MEF(s) | Mouse embryonic fibroblast(s) |
| MSC(s) | Mesenchymal stem cell(s) |
| PBS | Phosphate buffer saline |
| PCC | Pearson correlation coefficient |
| Rho | Rhodamine 123 |
| RSEM | RNA-Seq by Expectation-Maximization |
| SCF | Stem cell factor |
| SEM | Standard error of mean |
| SFEM | Serum free medium |
| siRNA | Small interfering RNA |
| TKI | Tyrosine kinase inhibitor |
| α-MEM | Alpha minimum essential medium |

# Introduction

## Chronic myeloid leukemia

Chronic myeloid leukemia (CML), one of the myeloproliferative syndromes, caused by t(9;22)(q34;q11.2), a reciprocal translocation of Bcr gene on chromosome 22 and Abl gene on chromosome 9, results in the Bcr/Abl fusion transcript. The oncoprotein produced by the transcript has constitutively activated tyrosine kinase activity that plays a role in cell proliferation, division, stress response and malignant transformation. In murine study, recipient mice transplanted with Bcr/Abl-transduced bone marrow cells developed a myeloproliferative syndrome that recapitulated features of CML (Daley et al., 1990). The cell of origin is believed to be the hematopoietic stem cells (HSCs), phenotypically defined as $lin^{-}CD34^{+}$ population, or progenitor cells but regain self-renewal ability after the mutation (Dick, 2008). Both can lead to expansion of a specific clone, known as cancer-propagating cell, and repopulating in the normal hematopoietic system with leukemic cells. Recently, Bcr/Abl transcript was found in endothelial cells with angiogenic property from CML patients (Gunsilius et al., 2000), and in vitro (Fang et al., 2005). These suggest that oncogenic hit may even occur in a more primitive stage, hemangioblast.

CML accounts for approximately 15 % of all cases of leukemia, or around 5,000 new cases per year. The disease is characterized by anemia, granulocytosis and granulocytic immaturity, basophilia, often thrombocytosis and splenomegaly. In 2001, imatinib was introduced as a treatment for CML that specifically inhibits tyrosine kinase domain in Abl, c-kit and PDGFR (Druker et al., 2001). Despite dramatic effect on reducing leukemic cells, an active leukemia will recur when imatinib therapy is discontinued. The

recurrent of disease indicates a particular population, so called leukemia stem cells, exists in the bone marrow.

**Leukemia stem cells (LSCs) are inherently resistant to tyrosine kinase inhibitor (TKI)**

Even though leukemia stem/progenitor cells can persist imatinib treatment (Corbin et al., 2011), a reduction in leukemic cell burden to the level of complete cytogenetics response indicates that imatinib affects on differentiated leukemic cells. It is unclear regarding the stage of which LSCs become sensitive to TKI. Studies described various mechanisms of how LSCs persist after imatinib treatment. Evidences showed LSCs acquire additional mutation in the Bcr/Abl protein which is almost always associated with imatinib resistance (Branford et al., 2003), gene amplification (Gorre et al., 2001; le Coutre et al., 2000; Weisberg and Griffin, 2000), disturbance in signaling pathway (Naka et al., 2010) and a difference in Bcr/Abl expression (Jiang et al., 2007; Kumano et al., 2012). However, naive CML stem cells from patients prior to treatment have been shown to be insensitive to TKI despite an inhibition of kinase (Corbin et al., 2011). Interestingly, imatinib did not select the expansion of normal fraction of $lin^{-}CD34^{+}CD38^{-}$, but the leukemic $lin^{-}CD34^{+}CD38^{-}$ cells were also able to expand. The findings suggest that LSCs are insensitive to imatinib and cannot be eliminated by TKI alone.

**Bcr/Abl contributes to the distinctive features of CML LSCs**

Owing to the complexity in the molecular signaling in CML, several fairly diverse functions have been attributed to Bcr/Abl. The fusion of Bcr sequence 5' of the Abl SH3 domain abolish the physiologic inhibition of the Abl kinase which play the central role in leukemogenesis. Although Bcr has its own kinase activity and its substrates identified so

far are Bap-1 (Reuther et al., 1994) and possibly Bcr itself (Laneuville, 1995), its kinase activity appears to be minor in disease pathogenesis. In addition, Bcr can be phosphorylated on several tyrosine residues (Wu et al., 1998), especially tyrosine 177, which binds Grb-2, an important adapter molecule involved in the activation of the Ras pathway (Ma et al., 1997). Even though these data do not support a role of Bcr in signal transduction, its true biological relevance remains to be elucidated.

Overall, it appears that the cytoplasmic location of the Bcr/Abl protein allows access to many cellular substrates that are unavailable to the predominantly nuclear Abl protein and serves a complex role as a cellular module that relays signals from extracellular and intracellular sources and that determines the activation of proliferation and survival pathway.

Three major mechanisms that have been affected by the malignant transformation of Bcr/Abl.

1. Alteration of adhesion to stroma cells and extracellular matrix (Gordon et al., 1987)
2. Constitutively active mitogenic signal transduction pathways (Puil et al., 1994)
3. Inhibition of apoptosis(Bedi et al., 1994)

**Impaired adhesion properties of CML stem/progenitor cells**

(Verfaillie et al., 1997) and (Gordon et al., 1987) have shown CML progenitor cells display impaired adhesive interaction to bone marrow stroma cells and fibronectin. A reduction in progenitor-stroma interactions leaded to negatively regulation in normal cell proliferation resulting in rapidly growth of CML cells. In the study, interferon-α (IFN-α), an old-day therapeutic agent in CML, appears to reverse the adhesion defect through a

recovery of β1 integrin function (Bhatia et al., 1994). Upon binding to their ligands, integrins initiate signal transduction through several adaptor proteins in addition to Crkl, one of the major Bcr/Abl downstream signaling substrates found in peripheral blood cells of patients with CML (Oda et al., 1994). Due to the specificity of its phophorylation to Bcr/Abl signaling, Crkl is an excellent method to determine Bcr/Abl activity. It involved in the regulation of cellular motility (Uemura and Griffin, 1999) and in integrin-mediated cell adhesion (Sattler et al., 1996) by association with other focal adhesion proteins such as paxillin and the focal adhesion kinase (Fak). Hence, it is conceivable that CML cells escape the normal growth inhibitory signal through the perturbed adhesion properties. Because Abl1 has been implicated in the intracellular transduction of such signals, this process may be further disturbed by the presence of a large pool of Bcr/Abl protein in the cytoplasm. Although there is plenty of evidence that Bcr/Abl influences integrin function, it is still unable to define the precise nature of the biological consequences.

**Inhibition of apoptosis**

Growth factor-dependent murine (Daley and Baltimore, 1988) and human hematopoietic cell lines (Sirard et al., 1994) retrovirally transduced with Bcr/Abl were protected from apoptosis after growth-factor withdrawal. Although an effect is critically dependent on tyrosine kinase activity and that correlates with the activation of Ras (Cortez et al., 1995; Puil et al., 1994), inhibition of the kinase activity by TKI did not reverse the protection in certain cell types for example, CML stem cells (Corbin et al., 2011) and CML iPSCs (Kumano et al., 2012) reflected the innate resistant within the stem cell population. Erk1/2, Akt, Jnk and Stat5 appear to compensate for the inhibition of Bcr/Abl kinase in

the CML iPSCs (Kumano et al., 2012). Although several studies showed that Bcr/Abl enhances cell survival and resists apoptosis, (Kumari et al., 2012) demonstrated that only cells expressed Bcr/Abl at low level could persist TKI. Thus, precise biological effects of Bcr/Abl in CML are still not well understood.

Amarante-Mendes and Dubrez suggest that Bcr/Abl may block the release of cytochrome C from the mitochondria and thus the activation of caspases (Amarante-Mendes et al., 1998; Dubrez et al., 1998). This effect upstream of caspase activation might be mediated by the Bcl-2 family of proteins. Bcr/Abl has been shown to up-regulate Bcl-2 through a Ras (Sanchez-Garcia and Martin-Zanca, 1997) or a PI3 kinase-kinase pathway (Skorski et al., 1997) in Baf/3 and 32D cells, respectively. Recently, (Duy et al., 2011) identified BCL6, a known proto-oncogene that is often translocated in diffuse large B-cell lymphoma, as a central component of resistance to TKI-induced apoptosis via p53 dependent manner. Another possible mechanism is the phosphorylation of the pro-apoptotic protein, Bad. Two recent studies provided evidences that the survival signal provided by Bcr/Abl is at least partially mediated by Bad and requires targeting of Raf-1 to the mitochondria (Majewski et al., 1999; Neshat et al., 2000). Furthermore, based on results obtained in transduced cell study, it was suggested that Bcr/Abl inhibits apoptosis through Fas receptor/Fas ligand (McGahon et al., 1995).

In summary, the inconsistency among studies may reflect genuine differences between cell lines and primary cells. To gain insight into anti-apoptotic activity of Bcr/Abl, it would be crucial to define the triggers that initiate apoptosis in vivo.

**Activation of mitogenic signaling**

Bcr/Abl oncoprotein orchestrates many signaling pathways to transform cell into malignancy. It constitutively activates mitogenic signaling pathways including RAS/mitogen-activated protein kinase (MAPK) pathways, the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway, phosphoinositide-3 (PI3) kinase pathway and c-Myc pathway.

**RAS/MAP kinase pathway**

Observation in CML patients that activating mutations are uncommon in Philadelphia-positive leukemia, even in the blastic phase of the disease implies that Ras pathway is constitutively active, and further activating mutations do not play a role in blast crisis of CML (Watzinger et al., 1994). Stimulation of cytokine receptors such as IL-3 leads to the activation of Ras and the subsequent recruitment of the serine–threonine kinase Raf to the cell membrane (Marais et al., 1995). Raf initiates a signaling cascade through the serine–threonine kinases Map2k1/Erk, which ultimately leads to the activation of gene transcription (Cahill et al., 1996). On phosphorylation of tyrosine 177 within the Bcr SH2 domain provides a docking site for the adaptor protein Grb-2 (Pendergast et al., 1993). Grb-2, after forming complex with Sos protein, stabilizes Ras in its active GTP-bound form. It is possible that two other Bcr/Abl substrates, Shc and Crkl, also involve in Ras activation, however this piece of information relies on the data indicating that this pathway perhaps only activated in v-Abl- but not in Bcr/Abl-transform cells (Kabarowski et al., 1994).

Studies of the c-Jun have uncovered a second MAPK pathway involving Jun N-terminal

kinase (Jnk) or stress-activated protein kinase (Sapk). (Raitano et al., 1995) has shown an activation of Sapk/Jnk pathway by Bcr/Abl is required for malignant transformation. Hence, signaling from Ras may be relayed through Rac (Skorski et al., 1998) to Gckr (germinal center kinase related) (Shi et al., 1999) and further down to Jnk/Sapk. Some evidence showed that p38, the third MAPK signaling cascade, as well as other pathways with mitogenic potential are also activated in Bcr/Abl–transformed cells. Eventually, all signals are transduced to the transcriptional machinery of the cell.

**JAK-STAT pathway**

The JAK-STAT signaling pathway consisting of three main components: (1) a receptor (2) Janus kinase (JAK) and (3) Signal Transducer and Activator of Transcription (STAT) transmits information from chemical signals outside the cell, through the cell membrane, and eventually turn on the cellular transcription machinery. The first evidence that showed Bcr/Abl elicits its transforming activity through the activation of Stat5 transcription factor which leads to increased expression of genes driving cell cycle progression and promoting survival came from studies in v-Abl–transformed B cells(Danial et al., 1995). Although Stat5 has pleiotropic physiologic functions (Nosaka et al., 1999), its effect in Bcr/Abl–transformed cells appears to be primarily anti-apoptotic and involves transcriptional activation of $Bcl_{XL}$ (Horita et al., 2000; Sillaber et al., 2000). In contrast to the physiologic activation of the JAK-STAT pathway by extracellular stimuli, Bcr/Abl perhaps bypass a phosphorylation of Jak proteins and directly activate Stat1 and Stat5 (Ilaria and Van Etten, 1996).

Observation from an ectopic expression of Bcr/Abl turns several growth factor–

dependent cell lines into factor independent (Daley and Baltimore, 1988; Kabarowski et al., 1994) explains the effect of Bcr/Abl on the JAK-STAT pathways in the cellular response to growth factors. In some experimental systems, there is evidence for an autocrine loop dependent on the Bcr/Abl–induced secretion of growth factors including IL-3 and G-CSF in early progenitor cells (Holyoake et al., 2001; Jiang et al., 1999). However, during the chronic phase, CML progenitor cells are still dependent on external growth factors for their survival and proliferation (Amos et al., 1995), though less than normal progenitors (Jonuleit et al., 1998).

**PI3 kinase pathway**

Bcr/Abl interacts with p85α subunit of PI3 kinase and forms multimeric complexes with Cbl, Crk and Crkl (Sattler et al., 1996) to initiate its activity that is required for the proliferation of Bcr/Abl–positive cells (Skorski et al., 1995). The next downstream cascade, the serine–threonine kinase Akt, had previously been implicated in anti-apoptotic signaling (Franke et al., 1997). (del Peso et al., 1997) has reported Akt to be a downstream cascade of the IL-3 receptor and identified a pro-apoptotic protein, Bad, as its key substrate. In addition, Phosphorylated Bad is inactive because it is no longer able to bind anti-apoptotic proteins such as $Bcl_{XL}$, and it is trapped by cytoplasmic 14-3-3 proteins. Altogether this indicates that Bcr/Abl might be able to mimic the physiologic IL-3 survival signal in PI3 kinase-dependent manner. In addition, inositol phosphatase is also activated in response to growth factor signals and by Bcr/Abl (Lioubin et al., 1996; Wisniewski et al., 1999). Thus, Bcr/Abl appears to have a profound effect on phosphoinositol metabolism, which might again shift the balance to a pattern similar to

physiological growth factor stimulation.

**c-Myc pathway**

Mutations, overexpression, rearrangement and translocation of c-Myc gene have been associated with a variety of hematopoietic tumors, leukemia and lymphoma. The transcription factor encoded by this gene bind on enhancer box sequences (E-box) and recruit histone acetyltransferases to regulate expression of its targets that plays a role in cell cycle progression, apoptosis and cellular transformation, though its target genes are largely unknown and "c-Myc target signatures" among studies shows little overlap (Chandriani et al., 2009). (Lin et al., 2012) has reported that in tumor cells expressing high levels of c-Myc, the transcription factor accumulates in the promoter regions of active genes and causes transcriptional amplification, producing increased levels of transcripts within the cell's gene expression program. Thus, rather than binding and regulating a new set of genes, c-Myc amplifies the output of the existing gene expression program. Activation of c-Myc by Bcr/Abl depends on the SH2 domain, however the link between c-Myc to the SH2 domain of Bcr/Abl is still unknown. Results obtained in v-Abl–transformed cells suggest that the signal is transduced through Ras/Raf, cyclin-dependent kinases (Cdks), and E2F transcription factors that ultimately activate the c-Myc promoter (Zou et al., 1997). Recent studies reported two potential effects of Abl on c-Myc: first, Abl1 signaling can indirectly augment acetylation of c-Myc by p300 (Sanchez-Arevalo Lobo et al., 2013) and Fbw7 (Reavie et al., 2013); second, Abl can directly phosphorylate c-Myc on tyrosine (Sanchez-Arevalo Lobo et al., 2013). In summary, the activation of c-Myc by Bcr/Abl is likely through indirect mechanism.

**Gene expression profile of CD34$^+$ from chronic phase CML mononuclear cells**

Evidences from a molecular signature of enriched CD34$^+$ cells from bone marrow of untreated patients with CML in chronic phase in comparison with normal CD34$^+$ shed the light on the genes encoding cell adhesion molecules, transcription factors, regulators of stem-cell fate, and inhibitors of cell proliferation are affected by Bcr/Abl. The N-cadherin (Cdh2) and others adhesion molecules such as L-selectin, CD44 and chemokine receptor CXCR4 was significantly lower as compared to normal donors. Fibronectin detachment analysis and transwell migratory assays confirmed the impaired adhesive and migratory properties (Bruns et al., 2009). The interferon regulatory factor 8 (Irf8), a pro-apoptotic gene that could induce apoptosis in Bcr/Abl-positive myeloid cells in mice (Diaz-Blanco et al., 2007) and its ability to antagonize Bcr/Abl by down regulation of Bcl-2, was downregulated in primary CML CD34$^+$. Moreover, novel transcriptional changes were identified including upregulation of genes involved in the transforming growth factor β pathway, leptin receptor, sorcin, tissue inhibitor of metalloproteinase 1 and the neuroepithelial cell transforming gene 1(Diaz-Blanco et al., 2007).

**In vitro hematopoietic differentiation of human iPSCs**

Several reports have been published on generation of highly enriched populations of blood cell types by in vitro differentiation of human ESCs by a) embryoid body (EB) formation, wherein three dimensional spheres of human ESCs are formed and differentiated into various hematopoietic precursors in the presence of hematopoietic cytokines [reviewed in (Moore et al., 2006)] b) cytokine based 2D differentiation system without use of feeder cells (Park et al., 2010) and, c) co-culture of hESCs on stromal

feeder cells such as OP9, S17, MS5 (mouse bone marrow stromal cell line), mFLSC (mouse fetal liver derived stromal line), FH-B-hTERT (immortalized human fetal liver hepatocyte line) and primary human stromal cell lines derived from AGM (aorta-gonard-mesonephros), FL, fetal BM (Moore et al., 2006; Vodyanik et al., 2005). It has been shown that following differentiation hESCs undergo series of changes leading to the formation of $CD34^+$ cells (Vodyanik et al., 2005). Hematopoietic progenitors within $CD34^+$ population can be identified by the expression of CD43, which discriminates hematopoietic cells from $CD34^+CD31^+CD43^-$ endothelial cells, and $CD34^+CD31^-CD43^-$ mesenchymal cells. Within $CD43^+$ population the expression of CD235a and CD41a marks erythromegakaryocytic progenitors and further two distinct populations of multipotent hematopoietic progenitors emerge based on expression of CD45 ($lin^-CD34^+CD43^+45^-$ and $lin^-CD34^+CD43^+CD45^+$) (Vodyanik et al., 2006). A study by Choi, et al demonstrated that the iPSCs follow a similar differentiation pattern as that of hESCs that was unaffected by the source of fibroblasts or combinations of reprograming factors used for reprograming (Choi et al., 2009b). Although few studies (Feng et al., 2010) observed reduced hematopoietic lineage differentiation efficiency from iPSC lines, most of the published literature has supported the fact that similar to ESCs, iPSC lines can be efficiently differentiated into various blood cells (Chang et al., 2011; Choi et al., 2009b; Niwa et al., 2009) with some variation in differentiation rates between individual iPSC clones.

**Generation of hematopoietic stem cell/progenitor cells ($lin^-CD34^+45^+$) and primitive CML cells from induced pluripotent stem cells (iPSCs)**

It is well known that the first wave of hematopoiesis occur in the yolk sac and later the definitive hematopoiesis develops in the aorta-gonad-mesonephros (AGM) in the fetus. An in vitro model that captures this developmental stage has been described by using human embryonic stem cell (Vodyanik et al., 2005). This model provides an in vitro method to obtain the ESCs-derived $CD34^+$ having HSC phenotype and functionality by using ESCs/OP9 coculture system. Recently, the advent of reprogramming technology (Takahashi and Yamanaka, 2006; Yu et al., 2009) using an ectopic expression of transcription factors, including Sox2, Oct4, Klf4, and c-Myc can turn somatic cells of the body into a special type of stem cell, called iPSC. Other combinations of transcription factors and ways of generating iPSCs have been developed over the past 2 years, especially the non-integrating system. Owing to its non-integrating feature, this method is favorable for cancer study where integrated transgenes may interfere the effect of interested genes. The potential of continuous self-renewal without losing pluripotency and ability to differentiate into all types of adult cells belonging to three primitive germ layers, for example blood, heart, brain or kidney cells, make human iPSC attractive complementary cell sources for large scale production. Due to the molecular and functional similarity between ESCs and iPSCs (Choi et al., 2009b), generation of patient specific iPSC-derived $CD34^+$ by applying the same strategies as ESC to the generate unlimited supply of LSCs to outnumber the inadequate source obtained from bone marrow and peripheral blood, would have a rampant impact on the field of pharmacogenetics as individual specific responses to several new drugs could be

monitored on patient specific stem cells, thus facilitating rapid drug screening and discovery of new drugs. In addition, iPSCs provide a unique opportunity to study human diseases in vitro, thus helping to unravel the complicated genotype-phenotype relationship in several monogenic disorders. iPSCs generated from patients with blood diseases can be used to generate blood cells carrying disease-specific genetic traits at cellular level to recapitulate the disease phenotype under in vitro conditions, and provide insights to the pathophysiology and emergence of the disease. Previous study from our group (Hu et al., 2011) has derived iPSC from chronic phase CML bone marrow mononuclear cells using non-integrating episomal vector containing Sox2, Oct4, Klf4, c-Myc, Nanog, Lin28 and SV40LT. The CML iPSCs had a complex karyotype with four-way translocation between chromosomes 1, 9, 22 and 11-t(1;9;22;11)(p34.1;q34;q11.2; q23)- that was presented in the patient BM with Bcr/Abl expression at the mRNA level. In addition, $CD43^{+}$-derived from CML iPSCs displayed hematopoietic progenitor activity in semisolid media, however, in-depth study of these cells has to be done. Elegant study by (Choi et al., 2009a) has shown more define subpopulation within $CD43^{+}$ cells, $lin^{-}$ $CD34^{+}CD43^{+}45^{+}$, possess hematopoietic stem/progenitor by further expansion of them in GM-CSF condition and then generated different types of mature myeloid cells including neutrophils, eosinophils, macrophages, dendritic cells etc.

It is known that mature cells derived from diseased human iPSC capture the parental diseased phenotype (Moretti et al., 2010; Tulpule et al., 2013), however the iPSC for malignancy model is not well establish. (Hochedlinger et al., 2004) has shown that embryonic stem cells derived from melanoma by using nuclear tranplantation retain its tumorigenic activity with higher penetrance and shorter latency when compared with the

donor mouse. The finding shed light on the question whether $lin^{-}CD34^{+}CD43^{+}45^{+}$ derived from leukemia iPSCs would have distinct leukemic phenotype.

**Platform for drug screening**

The prevalence of chronic and senile-associated disorders is increasing due to population aging and better survival of patients with genetic diseases. The development of new treatments has proven to be challenging because experimental tools, including in vitro cultures and animal models, recapitulate only some of the specific traits of human disease. Heart failure, neurodegenrative disorders, atherosclerosis and cancers are all cases in point. As a result, and despite huge investments by the pharmaceutical industry, only few new therapeutic agents are currently entering the market. Human iPSCs provides a novel concept to not only for disease modeling, but also as a platform for drug discovery and toxicity tests. Thus enabling researchers to undertake studies for treating diseases “in a dish”, which was previously inconceivable (Bellin et al., 2012).

## Chapter 1: Generation of leukemia stem-like cells from CML iPSCs

### Abstract

A key behind the treatment failure of chronic myeloid leukemia is its leukemia stem cell. Their abilities to persist tyrosine kinase inhibitor and acquire additional mutation allow residual disease to relapse. A strategy to target such population is essential to cure the disease. Due to a low number of LSCs can be obtained from patients, only few progresses have been done. Here we used CML-induced pluripotent stem cells to generated $lin^-$ $CD34^+45^+$($iCD34^+$) vitro. The cells have colony-forming activity in methycellulose, long-term culture-initiating ability, efflux rhodamine dye, ALDH activity. In addition, $iCD34^+$ derived from CML iPSCs showed distinctive CML leukemia stem cell properties including adhesion defect, rapid limited cytokine independent proliferation and tyrosine kinase inhibitor resistance. Comparative study between CML and normal $iCD34^+$ by RNA-sequencing shows similarity in differentially expressed genes compared to study between HSCs and LSCs. All together, these data suggest that CML $iCD34^+$ cells obtained from CML iPSCs potentially represent functional equivalent of somatic LSCs, and thus the iPSC technology could be used to generate large numbers of patient-specific CML stem-like cells for in vitro studies of human disease mechanisms as well as for drug screening.

### Introduction

Chronic myeloid leukemia is a myeloproliferative disorder characterized by unregulated growth of predominantly myeloid cells and their accumulation in the bone marrow and peripheral blood. CML originates in HSCs with t(9;22)(q34;q11.2) translocation, which

produces Bcr/Abl constitutively active kinase driving the expansion of leukemic progeny (Holtz et al., 2002; Holyoake et al., 2001; Ramaraj et al., 2004). However, studies of CML LSCs in humans are limited by the very low number of Bcr/Abl$^+$ cells within the most primitive hematopoietic cell compartments (Holyoake et al., 1999; Holyoake et al., 2001; Vargaftig et al., 2012) in contrast to dominance of leukemic cells in more mature and terminally differentiating compartments.

Reprogramming somatic cells to pluripotency allows for generation of iPSCs capable of self-renewal, large-scale expansion, and differentiation toward derivatives of all three germ layers, including blood (Takahashi and Yamanaka, 2006; Yu et al., 2009). Because iPSCs capture the entire genome of diseased cells and retain their disease phenotypes, they are already used successfully to model murine (Hochedlinger et al., 2004) and human genetic diseases (Grskovic et al., 2011). Recently we developed a method for generation of transgene-free iPSCs from bone marrow mononuclear cells and used this method to obtain iPSCs from the patient in chronic phase of CML (Hu et al., 2011).

Here we used these iPSC lines to generate CML stem-like cells (lin$^-$CD34$^+$CD45$^+$) with CML leukemia stem cell properties. These cells displayed normal hematopoietic stem cell properties as well as distinctive LSC features, including adhesion defect, imatinib resistance, and rapid limited cytokine independent proliferation. Following maturation expansion with cytokines, lin$^-$CD34$^+$CD45$^+$ cells lost CD34 expression and regained sensitivity to imatinib. This method makes it possible to produce CML leukemia stem cell equivalent in vitro on a large scale that can be used to study biology of CML stem

cell.

## Materials and Method

### iPSCs maintenance and differentiation

In this study, we used transgene-free BM1K, BM9, CML15 and CML17 iPSCs produced by reprogramming of bone marrow mononuclear cells from normal and CML patients in the chronic phase (Hu et al., 2011). Undifferentiated iPSCs were maintained in cocultures with mouse embryonic fibroblasts (MEFs). Hematopoietic differentiation was induced by transferring the iPSCs onto overgrowth OP9 feeder as we have previously described in details (Choi et al., 2009b; Vodyanik et al., 2005). $CD43^+$ cells were collected on day 8 or 9 of differentiation using MACS and cultured in α-MEM supplemented with 10% FBS, 50 μg/ml ascorbic acid, 100 μM monothioglycerol (complete serum supplemented medium; CSSM) and 200 ng/ml GM-CSF to expand selectively myeloid progenitors (Choi et al., 2009a). Enriched myeloid progenitors were cultured for an additional 4 days in the same media supplemented with 10 ng/ml IL-3, 100 ng/ml IL-6, 100 ng/ml Flt3L, 200 ng/ml GM-CSF and 100 ng/ml SCF.

### Purification of $lin^-CD34^+CD45^+$ from iPSCs-derived $CD43^+$ and primary bone marrow

$CD43^+$ hematopoietic cells were collected from differentiated iPSC cultures using MACS and labeled with CD235a/CD41a FITC, CD45 APC and CD38 PE (BD Pharmingen). $Lin^-CD45^+CD38^+$ and $lin^-CD45^+CD38^-$ subpopulations were obtained by fluorescence-activated cell sorter (FACSAria) (Vodyanik et al., 2006). Bone marrow mononuclear cells from CML patients in the chronic phase were purchased commercially (AllCells or

Applied StemCells), or obtained from the patients at UW hospital (Madison, WI) with approval from the University of Wisconsin Institutional Review Board. Donors had previously signed an Institutional Review Board-approved consent. Mononuclear cells labeled with the lineage-specific markers CD2, CD3, CD14, CD15, CD16, CD19, CD20, CD24, CD56, CD66b, and Glycophorin A (FITC-conjugated antibodies), CD34 APC and DAPI to exclude dead cells. Live $lin^-CD34^+$ cells were isolated using FACSAria (BD).

**Hematopoietic colony-forming assay**

Hematopoietic clonogenic assays were performed using serum-containing StemMACS HSC-CFU media (Miltenyi Biotec, CA). Optimal number of cells, 100-1,000 cells, were plated with and without 10 μM imatinib unless specified. Colonies were scored after 14–21 days of incubation according to morphological criteria as CFC-E, CFC-GEMM, CFC-GM, CFC-G, and CFC-M.

**Long-term culture initiating cell assay (LTC-IC)**

Sorted iPSC-derived $lin^-CD34^+CD45^+$ cells were plated in a 6-well plate at $10^4$ cells/well containing 5-7 day-old pre-cultures 1:1 ratio mixture of murine M2-10B4 cells and OP9 stromal cells at 1 x $10^5$ cells in serum-free LTC-IC medium consisting of IMDM, 10% BIT (Stemcell Technologies), EXCYTE, 1 μM mercaptoethanol, 10 μM hydrocortisone, 50 ng/ml SCF, 5 ng/ml IL-3, and 50 ng/ml IL-6. Cultures were maintained at 37 °C in a humidified atmosphere with 5% $CO_2$, and changed half medium weekly. After 5 weeks, cells were harvested and analyzed for CFC potential as described above.

**Rhodamine 123 exclusion assay**

FACS isolated $lin^-CD34^+CD38^-$ and $CD38^+$ cells were incubated with 0.5 μg/ml rhodamine (Rho) (Molecular Probes, Eugene, OR) in 1 ml of alpha MEM medium containing 2% FBS (assay medium) for 30 minutes at 37 °C. After washing, cells resuspended in assay medium and incubated for 40 minutes at 37 °C with and without 50 μM verapamil (Sigma) to reveal Rho exclusion activity. Cells were labeled with CD45-APC and 7-AAD (Sigma) and analyzed by flow cytometry. $Rho^{low}$ cells were defined as those showing less fluorescence in the FL-1 channel than exhibited by verapamil-treated samples.

**Aldehyde dehydrogenase activity**

Aldehyde dehydrogenase (ALDH) staining of $lin^-CD45^+CD38^-$ and $Lin^-CD45^+CD38^+$ cells was performed with Aldefluor kit (Stem Cell Technologies) according to manufacturer instructions. Control samples were established using diethylaminobenzaldehyde (DEAB), an ALDH inhibitor. Cells also were labeled with CD45-APC, and dead cells were excluded using 7-AAD staining. Samples were analyzed by flow cytometry.

**Cell proliferation assay**

Total $lin^-CD34^+$, $lin^-CD34^+CD38^-$ and $lin^-CD34^+CD38^+$ were plated in triplicate in 96-well plate at 300 cells/well. Cells were cultured in either CSSM or serum-free expansion media (SFEM) containing IMDM, 10% BIT, 2-mercaptoethanol, and EXCYTE, with or without 5 μM imatinib. When specified, the following cytokines were added: 10 ng/ml

IL-3, 100 ng/ml SCF, 100 ng/ml Flt3L, 100 ng/ml IL-6 and 200 ng/ml GM-CSF. Viable cell count was determined by trypan blue using hemocytometer.

**$IC_{50}$ assay**

The $lin^-CD34^+$ and $lin^+CD34^-$ cells were plated at $10^3$ cells/well in 96-well plate in CSSM containing 10 ng/ml IL-3, 100 ng/ml IL-6, 200 ng/ml GM-CSF, 100 ng/ml SCF, and 100 ng/ml Flt3L with 0 – 100 μM imatininb. After 24 h of culture, viable cell count was performed using trypan blue. $IC_{50}$ assay was also performed with iPSCs cultured on matrigel in TeSR medium and iPSC-derived MSCs cultured in serum-free medium (Vodyanik et al., 2010). The viability of MSCs and iPSC cultured with 0 – 100 μM imatininb was evaluated using trypan blue. The $IC_{50}$ was determined as the concentration of drug where cell death was 50% of that in relevant control wells (Sebaugh, 2011). Data from three assays performed in triplicate were used for statistical analysis and graphs plot for $IC_{50}$ determinations. Relative $IC_{50}$ were determined by fitting an exponential dose-response curve to the cell proliferation data by using GraphPad Prism software (GraphPad, San Diego, CA).

**CFSE tracking of cell division**

Differentiated cells were labeled with 1 μM carboxy-fluorescein diacetate succinimidyl diester (CFSE; Molecular Probes, Eugene, OR) as described in detail previously (Copland et al., 2006; Holtz et al., 2002). These cells were then incubated overnight in differentiation media supplemented with growth factors to allow excess unbound dye to leak out of the cells. Cells cultured in the presence of 10 μg/ml mitomycin C (Sigma

Aldrich) were used to establish the $CFSE_{max}$, (undivided cell population). The next day, $CFSE^{bright}$ cells were sorted by FACArias to exclude non-labeled and $CFSE^{dim}$ populations. These cells were then cultured for 4 days in DM supplemented with growth factors with or without 5 μM. At the end of the culture period, cells were stained with anti–CD34-APC and 7AAD for flow cytometry analysis using FACSCalibur. The percentage of cells in each generation was determined by using FlowJo software (Tree Star, Ashland, OR), with the position of the parent generation set on the basis of the fluorescence profile of undivided cells.

**Flow cytometric analysis.**

Cells were prepared in PBS-FBS (PBS containing 0.05% sodium azide, 1 mM EDTA, and 2% FBS), and labeled with a combination of monoclonal antibodies and 7-aminoactinomycin D (7-AAD) for dead cell exclusion. Samples were analyzed using a FACS-Calibur flow cytometer (BD Biosciences) with CellQuest acquisition software (BD Biosciences). Analysis was performed with FlowJo software (Tree Star, Ashland, OR) as previously described. Control staining with appropriate isotype-matched control antibodies (BD Biosciences) was included to establish thresholds for positive staining. All antibodies used in this study were listed in Table 1.

**Adhesion assay**

Ninety-six-well plate were coated with either fibronectin (BD bioscience) or BSA (Sigma) at 4 °C overnight. $iCD34^{+}$ cells were incubated in serum-free medium with low concentration of growth factors (GFs) consisting of IMDM supplemented with BIT

(Stemcell Technologies), 1 μM mercaptoethanol, EXCYTE, 1 ng/ml SCF, 1ng/ml IL-3, 1 ng/ml IL-6, 1 ng/ml Flt3L and 1 ng/ml GM-CSF, with and without 5 μM imatinib mesylate for 24 hours. Cells were then washed and resuspended in IMDM before plating onto either fibronectin-coated or BSA-coated wells at 1,000 cells/well for 2 hours. Subsequently, nonadherent and adherent fractions were separated as describe (Holtz et al., 2002). Both fractions were plated in serum-containing StemMACS HSC-CFU media (Miltenyi Biotec, CA), and the percentage of adherent CFCs was calculated.

**Hemangioblast and Mesenchymoangioblast assay and MSC-derivation**

Hemangioblasts and mesenchymoangioblast assays were performed as described in details (Vodyanik et al., 2010). Mesenchymal stem cells (MSCs) are derived from mesenchymoangioblasts and expanded as described previously.

**Western Blotting**

Cells were cultured in serum-free medium without growth factor in the presence or absence of 5 μM imatinib for 4 hours prior to harvesting. Lysates were prepared in buffer containing 0.5% Nonidet P-40 (Sigma Diagnostics) and 0.5% sodium deoxycholate supplemented with phenylmethylsulfonyl fluoride (1 mM), protease inhibitors mixture, and phosphatase inhibitors (50 mM NaF, 0.5 mM $Na_3VO_4$). Proteins were resolved on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to nitrocellulose membrane. Cells were immunoblotted for phospho-Crkl using rabbit polyclonal antibody (Cell Signaling Technology) and horseradish peroxidase– or alkaline phosphatase–conjugated secondary antibodies (Jackson Immuno

Research Laboratories). The protein expression level was determined by densitometry with the use of Image-Quant software (Amersham Pharmacia Biotech, Piscataway, NJ). Each experiment included the CML cell line K562 as a positive control.

**Gene expression analysis by real-time PCR**

RNA was isolated from the subpopulations cells using PureLink RNA mini kit (Life Technologies) according to the manufacturers' instruction. cDNA was prepared from 0.7 ug total RNA using oligo-dT primer with Advantage RT-for-PCR kit (Clontech). Quantitative real-time PCR analysis was performed for all the cDNA samples using self-designed specific primers (Table 2) and PlatinumSYBR Green qPCR SuperMix-UDG kit (Life Technologie). The reactions were run on a Mastercyclerep realplex thermal cycler (Eppendorf) and expression levels were calculated by minimal cycle threshold values (Ct) normalized to the reference expression of GAPDH in each sample (Pfaffl, 2001). All qPCR products were analyzed on 1.2% agarose gels to confirm the specificity of detection.

**Statistical analysis**

Data obtained from multiple experiments were reported as the mean ± SEM. Significance levels were determined by one-tailed *Student-t* test analysis.

**Table 1: List of antibodies**

| Antigen | Clone | Vendor |
|---|---|---|
| Annexin V | | BD Pharmingen |
| CD117 | YB5.B8 | BD Pharmingen |
| CD11c | 3.9 | Caltag |
| CD14 | M5E2 | BD Pharmingen |
| CD15 | HI98 | BD Pharmingen |
| CD16 | 3G8 | BD Pharmingen |
| CD19 | HIB19 | BD Pharmingen |
| CD1a | HI149 | Caltag |
| CD2 | RPA-2.10 | BD Pharmingen |
| CD20 | 2H7 | BD Pharmingen |
| CD235a | GA-R2(HIR2) | BD Pharmingen |
| CD24 | ML5 | BD Pharmingen |
| CD3 | UCHT1 | BD Pharmingen |
| CD31 | WM69 | BD Pharmingen |
| CD33 | WM53 | BD Pharmingen |
| CD34 | 581 | BD Pharmingen |
| CD38 | HIT2 | BD Pharmingen |
| CD41a | HIP8 | BD Pharmingen |
| CD43 | 1G10 | BD Pharmingen |
| CD45 | HI30 | BD Pharmingen |
| CD45RA | 5H9 | BD Pharmingen |
| CD56 | B159 | BD Pharmingen |
| CD66b | G10F5 | BD Pharmingen |
| CD90 | 5E10 | BD Pharmingen |
| mCD29 | HM beta 1-1 | Serotec |
| TRA-1-85 | TRA-1-85 | R&D systems |
| pCrkl(tyr207) | | Cell Signaling |
| Crkl (c-20) | | SantaCruz Biotechnology |

Table 2: List of primers

| Gene | Forward | Reward |
|---|---|---|
| GAPDH | GTGGACCTGACCTGCCGTCT | GGAGGAGTGGGTGTCGCTGT |
| OLFM4 | CCAGCTGGAGGTGGAGATAAG | TCAGAGCCACGATTTCTCGG |
| Bcr/Abl | ACGTTCCTGATCTCCTCTGACTATG | TCAGACCCTGAGGCTCAAAG |

## Results

### *CML iPSCs and BM iPSCs show similar pattern of mesodermal and hematopoietic development and efficacy of hematopoietic differentiation*

In present study we used OP9 differentiation system to induce hematopoietic development from two CML iPSC lines (CML15 iPSC and CML17 iPSC). These cell lines were generated from bone marrow mononuclear cells obtained from the patient with complex 4-way translocation (1;9;11;22) in chronic phase of CML who was not previously treated with imatinib (Hu et al., 2011). Using PCR analysis we found that this translocation was associated with p210 isoform (figure 1A). iPSC lines derived from normal bone marrow mononuclear cells (BM1K iPSC and BM9 iPSC) were used as a control (Hu et al., 2011). Previously, we demonstrated that undifferentiated iPSCs that are transferred to OP9 feeders undergo a series of changes leading to the formation of the apelin receptor ($APLNR^+$) mesoderm (Vodyanik et al., 2010), $CD31/144^+CD73^-$ $CD235a/43^-$ definitive hemogenic endothelial progenitors, and a full spectrum of $CD34^+$ hematopoietic progenitors, all of which can be identified by expression of CD43 (Choi et al., 2009b; Vodyanik et al., 2006). $APLNR^+$ mesodermal cells contain common precursors for mesenchymal stem cells (MSCs) and endothelial cells, mesenchymoangioblasts, and common precursors for endothelial and primitive blood cells, hemangioblasts. Both types of these progenitors can be identified using FGF2-dependent serum-free clonogenic assay. As shown in figure 1B, CML iPSCs and control BM iPSCs cocultured on OP9 produced similar subsets of mesodermal cells, hematoendothelial and hematopoietic progenitors and MSCs. No significant differences were observed in the number and composition of these progenitors generated from CML

or normal iPSCs.

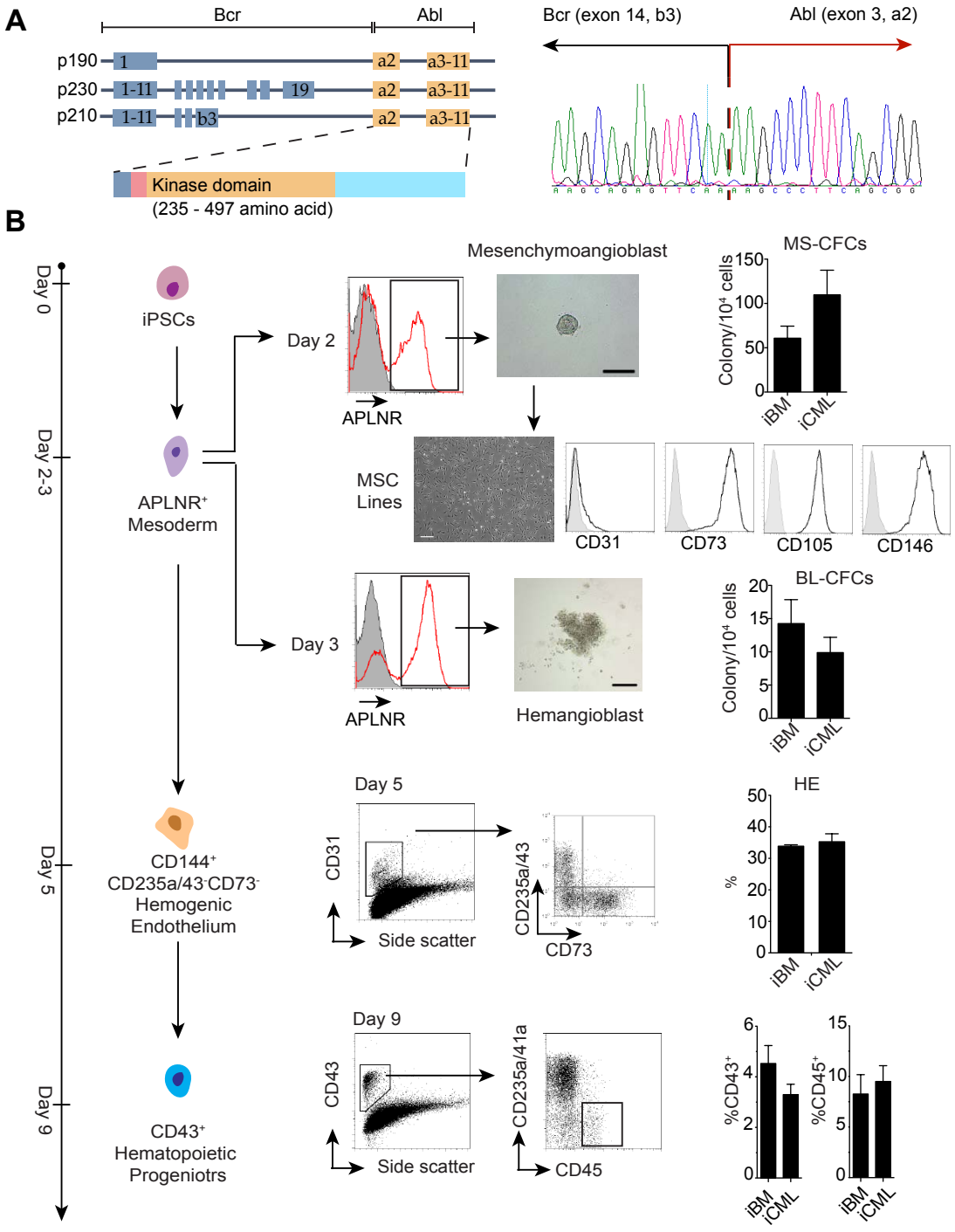


**Figure 1. Bcr/Abl rearrangement in CML iPSCs and their mesodermal lineage differentiation**

A. Schematic diagram showed 3 different isoforms of Bcr/Abl protein. CML iPSCs expressed typical p210 isoform without mutation in the kinase domain (235 – 497 amino acid). Flow chromatogram depicts the site of translocation junction.

B. Schematic diagram shows the differentiation of iPSCs-derived cells in coculture with OP9. Mesodermal cells are formed during the first 2-3 days of differentiation. These cells can be identified by expression of apelin receptor (APLNR). $APLNR^{+}$ cells have mesenchymonagioblast and hemangioblast potentials (BL-CFCs), which can be identified in serum-free FGF2 supplemented semisolid medium. Mesenchymoangioblast (MS) colonies where subsequently subcultured to generate mesenchymal stem cell (MSC). Histogram showed the typical surface markers of MSC. Closed histogram = isotype control. Immediate precursors of definitive hematopoietic cells hemogenic endothelial progenitors (HE) can be identified at day 5 of differentiation as $CD31^{+}CD235a/43^{-}CD73^{-}$ cells. Histogram shows the percentage of HE in total $CD31^{+}$ population. All hematopoietic progenitors with colony-forming potential can be identified by expression of CD43. These progenitors include $lin^{-}$ $CD34^{+}CD45^{+}$ cells highly enriched myeloid colony forming cells. Histogram showed percentage of $CD43^{+}$ in differentiation cultures and percentage of $CD45^{+}$ within the gated $CD43^{+}$ population of cells.

***Generation of LSC-like cells from CML iPSCs***

CML LSCs have been identified within the most primitive hematopoietic compartment as cells with long-term culture initiating cell (LTC-IC) or *in vivo* repopulating activities (Corbin et al., 2011; Li et al., 2012; Petzer et al., 1996; Sloma et al., 2010; Udomsakdi et al., 1992). Similar to normal HSCs, CML LSCs express markers of primitive hematopoietic cells including CD34 and CD90 (Udomsakdi et al., 1992). They are also negative for hematopoietic lineage markers ($lin^-$) and CD45RA. Due to dynamics in disease progression from chronic to blastic phase, CML LSCs are thought to be enriched in different compartments, $lin^-CD34^+CD38^-$ in chronic phase or $lin^-CD34^+CD38^+$ compartment in the blastic phase (Gerber et al., 2011; Goardon et al., 2011; Goff et al., 2013; Holyoake et al., 2001). In addition, CML LSCs display aldehyde dehydrogenase (ALDH) activity and ability to efflux Rhodamine-123 (Udomsakdi et al., 1992) and are resistant to imatinib (Corbin et al., 2011; Graham et al., 2002; Holtz et al., 2002). To investigate whether LSC-like cells can be generated from CML iPSCs we performed phenotypic and functional analysis of the $CD43^+$ hematopoietic cells generated from them at day 9 of differentiation on OP9. Similar to our findings with normal iPSCs (Choi et al., 2009b; Vodyanik et al., 2005; Vodyanik et al., 2006), $CD43^+$ cells derived from CML iPSCs were composed of $CD235a^+CD41a^+$ erythromegakaryocytic progenitors and $CD34^+CD43^+CD45^-$ and $CD34^+CD43^+CD45^+$ multipotent progenitors. The later cells are highly enriched in myeloid progenitors (Choi et al., 2009b; Vodyanik et al., 2006). As shown in (Figure 2B) day $9^{th}$, $CD45^+$ cells obtained at day 9 of differentiation expressed CD34, CD90 and CD117 primitive hematopoietic cell markers and were lacking lineage markers, CD38 and CD45RA, i.e. had typical $lin^-CD34^+CD38^-CD45RA^-CD90^+CD117^+$

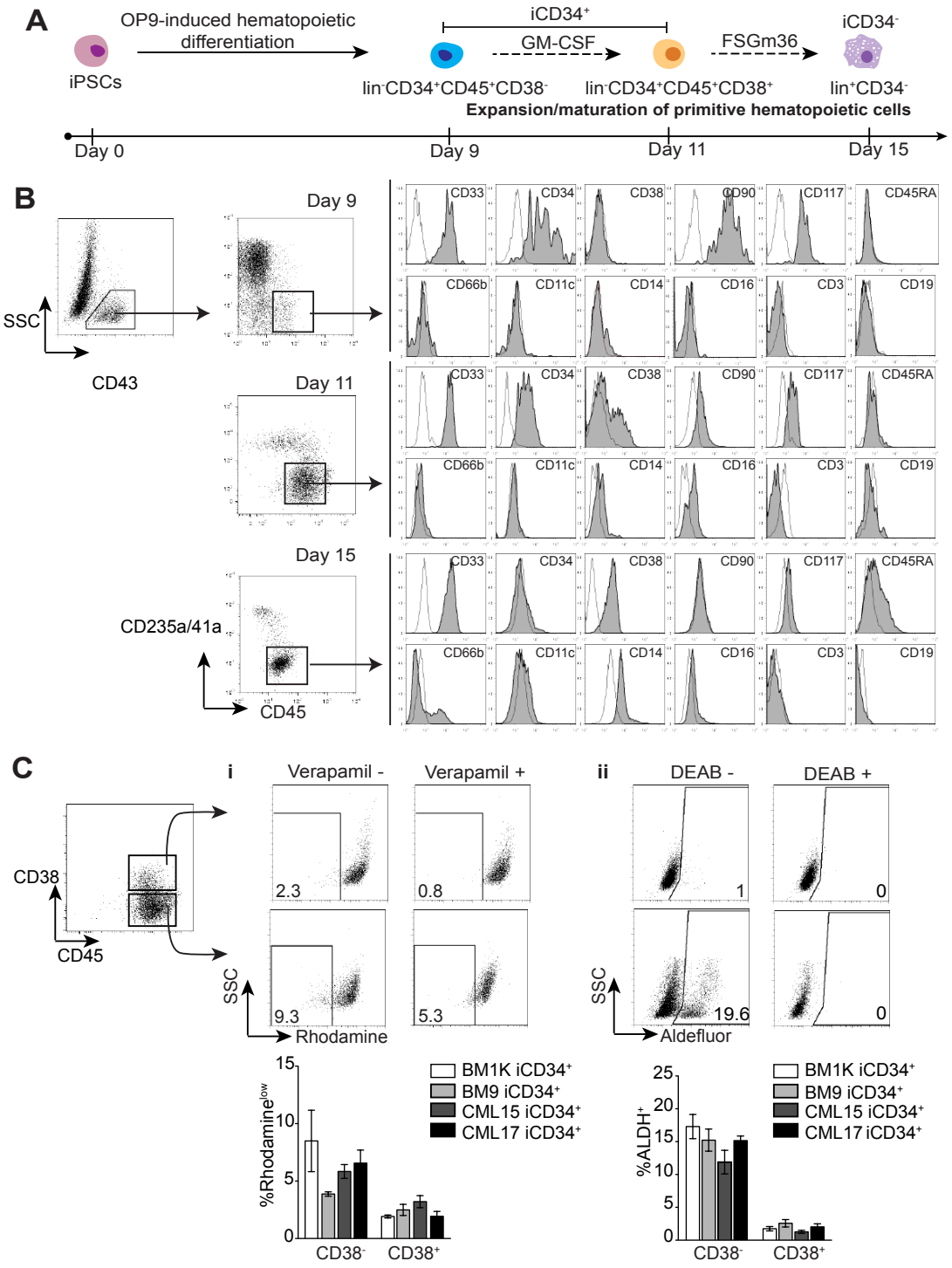


**Figure 2. Generation of lin$^-$CD34$^+$CD43$^+$CD45$^+$ primitive hematopoietic cells from CML iPSCs**

A. Schematic diagram of hematopoietic differentiation from iPSCs. Phenotype and its

designation are shown under and on the top of corresponding cells, respectively. Prefix-i indicates iPSC-derived. FSGm36 = Flt3L, SCF, GM-CSF, IL3, IL6.

B. A phenotype of $CD45^+$ cells obtained from CML iPSCs after differentiation on OP9 (day 9) and following their expansion/differentiation in stroma-free medium with growth factors (as indicated in A). Representative results from 3-4 independent experiments are shown.

C. Rhodamine (Rho) efflux **(i)** and ALDH activity **(ii)** in isolated CML $iCD34^+$ cells. Representative dot plots show RhO efflux and ALDH activity in $CD38^+$ and $CD38^-$ populations. Graph under corresponding dotplot shows quantification of Rho efflux and ALDH assay. The values are mean ± SEM of (% of $Rho^{low}_{verapamil-}$ - $Rho^{low}_{verapamil+}$) and (% of $ALDH^+_{DEAB-}$ - $ALDH^+_{DEAB+}$) from 3 experiments respectively. DEAB = diethylaminobenzaldehyde, ALDH = aldehyde dehydrogenase.

HSC phenotype. These primitive hematopoietic progenitors can be expanded by selecting $CD43^+$ cells from CML iPSCs differentiated on OP9, and culture them with GM-CSF for 2 days (Choi et al., 2009a). After expansion, $CD45^+$ cells retained expression of CD34, CD117, and CD90 stem cell markers and remained $lin^-$ and $CD45RA^-$, however some of them (approximately 25%) acquired expression of CD38 marker (Figure 1B, day 11). CML iPSCs-derived $lin^-CD34^+CD45^+$ (hereafter referred to as induced $CD34^+$; CML $iCD34^+$) cells were able to efflux rhodamine-123 (Rho) and expressed a high level of ALDH (Figure 2C). $Rho^{low}$ and $ALDH^{high}$ cells were enriched within the $CD38^-$ compartment as compared to $CD38^+$ compartment. The pattern of Rho and ALDH activity in CML $iCD34^+$ cells was similar to control BM iPSCs-derived $lin^-$

$CD34^+CD45^+$ (BM $iCD34^+$) cells (Figure 2C). Both, CML $iCD34^+$ and BM $iCD34^+$ cells were highly enriched in myeloid CFCs. However, the number of myeloid colonies within the $CD38^-$ compartment was significantly higher in CML $iCD34^+$ cells than BM $iCD34^+$ cells (Figure 3A).

Although CML LSCs share many properties with HSCs, they have abnormally increased long-term survival and proliferation, adhesion defect and innate resistant to tyrosine kinase inhibitors (Bhatia et al., 2000; Corbin et al., 2011; Verfaillie et al., 1997). Using LTC-IC assay and limiting dilution analysis we found that CML $iCD34^+$ cells produced a much higher number of LTC-IC-derived CFCs than BM $iCD34^+$ cells, indicating increased long-term survival (Figure 2C). In addition, CML $iCD34^+$ cells demonstrated significantly high proliferative potential in serum-containing media supplemented with growth factors compared to BM $iCD34^+$ cells (Figure 2D). Similar, CML $iCD34^+$ cells proliferated faster in serum-free media supplemented with growth factors. Previous studies demonstrated that $CD34^+$ cells isolated from CML patients exhibit an abnormal ability to proliferate in the absence of added growth factors. This abnormal proliferation seems to be dependent on autocrine loop initiated by secretion of IL-3 and G-CSF in early progenitor cells (Jiang et al., 1999; Jiang et al., 2007). To evaluate whether CML $iCD34^+$ behave in a similar fashion, we cultured them in serum-free medium without growth factors. As shown in (Figure 3D), CML $iCD34^+$ cells demonstrated a proliferative advantage relative to control BM $iCD34^+$ cells by withstanding growth factor deprivation in a serum-free condition.

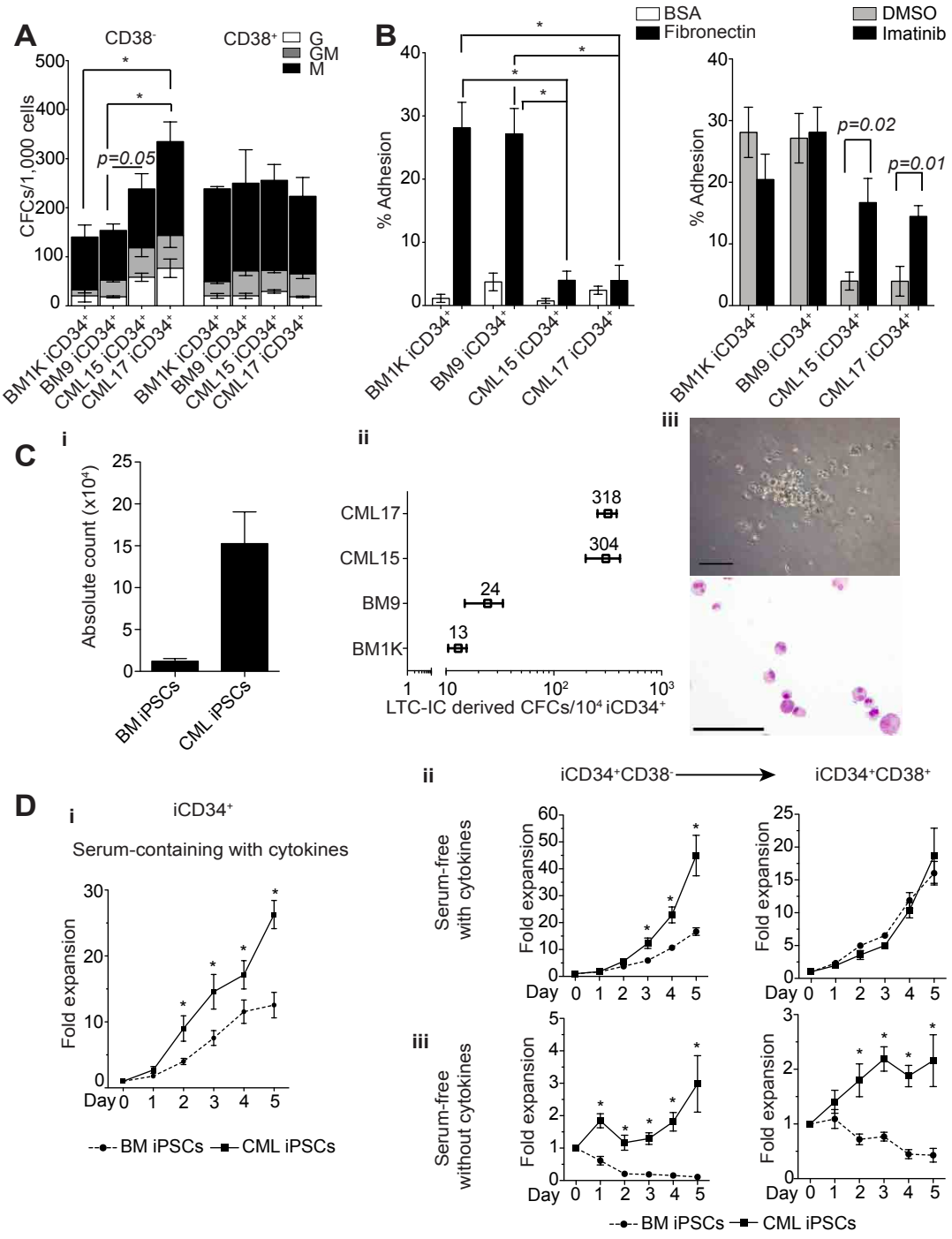


**Figure 3. Stem/progenitor cell properties of BM and CML iCD34$^+$ cells**

A. Colony formation from BM and CML iCD34$^+$ cells. Results are mean ± SEM from 3 independent experiments. * indicates significant differences ($p < 0.01$).

B. Adhesion of BM and CML iCD34$^+$ cells on fibronectin or control BSA-adsorbed plates. Before adhesion assay, cells were incubated for 24 h in serum-free medium containing low concentration of growth factors without (**left graph)** or with 5 μM imatinib (**right graph).** Results are mean ± SEM from 3 independent experiments. * indicates significant differences ($p < 0.01$). BSA = bovine serum albumin.

C. LTC-IC potential of iCD34$^+$ cells. **(i)** Absolute number of CD45$^+$ cells expanded from BM and CML iCD34$^+$ at the end of 5 weeks. **(ii)** Frequency of LTC-IC derived CFCs in BM and CML iCD34$^+$ cells. Numbers next to the plot represent 95% CI. **(iii)** Representative colony and Wright stained cytospin from the colony formed by CML iCD34$^+$ cells after 5 weeks of LTC-IC culture are shown. Scale bar = 100 μm.

D. Expansion of CML and BM iCD34$^+$ cells in serum-containing **(i)** and serum-free conditions with **(ii)** and without **(iii)** growth factors. Where indicate, SCF, IL3, IL6, Flt3L and GM-CSF were added. Results are mean ± SEM from 6 independent experiments (3 from each BM1 and BM9 iCD34$^+$, and 3 from each CML15 and CML17 iCD34$^+$). * indicates significant differences ($p < 0.05$).

It has been established that somatic CD34$^+$ cells with active Bcr/Abl signaling have reduced adhesion to fibronectin, which can be at least partially restored with imatininb treatment (Bhatia et al., 2001; Verfaillie et al., 1997). When we cultured CML iCD34$^+$ cells and control BM iCD34$^+$ cells in serum-free media supplemented with low concentration of growth factors and transferred them to fibronectin or bovine serum albumin (BSA)-coated plate, we found CML iCD34$^+$ cells showed impaired adhesive property, whereas BM iCD34$^+$ did not. The impaired adhesion of CML iCD34$^+$ cells was

partially rescued by imatinib (Figure 2B). Overall, these findings provide strong evidence that $lin^{-}CD34^{+}CD45^{+}$ cells derived from CML iPSCs behave similar to their somatic counterpart.

***Induced LSC-like cells are resistant to imatinib***

The dependence of CML cells on Bcr/Abl signaling enable the suppression of disease by TKIs and long-term survival of the patients. However, resistances of CML LSCs to imatinib preclude a complete cure of CML. Analysis of Bcr/Abl expression in undifferentiated CML iPSCs and their progeny by PCR and western blot revealed the highest level of Bcr/Abl expression in undifferentiated iPSCs with a major drop observed when cells become mesoderm (Figure 4). Following differentiation of CML iPSC-derived mesoderm into the mesenchymal stem cells (MSCs) and CML $iCD34^{+}$ cells the expression of Bcr/Abl maintained at lower level as compared to undifferentiated iPSCs. Despite the high level of Bcr/Abl expression, CML iPSCs did not show an increased proliferation rate as compared to control iPSCs. While imatinib down-regulated *Bcr/Abl* at mRNA and protein levels and phosphorylation of downstream Bcr/Abl target, p-Crkl, CML iPSCs growth rate was unaffected by imatinib (Figure 5A-E). Similarly, imatinib has no effect on growth of MSCs derived from CML iPSCs (Table 1). These data indicate that Bcr/Abl expressed in undifferentiated iPSCs or their non-hematopoietic derivatives does not activate abnormal cellular proliferation and is indispensable for their survival.

Reduced sensitivity to imatinib is recognized as an important feature distinguishing CML

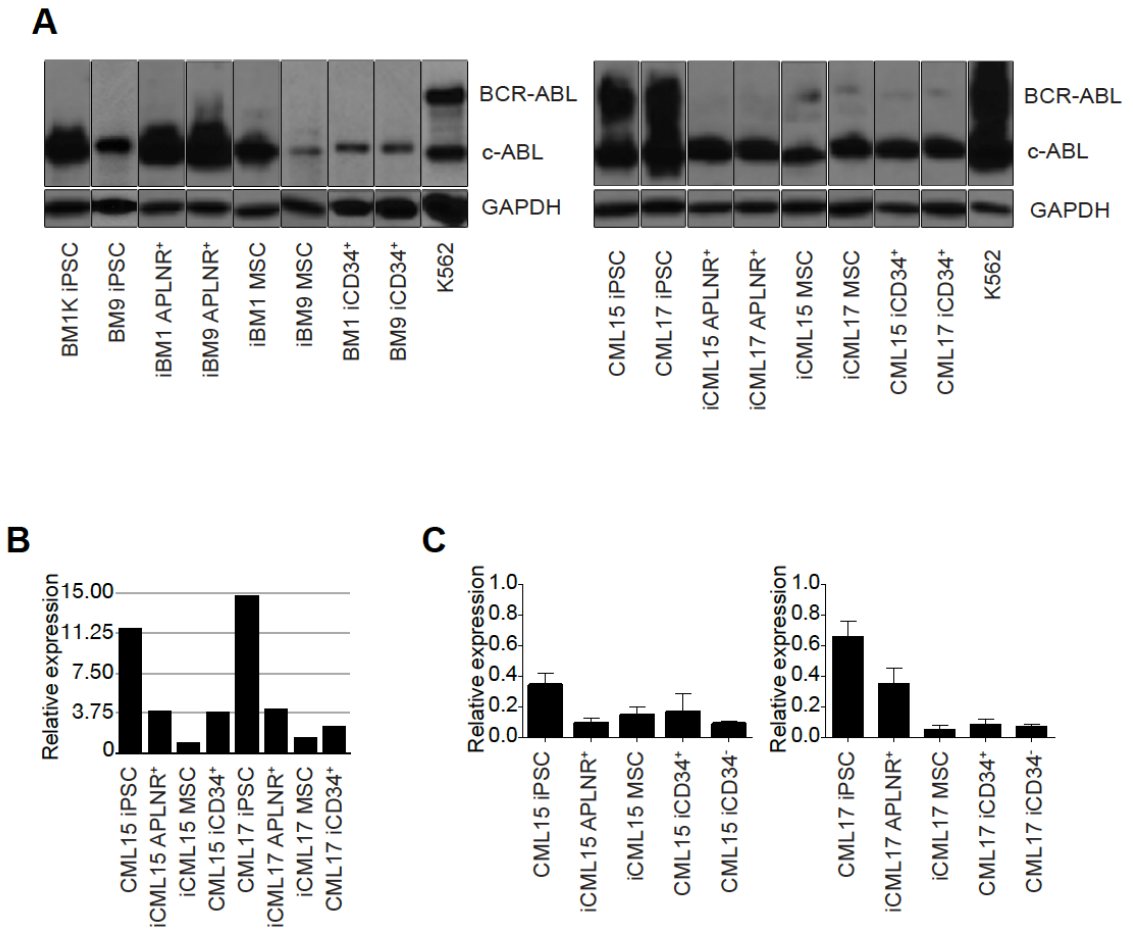


**Figure 4. Expression of Bcr/Abl in CML iPSCs and their derivatives**

A. Western blot showed expression of Bcr/Abl protein in normal BM iPSCs and CML iPSCs and their derivatives ($APLNR^+$ mesodermal cells isolated at day 3 of differentiation, mesenchymoangioblast-derived MSC cell lines (iMSC), $iCD34^+$ and $iCD34^-$ cells). GAPDH and K562 were used as a loading control and a positive control respectively.

B. Histogram showed expression level of BCR-ABL protein in CML iPSCs and their derivatives. The expression level was calculated relatively to K562.

C. qPCR quantification of *Bcr/Abl* mRNA expression in CML iPSCs and their derivatives. The expression level was calculated relatively to K562. Results are the

mean ± SEM from 3 experiments in duplicate.

LSCs from the more mature myeloid cells. Studies in CML patients demonstrated that Bcr/Abl kinase is active in primitive hematopoietic cells, and that imatinib inhibits this kinase without affecting survival of LSCs (Copland et al., 2006; Corbin et al., 2011; Graham et al., 2002; Holtz et al., 2002; Schemionek et al., 2010). To investigate whether this is similarly true for hematopoietic cells generated from CML iPSCs, we evaluated the phosphorylation status of the Bcr/Abl-specific substrate Crk-like protein (p-Crkl) in CML iCD34$^{+}$ and CML iCD34$^{-}$ cells using Western blot. As shown in Figure 6A, p-Crkl was active in CML iCD34$^{+}$ cells but not in control BM iCD34$^{+}$ cells. After treatment with imatinib, p-Crkl dramatically decreased in both primitive and more mature blood cells indicating that imatinib efficiently inhibits kinase activity independently of the stage of maturation. These data strongly support the conclusion that imatinib inhibits Bcr/Abl activity in the CML iPSC-derived blood cells. To find out whether hematopoietic cells generated from CML iPSCs have maturation stage-dependent sensitivity to imatinib, we evaluated its effect on CML iCD34$^{+}$ cells and mature CML iCD34$^{-}$ cells generated following expansion and maturation of CML primitive blood cells. To determine the effect of imatinib on CFCs, we cultured CML iCD34$^{+}$ in clonogenic medium with and without 10 μM imatinib. Because at high dose imatinib nonspecifically inhibits c-kit receptor which interrupts macrophage/monocyte and erythroid colony formation (Dewar et al., 2003; Oehler et al., 2003), we analyzed only G- and GM- CFCs. As shown in Figure 6C, the number of these CFCs from both CD38$^{+}$ and CD38$^{-}$ sub-fractions of CML iCD34$^{+}$ cells was not affected by imatinib. However, we noticed that imatinib reduced

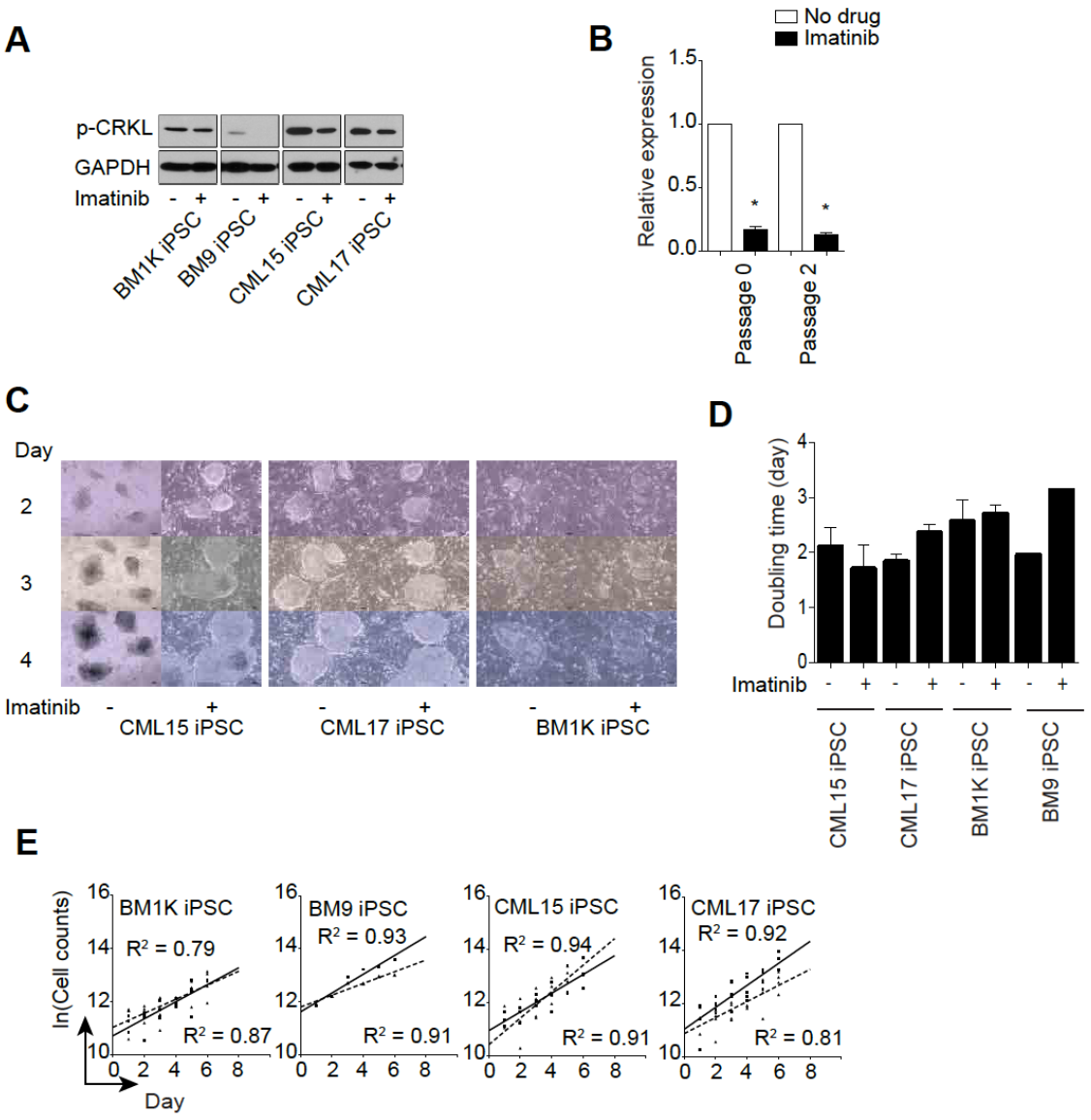


**Figure 5. The effect of imatinib effect on undifferentiated CML iPSCs**

A. Western blot showed inhibition of p-Crkl when imatinib was added to CML-iPSC cultures.

B. qPCR quantification of *Bcr/Abl* mRNA expression in CML iPSCs before and after culture with 10 μM imatinib for 2 passages. The expression level was calculated relatively to untreated control. Results are mean ± SEM of 3 experiments in duplicate.

C. Time-lapse pictures of colonies growing with and without imatinib.

D. Doubling time for cells growing with and without imatinib. Bars represent the mean ± SEM of 3 experiments.

E. Linear regression shows cell proliferation of BM and CML iPSCs with or without 10 μM imatinib. $R^2$ represent goodness of fit for each regression. Results are from 3-5 independent experiments (BM9 iPSC from 1 experiment).

**Table 3:** The 50% inhibition concentration ($IC_{50}$) for iPSCs and their derivatives.

| Cell type | $IC_{50}$ (uM) | $R^2$ |
|---|---|---|
| **BM1K** | | |
| iPSCs | 9.55 - 15.64 | 0.95 |
| MSCs | 12.32 - 18.82 | 0.97 |
| $CD34^+$ | 8.65 - 14.54 | 0.95 |
| $CD34^-$ | 12.39 - 24.81 | 0.92 |
| **BM9** | | |
| iPSCs | 15.09 - 24.07 | 0.98 |
| MSCs | 28.72 - 48.60 | 0.92 |
| $CD34^+$ | 10.12 - 16.18 | 0.97 |
| $CD34^-$ | 13.46 - 24.02 | 0.93 |
| **CML15** | | |
| iPSCs | 11.66 - 14.06 | 0.99 |
| MSCs | 17.16 - 34.10 | 0.91 |
| $CD34^+$ | 11.64 - 20.13 | 0.94 |
| $CD34^-$ | 2.80 - 4.29 | 0.96 |
| **CML17** | | |
| iPSCs | 12.85 - 18.07 | 0.98 |
| MSCs | 26.85 - 43.53 | 0.94 |
| $CD34^+$ | 6.20 - 11.42 | 0.92 |
| $CD34^-$ | 2.58 - 4.41 | 0.96 |

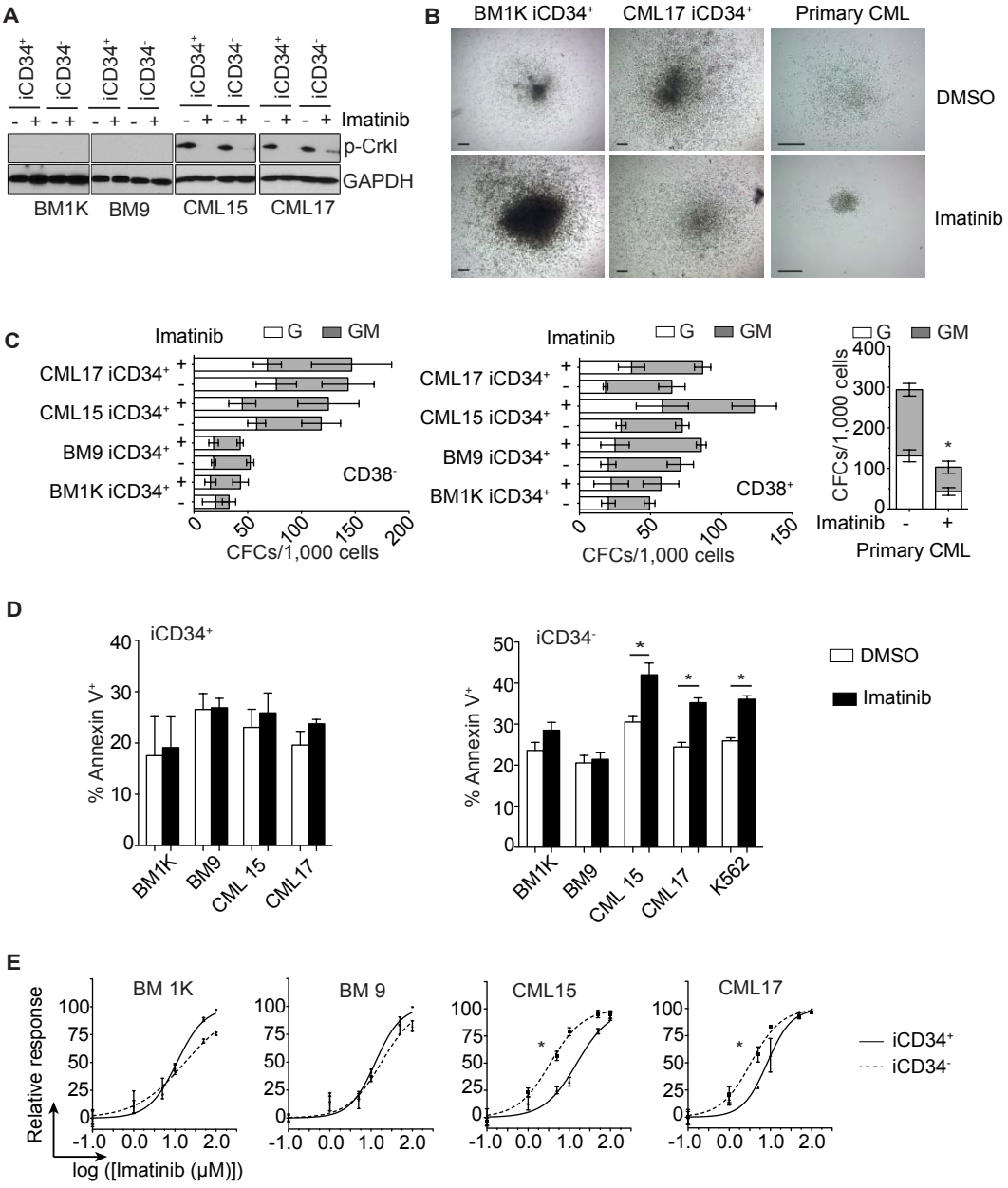


**Figure 6. CML iCD34$^+$ cells are resistant to tyrosine kinase inhibition**

A. Western blotting showed phospho-Crkl (p-Crkl) level in iCD34$^+$ and iCD34$^-$ cells in the presence or absence of imatinib. GAPDH was used as a loading control.

B. A representative colony pictures from BM and CML iCD34$^+$ cells, and parental CML bone marrow mononuclear cells cultured with and without 10 μM imatinib in a Methocult media. BM and CML iCD34$^+$ cells, scale bar = 100 μm. Parental CML, scale bar = 200 μm.

C. Colony formation from CD38$^+$ and CD38$^-$ within BM and CML iCD34$^+$ cells, and parental CML lin$^-$CD34$^+$ with and without 10 μM imatinib. Results are the mean ± SEM of 3 independent experiments. * indicates significant differences ($p < 0.05$).

D. Apoptosis in BM and CML iCD34$^+$, and their derivative iCD34$^-$ treated and non-treated with 5 μM imatinib for 24 hours. Apoptosis was evaluated by annexin V staining. Results are the mean ± SEM from 3 independent experiments. K562 was used as an imatinib-sensitive control. * indicate significant differences ($p<0.05$).

E. 50 % Inhibition concentration ($IC_{50}$) assay from BM and CML iCD34$^+$, and their derivative iCD34$^-$ was shown as relative response versus concentration of imatinib. * indicates significant $IC_{50}$ shift ($p<0.05$). Results are the mean ± SEM from 3 experiments performed in triplicate.

the colony size in CML iCD34$^+$ cells, while it has no effect or slightly enhanced the size on BM iCD34$^+$ cells (Figure 6B). The decrease of the colony size in CML iCD34$^+$ CFCs may be related to suppression of proliferation by imatinib or restoration of sensitivity to imatinib in more mature blood cells following colony expansion (Li et al., 2012). Although we found the lack of sensitivity of CML iCD34$^+$ CFCs to TKI, culture of parental bone marrow mononuclear cells in clonogenic medium with imatinib significantly reduced the number of CFCs, which is consistent with data reported in the

literature showing the inhibitory effect of imatinib on lin$^-$CD34$^+$CD38$^-$ and CD34$^+$CD38$^+$ CFCs obtained from CML patients in the chronic phase (Holtz et al., 2002; Konig et al., 2008). The differences in response to imatinib between somatic and CML iCD34$^+$ cells may indicate that CML iCD34$^+$ cells represent a more immature and homogenous population of primitive hematopoietic cells uniformly resistant to TKI, while somatic lin$^-$ CD34$^+$ cells are known to be heterogeneous (Nowak et al., 2010) and possibly include both a population of imatinib resistant and sensitive progenitors.

It has been shown that lin$^-$CD34$^+$ cells obtained from CML patients are resistant to apoptosis as compared to more mature cells (Corbin et al., 2011; Graham et al., 2002). Consistent with observation in somatic LSCs, imatinib did not induce apoptosis in CML iCD34$^+$ cells, while significant increase in apoptosis was observed in imatinib-treated CML iCD34$^-$ cells (Figure 6D). To confirm the maturation stage-dependent sensitivity to imatinib, we evaluated the effect of imatinib on viability of CML iCD34$^+$ using maximal inhibitory concentration 50% ($IC_{50}$) assay. We found that CML iCD34$^+$ required significantly higher concentration of imatinib to exert the effects as compared to CML iCD34$^-$ (Figure 6E and Table 1). We tested the accuracy of $IC_{50}$-derived from viable cell count by using Annexin V staining and found that both methods gave a value within the same range (Figure 7).

Next, we evaluated the effect of inhibition of Bcr/Abl on expansion of CML iCD34$^+$ cells. Consistent with observation in somatic CML lin$^-$CD34$^+$ cells (Corbin et al., 2011), imatinib had little effect on CML iCD34$^+$ cell proliferation in cytokine-supplemented cultures within the first 24 hours. However, significant reduction in cell expansion was

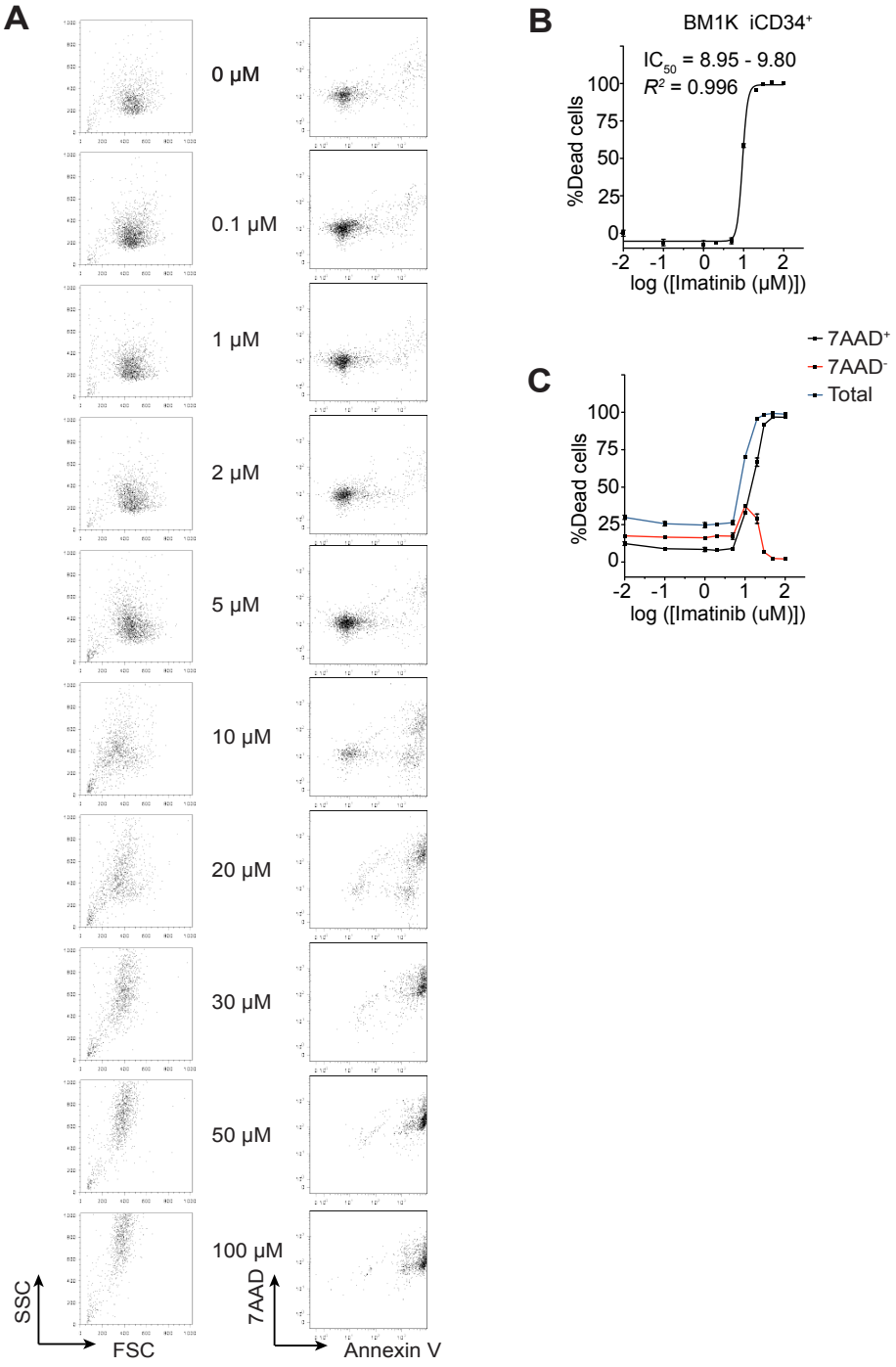


**Figure 7. Analysis of dead cells**

A. Representative dot plots show forward scatter (FSC) vs. side scatter (SSC) and 7AAD vs. Annexin V of BM1 iCD34$^{+}$ treated with various concentration of

imatnib for 24 hours.

B. Relative $IC_{50}$ of BM $iCD34^+$ shown in A, showed as relative response vs. concentration of imatinib. Results are the mean ± SEM from triplicate. $R^2$ represent goodness of fit for a regression.

C. Distribution of necrotic cell (Annexin $V^+7AAD^+$), apoptotic cell (Annexin $V^+7AAD^-$) and total dead cell (Annexin $V^+7AAD^{+/-}$)

observed following the next 4 days of culture of CML, but not control BM $iCD34^+$ cells growing in serum or serum-free conditions with cytokines. These differences were more obvious in serum-containing cultures (Figure 8A). Given that the proportion of mature cells following expansion increases, especially in serum-containing medium, we reasoned that inhibition of proliferation with advanced expansion could reflect that the cells had differentiated to the imatinib-sensitive stage. To prove this suggestion, we investigated the distribution of apoptotic cells within different compartments following expansion using CFSE labeling. We found that imatinib treatment of CML $iCD34^+$ cultures was associated with retention of $CD34^+$ cells in generations 2-6. On the other hand, non-treated cells showed gradual and substantial loss of CD34 expression and predominance of $CD34^-$ cells within the rapidly dividing compartment (Figure 8B). Staining of CFSE cultures with annexin V, revealed that the most primitive $iCD34^{bright}$ cells were resistant to imatinib-induced apoptosis regardless of proliferative potential, while substantial increase in apoptosis was observed in more mature $iCD34^{dim}$ and $iCD34^-$ cells treated with imatinib (Figure 8D). This implied that differentiated CML $iCD34^-$ cells became sensitive to imatinib and do not survive in the cultures, while CML $iCD34^+$ cells did not

undergo apoptosis upon imatinib treatment and are selected to dominate in culture.

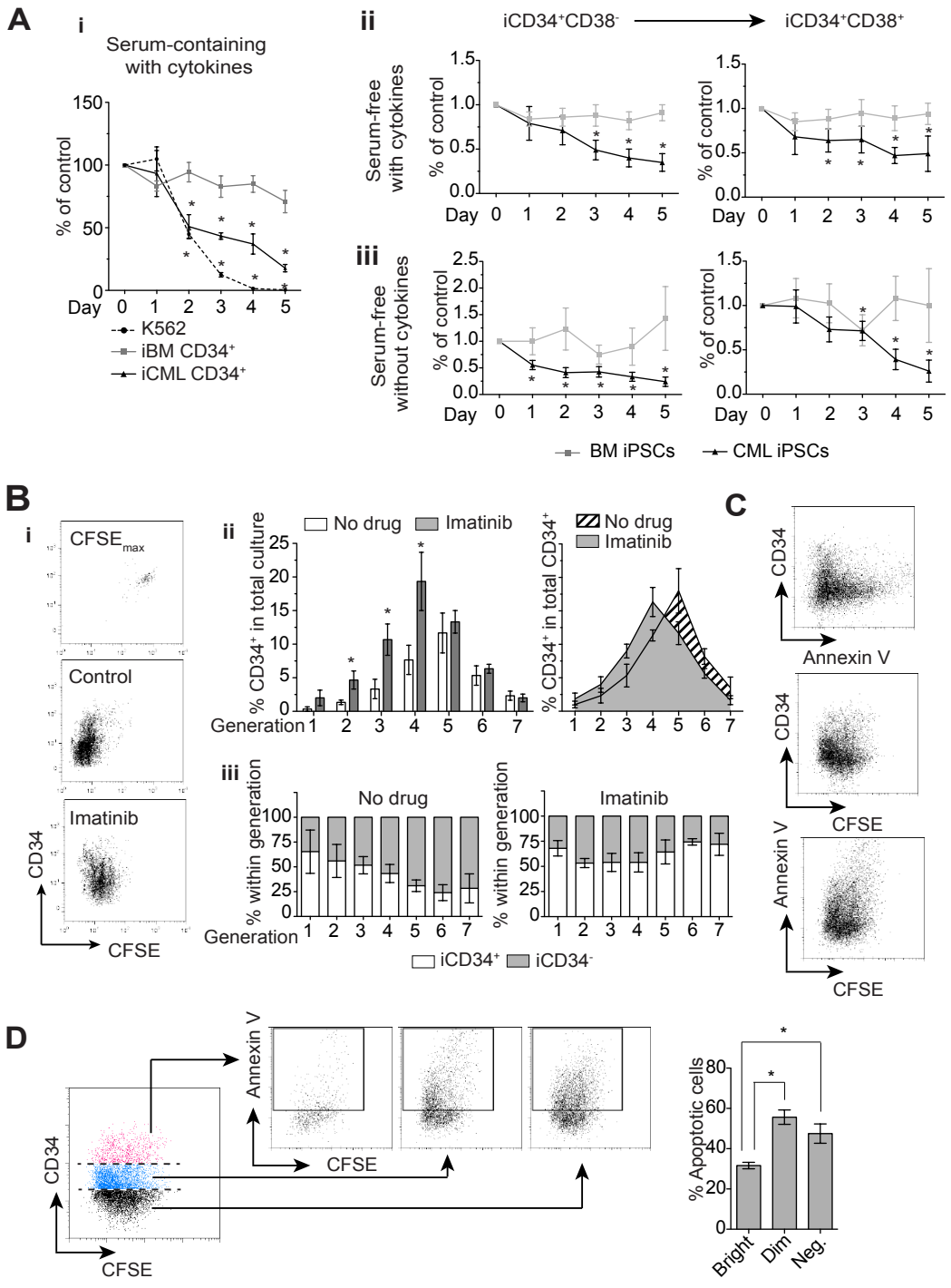


**Figure 8. Maturation induces CML iCD34$^+$ cells sensitive to tyrosine kinase inhibition**

A. Proliferation of BM and CML iCD34$^+$ in serum-containing with growth factors **(i)**, serum-free condition with **(ii**) and without growth factors **(iii)** in presence or absence of 5 μM imatinib**.** Where indicate, SCF, IL3, IL6, Flt3L and GM-CSF were added. Absolute cell count was shown as % of no imatinib control. Results are mean ± SEM from 6 independent experiments (3 from each BM1 and BM9 iCD34$^+$, 3 from each CML15 and CML17 iCD34$^+$). * indicates significant ($p < 0.05$)

B. CFSE tracking of CML iCD34$^+$ cells. (**i**) iCD34$^+$ cells were cultured with or without 5 μM imatinib for 4 days in serum-containing media supplement with growth factors. Mitomycin C-treated cells were used to set the $CFSE_{max}$. Dot plot shows CD34 vs. CFSE. (**ii**) The percentage of iCD34$^+$ cells in total culture **(left)**, and in total CD34$^+$ population **(right)**. (**iii**) The relative proportion of iCD34$^+$ and iCD34$^-$ cells within each generation. Histogram shows mean ± SEM of 3 experiments. * indicates significant ($p<0.05$)

C. Dotplots show expression of CFSE, CD34 and Annexin V by flow cytometry.

D. Flow cytometric analysis of apoptosis (7AAD$^-$Annexin V$^+$) and CD34 expression at the end of 4-day expansion with 5 μM imatinib. Histogram shows relative proportion of apoptotic cells within iCD34$^{bright}$, iCD34$^{dim}$, and iCD34$^-$ (neg.) population. Results are mean ± SEM of 3 experiments. * indicate significant ($p<0.05$).

Taken together, these results indicate that CML iCD34$^+$ cells reproduce many aspects of drug resistance observed in somatic primitive hematopoietic cells from CML patients in the chronic phase.

**Discussion**

Ex vivo culture of CML cells, ectopic expression of Bcr/Abl and mouse model have provided insights into CML pathogenesis, and led to development of targeted therapy of this neoplastic disease with Bcr/Abl-kinase inhibitor, imatinib. Due to spontaneous differentiation, the primitive hematopoietic cells cannot be expanded and maintained for long in these models. The major advantage of the method described in the current study is that it is unlimited and is scalable to provide sufficient primitive hematopoietic cells in vitro to study CML LSCs and use them for drug screening.

It is generally accepted that reprogramming technology offers novel opportunities in basic research, disease modeling, drug screening and cell therapies (Cherry and Daley, 2012; Colman and Dreesen, 2009; Sancho-Martinez et al., 2011; Zhu et al., 2011). Multiple studies already demonstrated the validity of iPSC model for studies of pathogenesis of human diseases, including among many others genetic diseases such as long-QT syndrome (Moretti et al., 2010), spinal muscular atrophy (Ebert et al., 2009), progeria (Liu et al., 2011a), and Hurler syndrome (Tolar et al., 2011). Although potential utility of iPSCs technology for study neoplasia has been suggested (An et al., 2012; Ye et al., 2009), only few groups reported generation of iPSCs from malignant cells. Hochedlinger and colleagues, who generated mouse pluripotent stem cells by transplanting nuclei from melanoma into oocytes (Hochedlinger et al., 2004), has achieved the first successful reprogramming of cancer cells. After discovery of pluripotency factors that can reprogram blood cells, iPSC lines have been generated from a patient with myeloproliferative disorder bearing JAK2-V617F mutation (Ye et al.,

2009) and from CML cell lines (Carette et al., 2010) and primary bone marrow cells obtained from CML patients in chronic phase (Hu et al., 2011; Kumano et al., 2012). These studies demonstrated that iPSC-generated from neoplastic cells carry diseases-specific genetic mutation and can generate blood cells affected by particular mutation.

In the present studies we demonstrated that $lin^{-}CD34^{+}CD45^{+}$ (CML $iCD34^{+}$) primitive hematopoietic cells with the phenotypic and functional properties of leukemia stem cells, including imatinib resistance, could be generated from CML iPSCs. In summary, our study provide a novel method to generate CML stem cells in vitro from patient-specific iPSCs that capture entire genetic profile including all of the genetic modifiers that have important, yet largely unknown, role in the pathology of CML disease progression and drug resistance. Future study will evaluate the mechanism underlying the innate resistance of CML LSC in chronic phase to imatinib and explore the new drug target by utilizing these cells.

iPSC-based model provides several important advantages for study CML pathogenesis. iPSCs can be expanded indefinitely and used to generate an unlimited number LSC-like cells for functional studies and drug screening. Because iPSCs are single cell origin, multiple iPSC lines can be generated from the same patient to capture the diversity of genetic alterations within leukemia cell populations and address the role of non-Bcr/Abl associated mutations in disease development. iPSCs obtained from the patients before and after acquisition of drug resistance or progression to blast crisis would be a valuable tool for addressing molecular mechanisms of CML progression and drug resistance. In

addition, our studies demonstrated that the biological effect of Bcr/Abl on cells depends on epigenetic environment. Although Bcr/Abl expression was detected in undifferentiated CML iPSCs and at all stages of their differentiation (mesoderm, hemogenic endothelium, blood cells), and in non-hematopoietic cells derived from CML iPSC such as MSCs, imatinib induced significant apoptosis only in mature $CD34^{-}CD45^{+}$ hematopoietic cells. We also revealed that both undifferentiated CML iPSCs and LSC-like cells were resistant to imatinib-induced apoptosis. However, inhibition of Bcr/Abl activity with imatinib has a different effect on proliferation of LSC-like cells and undifferentiated CML iPSCs, pointing out that epigenetic environment and signaling pathways interacting with Bcr/Abl kinase are different in two types of cells. These differences can be further exploited to define epigenetic changes and biochemical pathways involved in CML pathogenesis.

# Chapter 2: Induced Pluripotent Stem Cell Model of Chronic Myeloid Leukemia Revealed Olfactomedin 4 as a Novel Therapeutic Target in Primitive Leukemia Cells


## Abstract

The definitive cure of leukemia requires identification of novel therapeutic targets to eradicate leukemia stem cells (LSCs). However, rarity of LSCs within the pool of malignant cells remains a major limiting factor for their study in humans. Here we show that $lin^-CD34^+CD45^+$ cells with LSC features can be generated de novo from reprogrammed primary chronic myeloid leukemia (CML) cells. Similar to somatic LSCs, induced $lin^-CD34^+CD45^+$ ($iCD34^+$) cells were resistant to tyrosine kinase inhibitor (TKI) imatinib, but became sensitive after maturation. Molecular profiling of CML and control $iCD34^+$ cells treated and not treated with imatinib identified olfactomedin 4 (Olfm4) as the one of the top-ranked genes induced by TKI. The knockdown of Olfm4 by siRNA inhibited growth of $iCD34^+$ cells and primary somatic LSCs, and increased their responses to imatinib. This is the first study to show that iPSC can be used to produce LSC-like cells and identify their novel drug targets.


## Introduction

Chronic myeloid leukemia (CML), a myeloproliferative disorder characterized by Philadelphia chromosome -t(9;22)(q34;q11.2)-, originates within hematopoietic stem cells (HSCs). Constitutively active Bcr/Abl kinase produced by the translocated chromosome drives the transformation and expansion of leukemic progeny (Daley et al., 1990; Holtz et al., 2002; Holyoake et al., 2001; Ramaraj et al., 2004). Currently Abl

tyrosine kinase inhibitor (TKI) imatinib is employed as a first line of treatment for CML (Druker et al., 2006; Druker et al., 2001). Although imatinib therapy induces complete cytogenetic response in most of the patients, it does not cure CML, because leukemic stem cell (LSC) persist in the bone marrow even in patients with complete remission (Cortes et al., 2004; Graham et al., 2002). Several resistant mechanisms have been proposed to explain disease persistence including, acquired mutations affecting Bcr/Abl desensitize its kinase domain to imatinib (Branford et al., 2003), enhanced expression of the drug efflux pump and P-glycoprotein (Chaudhary and Roninson, 1991). Highly expression of Bcr/Abl protein in the CML stem cell may reduce its sensitivity to imatinib, however, the correlation to the persistence of residual disease is still controversial (Sherbenou et al., 2007). Therefore, eradication of CML requires understanding the mechanisms of imatinib resistance and developing new drugs targeting alternative cell survival and renewal pathways in CML LSCs.

Patient-specific iPSC has taken a new turn for studying developmental dysfunction in genetic disease including malignancy. Directed differentiation of the iPSCs into specific tissues of interest enables insight into cell fate decisions and provides a scalable in vitro model of early disease development. In the previous chapter we demonstrated that primitive CML cells with LSC properties could be generated from CML iPSCs and potentially useful to study CML LSC biology particularly screening for novel therapeutic targets.

In this study, we performed comparative analysis of gene expression in $lin^{-}CD34^{+}CD45^{+}$

cells obtained from normal bone marrow- and CML- iPSCs treated and not treated with imatinib and identified olfactomedin 4 (Olfm4) as the top-ranked gene induced by imatinib. Olfm4 knockdown using siRNA inhibited growth of CML iPSCs-derived $lin^-$ $CD34^+CD45^+$ and increased cell dead when combine with imatinib. Importantly, Olfm4 knockdown in parental somatic CML LSCs also showed similar effects as well as reduction in long-term survival. This effect was potentiated by imatinib. Overall this study provides the first successful demonstration of the validity of iPSC model for CML LSC studies and identification of novel targets for drugs in CML LSCs.

## Materials and Method

### iPSCs maintenance and differentiation

In this study, we used transgene-free BM1K, BM9, CML15 and CML17 iPSCs produced by reprogramming of bone marrow mononuclear cells from normal and CML patients in the chronic phase (Hu et al., 2011). Undifferentiated iPSCs were maintained in cocultures with mouse embryonic fibroblasts (MEFs). Hematopoietic differentiation was induced by transferring the iPSCs on to overgrowth OP9 feeders as we have previously described in details (Choi et al., 2009b; Vodyanik et al., 2005). $CD43^+$ cells were collected on day 8 or 9 of differentiation using MACS and cultured in α-MEM supplemented with 10% FBS, 50 μg/ml ascorbic acid, 100 μM monothioglycerol (complete serum supplemented medium; CSSM) and 200 ng/ml GM-CSF to expand selectively myeloid progenitors (Choi et al., 2009a). Enriched myeloid progenitors were cultured for an additional 4 days in the same media supplemented with 10 ng/ml IL-3, 100 ng/ml IL-6, 100 ng/ml Flt3L,

200 ng/ml GM-CSF and 100 ng/ml SCF.

**Purification of lin$^-$CD34$^+$CD45$^+$**

CD43$^+$ hematopoietic cells were collected from differentiated iPSC cultures using MACS and labeled with CD235a/CD41a FITC, CD45 APC and CD38 PE (BD Pharmingen). Fluorescence-activated cell sorter (FACSAria) was used to sort lin$^-$CD45$^+$CD38$^+$ and lin$^-$CD45$^+$CD38$^-$ subpopulations (Vodyanik et al., 2006). Bone marrow mononuclear cells from CML patients in the chronic phase were purchased commercially (AllCells or Applied StemCells), or obtained from the patients at UW hospital (Madison, WI) with approval from the University of Wisconsin Institutional Review Board. Donors had previously signed Institutional Review Board-approved consent. Mononuclear cells labeled with the lineage-specific markers CD2, CD3, CD14, CD15, CD16, CD19, CD20, CD24, CD56, CD66b, and Glycophorin A (FITC-conjugated antibodies), CD34 APC and DAPI to exclude dead cells. Live lin$^-$CD34$^+$ cells were isolated using FACSAria (BD). All antibodies used in this study were listed in table 1.

**Hematopoietic colony-forming assay**

Hematopoietic clonogenic assays were performed using serum-containing StemMACS HSC-CFU media (Miltenyi Biotec, CA). Optimal numbers of cells, 100-1,000 cells, were plated with and without 10 μM imatinib unless specified. Colonies were scored after 14–21 days of incubation according to morphological criteria as CFC-E, CFC-GEMM, CFC-GM, CFC-G, and CFC-M.

**Long-term culture initiating cell assay (LTC-IC)**

$lin^-CD34^+$ cells were pretreated for 7 days with 5 μM imatinib. All cells remaining after culture were washed, and then plated in a 12-well plate containing inactivated murine M2-10B4 cells in LTC-IC medium consisting of IMDM, 10% BIT (Stemcell Technologies), EXCYTE, 1 μM mercaptoethanol, 1 μM hydrocortisone, FBS and horse serum. Cultures were maintained at 37 °C in a humidified atmosphere with 5% $CO_2$, and changed half medium weekly. After 5 weeks, cells were harvested and analyzed for CFC potential using bulk culture method as described in Stem Cell Technologies protocol (http://www.stemcell.com/en/Technical-Resources/db5a9/28412_ltc_ic-H.aspx).

**Cell proliferation assay**

Scramble or Olfm4 transfected $lin^-CD34^+CD45^+$ from iPSCs were plated in 96-well plate at 300 cells/well. Cells were cultured in CSSM supplemented with 10 ng/ml IL-3, 100 ng/ml SCF, 100 ng/ml Flt3L, 100 ng/ml IL-6 and 200 ng/ml GM-CSF. Viable cell yields were determined by counting trypan blue-excluding viable cells using hemocytometer.

**siRNA transfection**

iPSCs-derived $lin^-CD34^+CD45^+$ cells and somatic CML bone marrow $lin^-CD34^+$ cells were transfected with 100 nM of either siOLFM4 or AllStars Neg. siRNA AF 488 using HiPerfect transfection reagent according to the manufacturer's protocol (all from Qiagen) in SFEM supplemented with low GFs. The silencing efficiently was evaluated after 24 hours using qPCR.

**Culture of primary CML bone marrow lin$^-$CD34$^+$ cells.**

CML bone marrow lin$^-$CD34$^+$ cells were cultured at 37 °C, 5% $CO_2$, in a humidified incubator in SFEM supplemented with low GFs. When indicated 10 ng/ml G-CSF, 300 ng/ml Olfm4, and/or 5 μM imatinib were added. In some experiments, somatic CML lin$^-$ CD34$^+$ cells were cultured in serum free medium without GFs in a presence or absence of 300 ng/ml Olfm4.

**Gene expression analysis by real-time PCR**

RNA was isolated from the subpopulations cells using PureLink RNA micro kit (Life Technologies) according to the manufacturers' instruction. cDNA was prepared from 0.7 μg total RNA using oligo-dT primer with Advantage RT-for-PCR kit (Clontech). Quantitative real-time PCR analysis was performed for all the cDNA samples using self-designed specific primers (Table 2) and PlatinumSYBR Green qPCR SuperMix-UDG kit (Life Technologie). The reactions were run on a Mastercycle realplex thermal cycler (Eppendorf) and expression levels were calculated by minimal cycle threshold values (Ct) normalized to the reference expression of GAPDH in each sample (Pfaffl, 2001). All qPCR products were analyzed on 1.2% agarose gels to confirm the specificity of detection.

**RNA-Sequencing Analysis**

Total RNA from the subpopulations cells was isolated with PureLink RNA micro kit (Life Technologies) and was subjected to subsequent on-column DNaseI treatment. The quality of the total RNA was confirmed by capillary electrophoresis on the Bioanalyzer 2100 (Agilent Technologies). Poly A+ RNAs were linearly amplified using a modified

T7 amplification method (Sengupta et al., 2010) to generate highly consistent, strand-specific mRNA-Seq libraries. After cDNA library preparations, the samples were quantitated with the Qubit fluorometer (Invitrogen) and were then sequenced for forty-two cycles of single-read sequencing on the Illumina Genome Analyzer IIx. Image analysis and base calling were done with the Illumina Genome Analyzer Pipeline Software. After quality assessment and adaptor filtering, the raw sequencing reads were then aligned to the annotated 31,147 RefSeq genes (Human hg18 or NCBI36 build) using the Bowtie algorithm by allowing two mismatches (Langmead et al., 2009). At most, 200 alignments were allowed if a read maps to multiple genes (gene multi-reads) or maps to a single gene but multiple isoforms (isoform multi-reads). Isoform or gene relative expression levels were estimated and quantified by the RSEM algorithm in terms of ‘‘transcripts per million’’ (tpm) (Li et al., 2010). Hierarchical cluster analyses were carried out with PCC (Pearson correlation coefficient) as the distance metric. The average distance between each cluster pair was used as the basis to merge lower-level clusters into higher-level clusters. To visualize the gene-expression levels, a heat-map was composed using MultiExperiment Viewer v4.2 (http://www.tm4.org)

**Statistical analysis**

Data obtained from multiple experiments were reported as the mean ± SEM. Significance levels were determined by one-tailed *Student-t* test analysis.

**Results**

***Identification of olfactomedin 4 as a novel target in LSC-like cells***

The ability to eradicate CML LSCs depends upon identification of unique drug targets.

To find out whether we can use the iPSC model to discover novel drug targets in primitive CML hematopoietic cells, we used RNAseq to perform molecular profiling of CML and control BM iCD34$^+$ cells treated or non-treated with imatinib. Compared to control BM iCD34$^+$ cells, untreated CML iCD34$^+$ cells showed significant differences in expression of genes regulating cell cycle and proliferation, adhesion and motility, and stem cell fate (Table 3). Many of these genes were similar to differentially expressed genes in somatic CD34$^+$ cells from patient in chronic phase CML compared to normal CD34$^+$ cells (Bruns et al., 2009; Diaz-Blanco et al., 2007). The highly expressed genes in the CML iCD34$^+$ cells compared to BM iCD34$^+$ cells are genes associated with cancer development such as *Myc, Dusp1* and *Rasa3*. The down-regulated genes, included *Actg1* and adhesion molecules, *CD44*. After treatment with 5 μM imatinib for 16 hours, the molecular profile of CML iCD34$^+$ cells became similar to BM iCD34$^+$ cells, consistent with the critical role of Bcr/Abl signaling in establishing a unique transcriptional signature of neoplastic cells in CML (Figure 9D).

To find candidate genes associated with imatinib resistance, we designed algorithm to select genes that are specifically induced or suppressed by imatinib in CML iCD34$^+$ cells (Figure 9A). First we chose genes that were induced by imatinib in CML iCD34$^+$, but not in BM iCD34$^+$ cells. Then we excluded from this group, genes that showed less than 2 fold change. Based on this algorithm we identified 127 candidate genes. Using a similar approach, we also identified 11 candidate genes, which were selectively down-regulated by imatinib (Figure 9A). Interestingly, one of the top-ranking up-regulated gene on this

**Table 4:** Differentially expressed genes in CML and control BM iCD34$^{+}$ cells

| | | |
|---|---|---|
| **BCR-Abl signaling** | | Fold change |
| DUSP1 | Dual specificity phosphatase 1 | -1.48 |
| G3BP2 | GTPase activating protein (SH3 domain) binding protein 2 | 1.27 |
| PIK3CB | Phosphoinositide-3-kinase, catalytic, beta polypeptide | 1.37 |
| MYC | v-myc myelocytomatosis viral oncogene homolog (avian) | 1.58 |
| **Proliferation and apoptosis** | | |
| RASA3 | RAS p21 protein activator 3 | -1.51 |
| MCM3 | Minichromosome maintenance complex component 3 | 1.16 |
| POLE3 | Polymerase (DNA directed), epsilon 3 (p17 subunit) | 1.16 |
| CASP2 | Caspase 2, apoptosis-related cysteine peptidase | 1.26 |
| CASP6 | Caspase 6, apoptosis-related cysteine peptidase | 1.3 |
| CASP3 | Caspase 3, apoptosis-related cysteine peptidase | 1.38 |
| ORC2 | Origin recognition complex, subunit 2 | 1.42 |
| POLE2 | Polymerase (DNA directed), epsilon 2 (p59 subunit) | 1.61 |
| ORC4 | Origin recognition complex, subunit 4 | 1.77 |
| CDK2AP1 | Cyclin-dependent kinase 2 associated protein 1 | 1.88 |
| **Self-renewal and early stem cells** | | |
| CD53 | CD53 molecule | -2.55 |
| PTEN | Phosphatase and tensin homolog | -1.22 |
| TAL1 | T cell acute lymphocytic leukemia 1 | 2.64 |
| ALDH1A1 | Aldehyde dehydrogenase 1 family, member A1 | 2.79 |
| MEIS1 | Meis homeobox 1 | 3.43 |
| **Motility** | | |
| PDE4B | Phosphodiesterase 4B, cAMP-specific | -2.06 |
| DOCK2 | Dedicator of cytokinesis 2 | -1.45 |
| ARPC3 | Actin related protein 2/3 complex, subunit 3, 21kDa | -1.27 |
| **Differentiation** | | |
| GATA3 | GATA binding protein 3 | -11.16 |
| BCL6 | B-cell CLL/lymphoma 6 | -7.21 |
| BLNK | B-cell linker | -2.93 |
| CEBPD | CCAAT/enhancer binding protein (C/EBP), delta | -1.37 |
| KLF1 | Kruppel-like factor 1 (erythroid) | 3.03 |
| **Growth factors & Receptor** | | |
| IL7R | Interleukin 7 receptor | -14.52 |
| CCR2 | Chemokine (C-C motif) receptor 2 | -2.83 |
| INSR | Insulin receptor | -1.41 |

| | | |
|---|---|---|
| GPR56 | G protein-coupled receptor 56 | 2.11 |
| F2R | Coagulation factor II (thrombin) receptor | 3.07 |
| **Adhesion & Cytoskeleton** | | |
| ACTG1 | Actin, gamma 1 | -1.54 |
| CD44 | CD44 molecule (Indian blood group) | -1.16 |
| PARVB | Parvin, beta | 1.13 |
| TEK | TEK tyrosine kinase, endothelial | 19.16 |
| **Fatty acid and lipid metabolism** | | |
| ACSL5 | Acyl-CoA synthetase long-chain family member 5 | 1.19 |
| FASN | Fatty acid synthase | 1.27 |

+ : CML Up regulation
- : CML Down regulation

list was *Olfm4* which has been reported to have anti-apoptotic activity in malignancy (Liu et al., 2012; Oh et al., 2011; Zhang et al., 2004). After confirming that imatinib selectively induces expression of *Olfm4* in CML iCD34$^+$ cells using qPCR (Figure 9F), we evaluated the effect of Olfm4 knock down on apoptosis in CML iCD34$^+$ cells using Olfm4-targeted small interference RNA (siOLFM4). As determined by annexin V staining, Olfm4 siRNA knockdown significantly augmented the imatinib-induced apoptosis in CML iCD34$^+$ cells (Figure 10Ai), indicating that this gene could be important for survival of primitive CML cells. In addition, Olfm4 siRNA significantly inhibited CML iCD34$^+$ CFCs and proliferation (Figure 10Aii - iii).

***OLFM4 supports proliferation of CML stem/progenitor cells***

To find out whether these findings can be translated to somatic cells, we evaluated the expression and function of Olfm4 in somatic CML bone marrow lin$^-$CD34$^+$ cells and lin$^-$

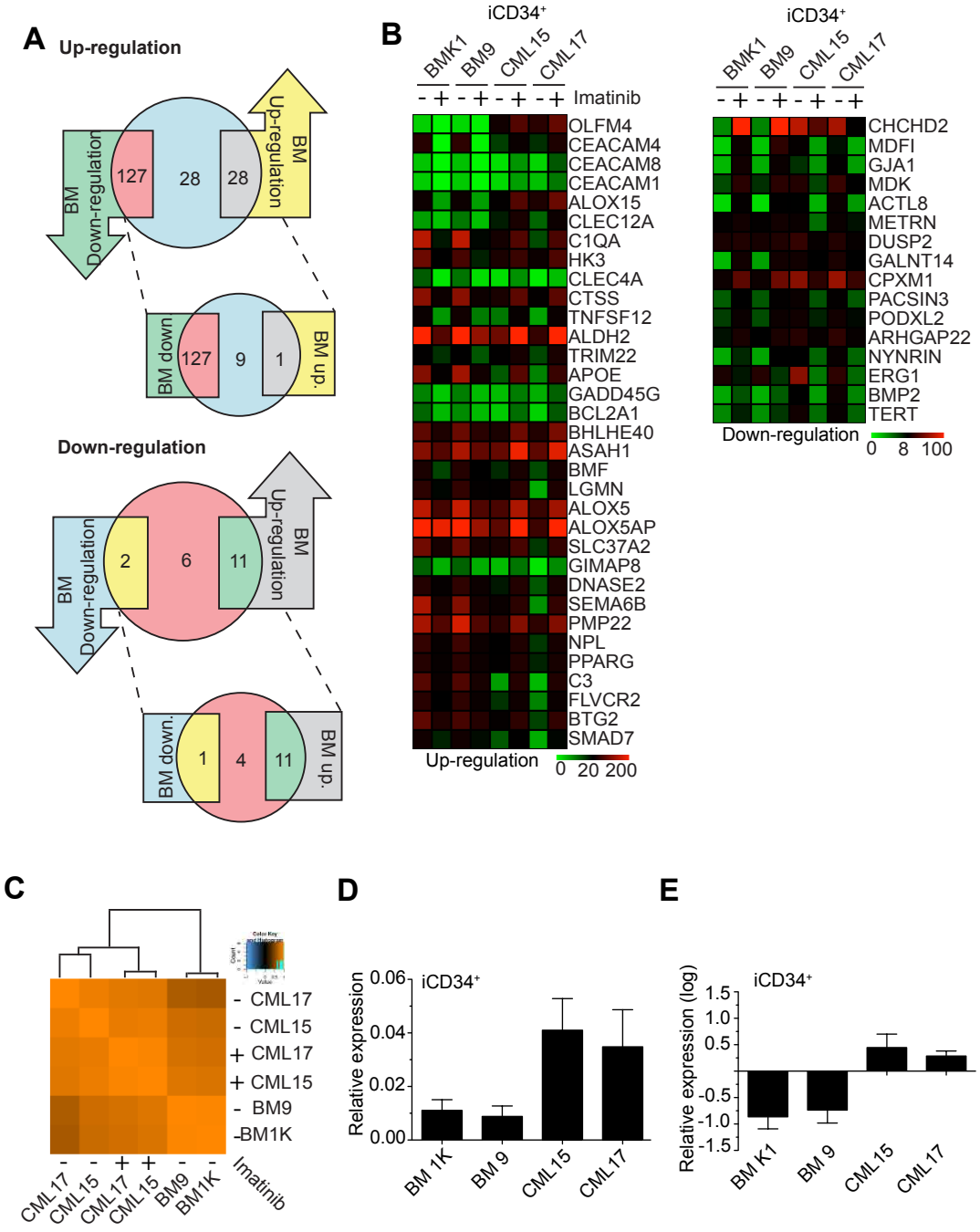


**Figure 9. Gene expression analysis reveals groups of imatinib-induced and suppressed genes in CML iCD34$^+$**

A. Venn diagram shows overlap between the genes differentially expressed in iCD34$^+$

cells.

B. Heat map shows expression of genes selectively induced or suppressed by imatinib in CML iCD34$^+$.

C. Pearson correlation of gene expression in iCD34$^+$ cells treated and non-treated with imatinib.

D. Expression of OLFM4 in CML and BM iCD34$^+$ by qPCR. The results are mean ± SEM of 3 experiments in duplicate. The expression level was calculated relatively to the sample GAPDH.

E. qPCR analysis of the effect of imatinib treatment on expression of OLFM4 in CML and BM iCD34$^+$. The results are mean ± SEM of 3 experiments in duplicate. The expression level was calculated relative to untreated control.

CD34$^-$ cells from bone marrow obtained from the other CML patients in chronic phase. Using PCR, we detected Olfm4 expression in CD34$^-$ fraction but not in CML lin$^-$CD34$^+$ cells. However we found G-CSF quickly induced Olfm4 expression in CML lin$^-$CD34$^+$ as early as 24 hours (Figure 10B) and the induction increased even more when imatinib was added (Figure 10C). Next, we evaluated a functional role of Olfm4 in parental CML lin$^-$ CD34$^+$ cells and lin$^-$CD34$^+$ cells from other 6 CML patients in chronic phase. Knockdown of Olfm4 in CML lin$^-$CD34$^+$ using siRNA (transduction efficiency is shown in Figure 11) dramatically reduced colony formation in all CML samples, which can be rescued by Olfm4 protein (Figure 12A). To determine the effect of Olfm4 knockdown on cells more primitive than CFCs, we performed LTC-IC assay. We found imatinib does not affect the primitive cell in any sample ($p>0.05$), but siOLFM4 dramatically reduces

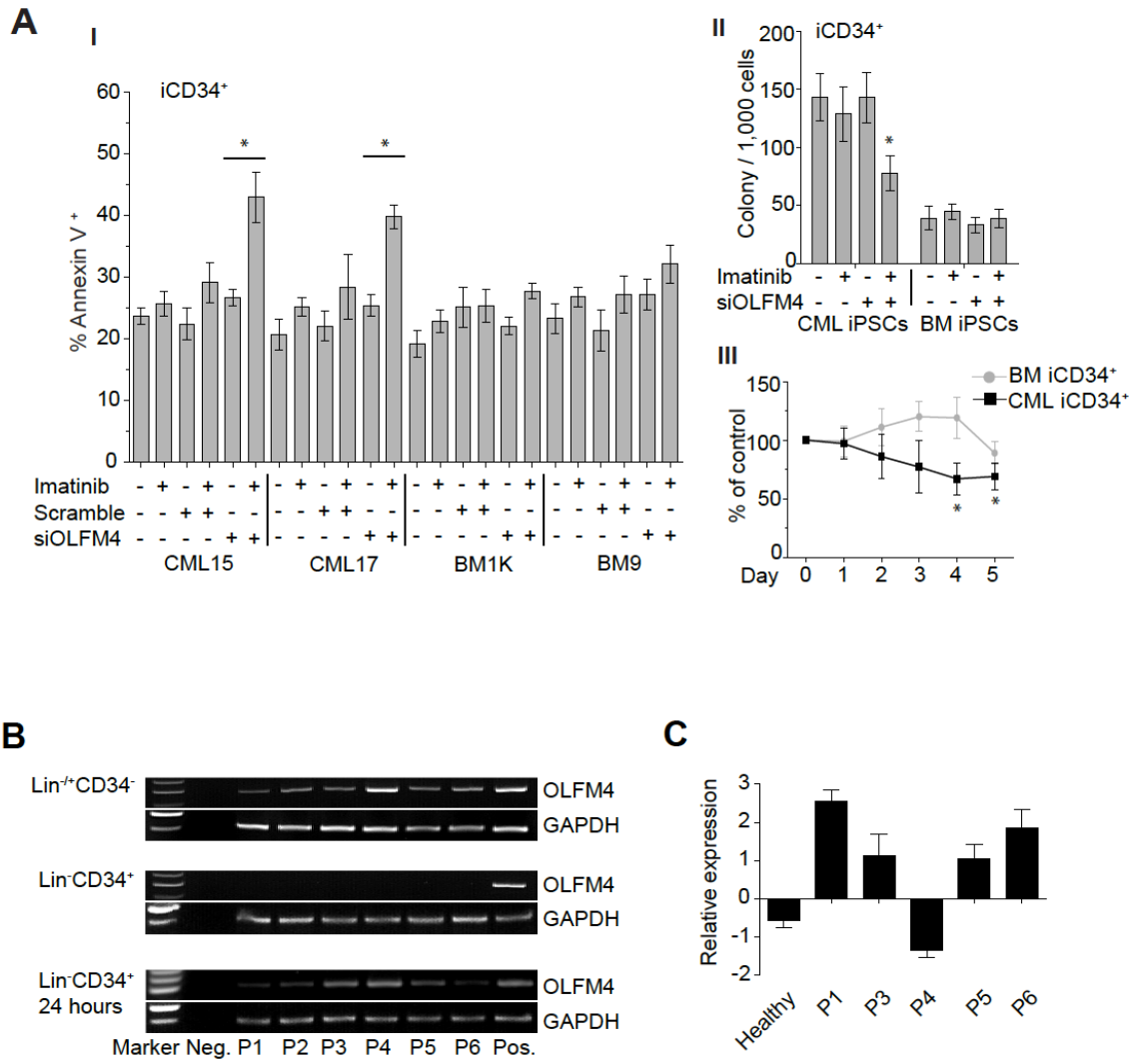


**Figure 10. OLFM4 knockdown potentiates imatinib-induced cell death and inhibit cell proliferation in somatic CML stem/progenitor cells ($lin^-CD34^+$)**

A. BM and CML $iCD34^+$ were transduced with siOLFM4 or negative control siRNA (scramble) or left untransduced. When indicated, 5 μM imatinib was added and incubated for 24 hours. (i) Cell death was measured by annexin V staining. Results are the mean ± SEM of 3-5 independent experiments. (ii) Colony formation (G + GM). Results are the mean ± SEM of 3 independent experiments. (iii) Cells numbers in serum-containing media supplemented with growth factors with or without 5 μM

imatinib for 5 days. Results are mean ± SEM of 3-5 experiments in duplicate. * indicates significant ($p < 0.05$).

B. PCR showing expression of OLFM4 in $lin^{-/+}CD34^{-}$, $lin^{-}CD34^{+}$, and $lin^{-}CD34^{+}$ after culturing in serum-free media supplemented with IL3, IL6, SCF, Flt3L, GM-CSF and G-CSF for 24 hours from primary CML bone marrow cells.

C. qPCR quantification of relative OLFM4 expression after treatment with 5 μM imatinib. Histogram represents mean ± SEM of 3 experiments.

number of LTC-IC-derive CFC to less than half of control (Figure 12B, $p<0.05$). We examined the effect of Olfm4 in CML $lin^{-}CD34^{+}$ cells using serum-free medium without growth factors, and we found Olfm4 protein enhanced cell proliferation (Figure 12C). Taken together, it suggested that CML $lin^{-}CD34^{+}$ cells quickly up-regulated Olfm4 to support their survival when their growth advantage was taken out. Although differentiated cells ($CD34^{-}$) are sensitive to TKI, they play an important role as a supportive niche for CML stem/progenitor cells by producing Olfm4 (Figure 12D).

**Discussion**

Ex vivo cultures of CML-derived cell lines and primary CML cells, ectopic expression of Bcr/Abl in $CD34^{+}$ cells and mouse models have provided important insights into CML pathogenesis and led to development of targeted therapy of this neoplastic disease with Bcr/Abl-kinase inhibitor, imatinib. Despite of these achievements, CML in many cases remains incurable due to the innate resistance of CML LSCs to TKI (Corbin et al., 2011; Graham et al., 2002; Holyoake et al., 2001). Thus, achieving curative therapy would

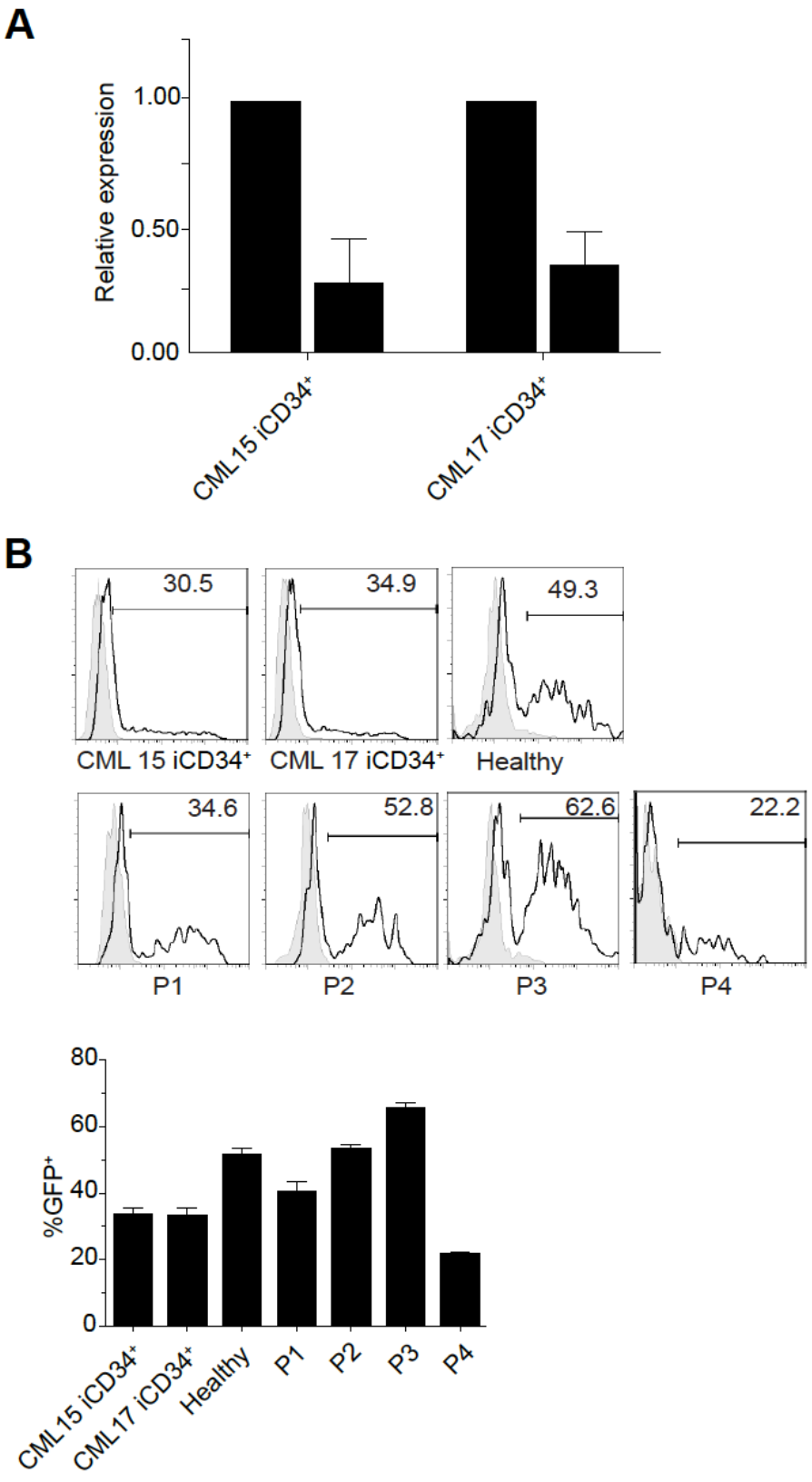


**Figure 11:** siRNA transfection efficiency

A. qPCR analysis of efficiency the OLFM4 knockdown using OLFM4 siRNA after 24 hours. Results are mean ± SEM of 3 independent experiments in duplicate. The expression level was calculated relative to negative control (scramble) siRNA.

B. Representative flow histogram showed transfection efficiency at single cell level. Alexafluor 488-conjugated scramble siRNA was transfected to CML iCD34$^{+}$ and CML lin$^{-}$CD34$^{+}$ for 24 hour. Graph shows the mean ± SEM of 3 independent experiments.

require selective targeting of CML LSCs with novel drugs. However, the lack of a sufficient source of primitive hematopoietic cells limits the ability to study CML LSCs and use them for drug discovery.

In the present studies we demonstrated that lin$^{-}$CD34$^{+}$CD45$^{+}$ (CML iCD34$^{+}$) primitive hematopoietic cells with the phenotypic and functional properties of leukemia stem cells, including imatinib resistance, could be generated from CML iPSCs. Using molecular profiling of control BM and CML iCD34$^{+}$ cells treated and non-treated with imatinib we identified Olfm4 gene as a novel anti-apoptotic factor in CML iCD34$^{+}$. Subsequent knockout of this gene in CML CD34$^{+}$ from CML patients in chronic phase, confirmed a similar role of Olfm4 in somatic CML primitive cells.

Human olfactomedin 4 (Olfm4, also called GW112 and hGC-1) gene encodes a secreted glycoprotein with multimer structure (Liu et al., 2006), which plays an important role in a

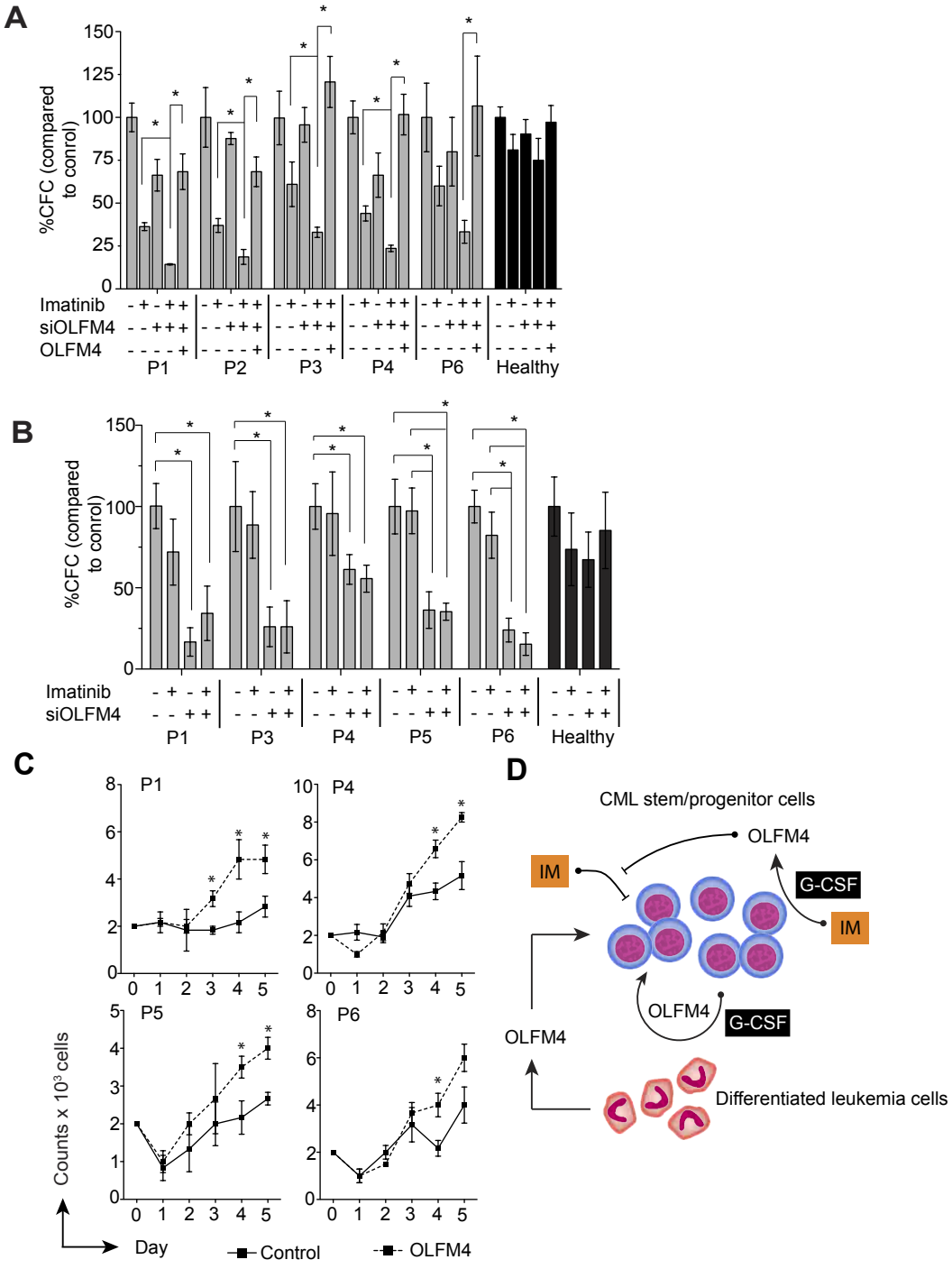


**Figure 12. OLFM4 provides mitogenic signal to CML stem/progenitor cells**

A. The lin$^-$CD34$^+$ cells were isolated from parental (P1) and unrelated bone marrow (P2-P6) CML in chronic phase. After transduction with siOLFM4 or scramble, cells were

cultured in serum-free media supplemented with low growth factors. When indicated, 5 μM imatinib was added to the media. After 24 hours, colony formation assay was performed. Results are the mean ± SEM of 3 independent experiments. * indicates significant ($p < 0.05$).

B. CML $lin^-CD34^+$ cells were transduced with siOLFM4 or scramble, and were then cultured in serum-free medium supplemented with low growth factors. When indicated, 5 μM imatinib or 300 ng/ml OLFM4 was added to the media. After 1 week, LTC-IC assay was performed. Results are the mean ± SEM of 3 independent experiments.

C. Expansion of CML $lin^-CD34^+$ in serum-free media without growth factors in the presence or absence of OLFM4 protein. Results are mean ± SEM from 3 independent experiments. * indicates significant differences ($p < 0.05$).

D. Proposed model of the role of OLFM4.

variety of cellular functions including cell adhesion, cell cycle and apoptosis (Tomarev and Nakaya, 2009). In human intestine, Olfm4 was identified as a robust marker of $LGR5^+$ stem cells and subset of cancer cells (van der Flier et al., 2009). Olfm4 is involved in cell growth and apoptosis in human malignancies (Liu et al., 2010; Oh et al., 2011; Park et al., 2012; Zhang et al., 2004) and is considered to be an inducible resistance factor to apoptotic stimuli (Koshida et al., 2007). Olfm4 interacts with GRIM-19 (Zhang et al., 2004), which is a component of respiratory complex I of mitochondria and has an anti-apoptotic role in prostate cancer cells (Huang et al., 2004). However, Olfm4 effects on apoptosis appear to be a cell-type dependent. Although Olfm4 was initially identified in myeloblasts and later in neutrophils (Clemmensen et al., 2012; Zhang et al., 2002), the

biological function of Olfm4 in normal and neoplastic hematopoiesis remains largely unknown. It has been shown that Olfm4 expression is upregulated in a subset of patients with acute myeloid leukemia and that overexpression of Olfm4 in HL-60 leukemia cell line induces their differentiation and apoptosis (Liu et al., 2010). Here for the first time, we demonstrated that Olfm4 is critical for survival of primitive blood cells from the chronic phase of CML, and could be explored as a novel drug target for CML LSCs.

iPSC-based model provides several important advantages for study CML pathogenesis. iPSCs can be expanded indefinitely and used to generate an unlimited number LSC-like cells for functional studies and drug screening. Because iPSCs are single cell origin, multiple iPSC lines can be generated from the same patient to capture the diversity of genetic alterations within leukemia cell populations and address the role of non-Bcr/Abl associated mutations in disease development. iPSCs obtained from the patients before and after acquisition of drug resistance or progression to blast crisis would be a valuable tool for addressing molecular mechanisms of CML progression and drug resistance. In addition, our studies demonstrated that the biological effect of Bcr/Abl on cells depends on epigenetic environment. Although Bcr/Abl expression was detected in undifferentiated CML iPSCs and at all stages of their differentiation (mesoderm, hemogenic endothelium, blood cells), and in non-hematopoietic cells derived from CML iPSC such as MSCs, imatinib induced significant apoptosis only in mature $CD34^{-}CD45^{+}$ hematopoietic cells. We also revealed that both undifferentiated CML iPSCs and LSC-like cells were resistant to imatinib-induced apoptosis. However, inhibition of Bcr/Abl activity with imatinib has a different effect on proliferation of LSC-like cells and undifferentiated CML iPSCs,

pointing out that epigenetic environment and signaling pathways interacting with Bcr/Abl kinase are different in two types of cells. These differences can be further exploited to define epigenetic changes and biochemical pathways involved in CML pathogenesis.

Overall our studies demonstrated for the first time the utility of iPSC-based models for study and successful identification of novel therapeutic targets in CML LSCs (Figure 13). Further exploration of this model will also be of value for the study acquired drug resistance, diversity of genetic alterations within tumor, and epigenetic mechanisms of leukemogenesis.

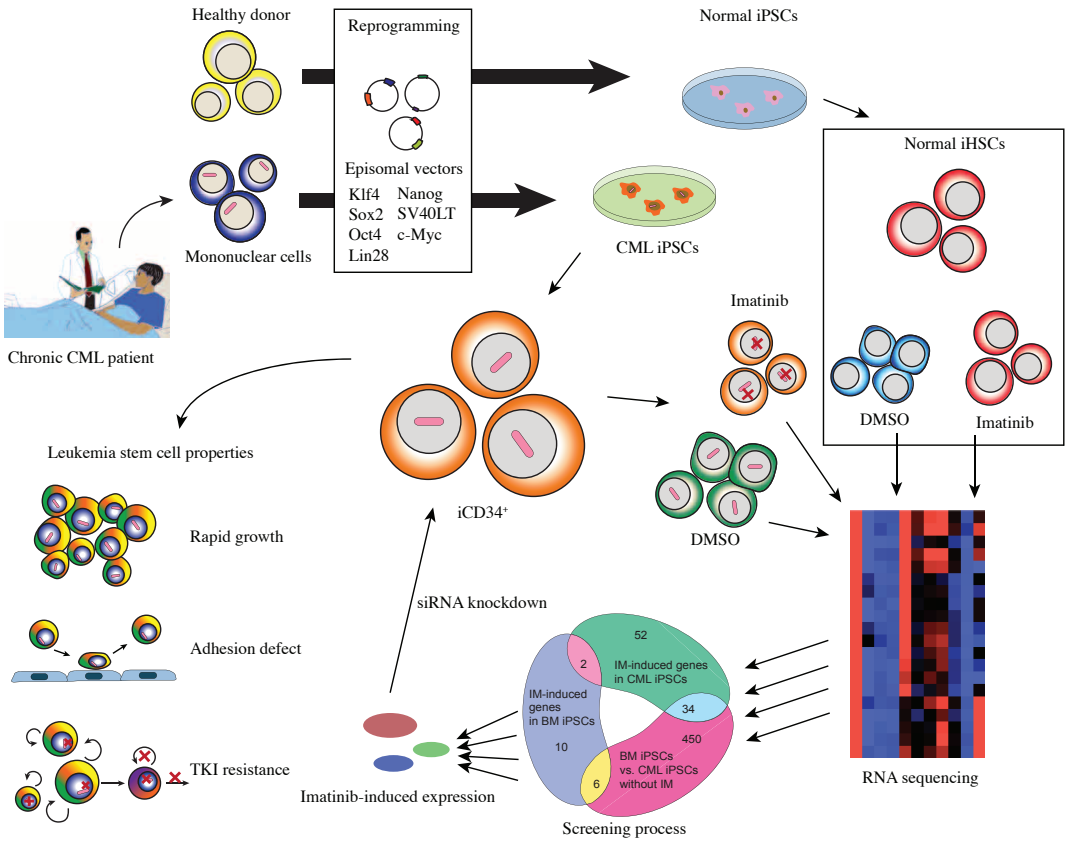


**Figure 13.** The iPSC model of CML: From bedside to patient

## Chapter 3: Overall conclusion and Future directions

### Conclusions

CML stem cell has long been targeting with tyrosine kinase inhibitors. In spite of vigorous attempt, recurrent and aggressive relapse are not uncommon. To aim at cure, understandings biology of CML stem cell is required to develop strategies targeting CML stem cell. However, autologous source of CML stem cell from patients are available at limited numbers. In the current work, we provide alternative method to generate CML stem-like cell (CML iCD34$^{+}$) in vitro from CML iPSC using an OP9 coculture system, which can be scaled up. In the first part of this study we demonstrate the functional similarity of CML iCD34$^{+}$ and somatic CML stem cell including adhesion defect, rapid limited cytokine-independent proliferation, and tyrosine kinase resistance.

The ability to generate iPSCs from patients, and an increasingly refined capacity to differentiate these iPSCs into disease-relevant cell types, promises a new paradigm in drug development. Later in the study, we exploit the advantage of iPSCs to identify novel drug targets and examine the probability of clinical success of the new target. We performed high throughput whole transcriptome sequencing from the TKI-insensitive CML and normal iCD34$^{+}$ to screen for genes that were selectively affected by imatinib in CML. Using bioinformatics algorithm; we identify Olfm4 as a top-ranked gene that could be a potential target for CML stem cells. Using small interfering RNA to knockdown Olfm4, CML iCD34$^{+}$ becomes sensitive to imatinib-induced apoptosis, proliferates slowly and has remarkable reduction in colony formation.

Examining a role of Olfm4 in primary CML lin$^-$CD34$^+$ (including parental cells and 5 other non-related samples), we found Olfm4 is rapidly induced by G-CSF. Imatinib itself could not induce Olfm4 expression, but it substantially enhanced G-CSF-induced Olfm4 expression in CML samples. Although a combination of imatinib and Olfm4 knockdown dramatically reduced CML progenitor cells compared to imatinib alone, only Olfm4 knockdown could reduce numbers of primitive CML cells. In addition, in a serum-free medium without GFs, Olfm4 supported proliferation of CML lin$^-$CD34$^+$. All together, Olfm4 has autocrine/paracrine activities that promote survival of primitive CML cells in vitro. Overall our studies demonstrated for the first time the utility of iPSC-based models for study and successful identification of novel therapeutic targets in primitive CML cells.

**Future directions**

The rapid progress in reprogramming technology facilitating the more efficient and accessible generation of patient-specific iPSCs has taken a new turn to the development of strategies for iPSC-based disease modeling and regenerative therapy. A major goal of human therapy for CML is to develop methods targeting the genetic aberrant HSCs that allow elimination or correction of the genetic defect. As a critical step in advancing gene-targeting strategies for the correction of disease mutations, unlimited source of appropriate cells is required. In according to this goal, we described the CML iPSC-derived iCD34$^+$ model to understand disease pathogenesis and screen for drugs in CML research. Such cells could be isolated at convenient in a cell-cultured dish. However, a lack of repopulating ability in animal still needs to be overcome. The following issues

would be future directions for therapeutic applications in hematopoietic diseases by using HSC and LSCs derived from normal and CML iPSCs.

**Ability to repopulate all hematopoietic lineages in animal**

Persistent of CML depends on the unique ability of few CML stem cells to self-renew and to generate differentiated progeny of all blood cell lineages those are insensitive to treatment. Long-term multi-lineage engraftment of iPSC-derived hematopoietic cells to reconstitute hematopoietic system of an animal model still remains as one of the most significant and challenging research areas. Based on our study, CML iPSC-derived hematopoietic cells showed phenotype, clonogenic progenitor capacity, and functional properties similar to somatic counter parts, but they failed to reconstitute in a recipient NSG mice injected intravenously which is a current major limitation of using iPSCs for hematopoietic stem cell study. However, the explanations for the engraftment failure lie on two reasons. First primary CML stem cells in chronic phase might be deficit in engraftment since some of the samples obtained from patients can repopulate in mice, and those engraftable samples could not develop leukemia in mice. Second is the defect in CML iPSC-derive hematopoietic cells themselves. (Amabile et al., 2013) and colleagues reported a novel in vivo system in which human iPSCs differentiate within teratomas that can reconstitute a human immune system when transplanted into immunodeficient mice. Although the study showed $CD34^{+}CD45^{+}$ cells could be detected in NSG recipient bone marrow, it showed partial hematopoietic lineages reconstitution in the transplanted mice and an engraftment was limited to a short time period. Speculation that the difficulty of producing hematopoietic cells capable of long-term reconstitution of

recipients might because of the non-physiologic microenvironment in which the cells were differentiated.

The transplantable stem cells derived from iPSCs will be the most accurate population for studying stem cell biology and drug screening targeting diseased stem cells. Therefore, future research should focus on how to make hematopoietic cells from iPSCs transplantable i.e. novel differentiation system, specific genes required for activation of self-renewal and homing, and novel animal model that provides appropriate environment for the cells. Such methods would provide a significant leap in hematopoietic field showing the HSC and LSC properties of iPSC-derived hematopoietic cells.

**Genetic correction in iPSCs and dendritic cell-based vaccine for leukemia**

The ability to genetically modify human stem cells is an invaluable approach for both modeling diseases and providing potential therapies. Current developments in gene modification methods are a critical step to achieve the possibility of creating genetically corrected human iPSCs for the eventual use of these cells in autologous cell replacement therapy (Hockemeyer et al., 2009). Promising preclinical studies have demonstrated correction of disease-causing mutations in a number of hematological, neuronal, and mesenchymal cells disorders (An et al., 2012; Chang and Bouhassira, 2012; Liu et al., 2011b). CML iPSC-derived iCD34$^{+}$ cells could be used to develop a drug delivery system that carry gene-editing tools to the mutant cells and correct the genetic defect in vivo.

Dendritic cell-base vaccine, Provenge, is currently used in the treatment of metastatic

castration-resistant prostate cancer (Kantoff et al., 2010) and several trials are underway to develop effective DC-vaccines several diseases. CML iPSCs-derived dendritic cells can be used to study the mechanism of how dendritic cells become anergic to leukemic cells. Exploration on such mechanisms will provide invaluable clues for the future immunotherapy. These advances highlight the exciting potential for stem cell research combined with gene therapy approaches to produce new therapies in the future.

**Mechanism of actions of OLFM4 in CML**

Olfm4 belongs to a family of olfactomedin domain-containing proteins, which consists of at least 13 members in mammals. The possibility that Olfm1 interact with the secreted antagonist of Wnt signaling pathway, Wif-1, (Nakaya et al., 2008) suggests that Olfm1 similar to another family of olfactomedin domain-containing proteins myocilin serves as a modulator of Wnt pathway (Kwon et al., 2009). (Heidel et al., 2012; Zhao et al., 2007) have shown a vital role of beta-catenin, dowstream signaling of Wnt pathway, in survival of CML stem cell. Loss of function or pharmacologic inhibition of beta-catenin result in deficient in long-term growth and maintenance of CML stem cell as well as a profound reduction in the ability to develop BCR/ABL-induced chronic myelogenous leukemia (CML) in vivo. However, the relationship of Olfm4 and Wnt signaling in CML has not been studied yet. Future investigation should address mechanisms of the anti-apoptotic and mitogenic effect of Olfm4 in CML. Such mechanisms would reveal alternative survival pathway of CML and opportunity to uncover novel drug targeting strategy.

iPSC-based model provides several important advantages for study CML pathogenesis.

iPSCs can be expanded indefinitely and used to generate an unlimited number LSC-like cells for functional studies and drug screening. Because iPSCs are single cell origin, multiple iPSC lines can be generated from the same patient to capture the diversity of genetic alterations within leukemia cell populations and address the role of non-Bcr/Abl associated mutations in disease development. iPSCs obtained from the patients before and after acquisition of drug resistance or progression to blast crisis would be a valuable tool for addressing molecular mechanisms of CML progression and drug resistance.

**Appendix: List of publications**

1. Hu K, Yu J, **Suknuntha K**, Tian S, Montgomery K, Choi KD, et al. Efficient generation of transgene-free induced pluripotent stem cells from normal and neoplastic bone marrow and cord blood mononuclear cells. Blood. 2011 Apr 7;117(14):e109-19.
2. Togarrati PP, **Suknuntha K**. Generation of mature hematopoietic cells from human pluripotent stem cells. Int J Hematol. 2012 Jun;95(6):617-23.
3. Choi KD, Vodyanik MA, Togarrati PP, **Suknuntha K**, Kumar A, et al. Identification of the hemogenic endothelial progenitor and its direct precursor in human pluripotent stem cell differentiation cultures. Cell Rep. 2012 Sep 27;2(3):553-67.
4. Wei Xie, Matt Schultz, Ryan Lister, Zhonggang Hou, Nisha Rajagopal, Pradipta Ray, John W. Whitaker, Shulan Tian, R. David Hawkins, Danny Leung, Hongbo Yang, Tao Wang, Ah Young Lee, Scott A. Swanson, Jiuchun Zhang, Yun Zhu, Audrey Kim, Joseph Nery, Mark A. Urich, Samantha Kuan, Chia-an Yen, Sarit Klugman, Pengzhi Yu, **Kran Suknuntha**, Nicholas E. Propson, Huaming Chen, Lee

E. Edsall, Ulrich Wagner, Yan Li, Zhen Ye, Ashwinikumar Kulkarni, Zhenyu Xuan, Wen-yu Chung, Neil C. Chi, Jessica Antosiewicz-Bourget, Igor Slukvin, Ron Stewart, Michael Q. Zhang, Wei Wang, James A. Thomson, Joseph R. Ecker, and Bing Ren. Epigenomic Analysis of Multi-lineage Differentiation of Human Embryonic Stem Cells. Cell. 2013.04.022

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