CELLULAR REPROGRAMMING

OF

HUMAN ACUTE MYELOID LEUKEMIA PATIENT SOMATIC CELLS

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ABSTRACT

Acute myeloid leukemia (AML) is a fatal cancer of the human hematopoietic system characterized by the rapid accumulation of non-functional, immature hematopoietic cells in the bone marrow (BM) and peripheral blood (PB) of affected patients. Limited sources of safe hematopoietic stem/progenitor cells (HSPCs) for transplantation and incomplete mechanistic understandings of disease initiation, progression and maintenance have impeded advances in therapy required for improvement of long-term AML patient survival rates. Toward addressing these unmet clinical needs, the ability to generate induced pluripotent stem cells (iPSCs) from human somatic cells may provide platforms from which to develop patient-specific (autologous) cell-based therapies and disease models. However, the ability to generate iPSCs from human AML patient somatic cells had not been investigated prior to this dissertation. Accordingly, I hypothesized that *cellular reprogramming of human AML patient somatic cells to iPSCs is possible and will enable derivation of autologous sources of normal and dysfunctional hematopoietic progenitor cells (HPCs).*

I first postulated that reprogramming AML patient fibroblasts (AML Fibs) to pluripotency would provide a novel source of normal autologous HPCs. Our findings revealed that AML patient-specific iPSCs devoid of leukemia-associated aberrations found in the matched bone marrow (BM) could be generated from AML Fibs, and demonstrated that this cellular platform allowed for the derivation of healthy HPCs capable of normal differentiation to mature myeloid lineages *in vitro*. During the tenure of these experiments we also redefined conventional reprogramming methods by

discovering that OCT4 transcription factor delivery combined with culture in pluripotent-supportive media was minimally sufficient to induce pluripotency in AML and normal Fibs.

Toward capturing and modeling the molecular heterogeneity observed across human AML samples *in vitro*, we next asked whether reprogramming of AML patient leukemic cells would enable generation of iPSCs and derivative HPCs that recapitulated dysfunctional differentiation features of primary disease. Our results demonstrated that conventional reprogramming conditions were insufficient to induce pluripotency in leukemic cells, but that generation of AML iPSCs could be reproducibly achieved in one AML sample when reprogramming conditions were modified. These AML iPSCs and their derivative HPCs harboured and expressed the leukemia-associated aberration found in the BM leukemic cells and similarly possessed dysfunctional differentiation capacities.

Together, this body of works provides the proof of principle that cellular reprogramming can be applied on a personalized basis to generate normal and dysfunctional HPCs from AML patient somatic cells. These foundational findings should motivate additional studies aimed at developing iPSC-based cell therapies and disease models toward improving AML patient quality of life and long-term survival rates.

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TABLE OF CONTENTS

CHAPTER 2

CHAPTER 3

LIST OF FIGURES AND TABLES

CHAPTER 1

CHAPTER 2

CHAPTER 3

CHAPTER 4

CHAPTER 5

LIST OF ABBREVIATIONS

AML	acute myeloid leukemia
APL	acute promyelocytic leukemia
AraC	cytarabine
ATRA	all-trans retinoic acid
bFGF	basic fibroblast growth factor
BM	bone marrow
BMP4	bone morphogenetic protein 4
CFU	colony forming unit
CML	chronic myeloid leukemia
DNMT	DNA methyltransferase
DZNep	3-deazaneplanocin A
EB	embryoid body
ECC	embryonal carcinoma cell
ESC	embryonic stem cell (preceding m=mouse, h= human)
FAB	French-American-British
Fib	fibroblast
FISH	fluorescence *in situ* hybridization
FLT3L	FLT3 ligand
G-CSF	granulocyte-colony stimulating factor
HAT	histone acetyltransferase
HDAC	histone deacetylase

HDF	human dermal fibroblast
HDM	histone demethylase
HMT	histone methyltransferase
HPC	hematopoietic progenitor cell
HSPC	hematopoietic stem/progenitor cell
ICM	inner cell mass
IGFII	insulin-like growth factor II
iMEF	irradiated mouse embryonic fibroblast
IL-3	interleukin-3
IL-6	interleukin-6
iPSC	induced pluripotent stem cell
LSC	leukemia stem cell
LTC-IC	long-term culture-intiating cell
MEF-CM	mouse embryonic fibroblast-conditioned media
MEF	mouse embryonic fibroblast
MPB	mobilized peripheral blood
OSKMN	OCT4, SOX2, KLF4, cMYC, NANOG (and any combination therein)
PB	peripheral blood
PSC	pluripotent stem cell (preceding m=mouse, h=human)
RBC	red blood cell
SC-IC	suspension culture-initating cell
SCF	stem cell factor

SCID	severe combined immunodeficient mice
SCNT	somatic cell nuclear transfer
TF	transcription factor
UCB	umbilical cord blood
VPA	valproic acid

CHAPTER 1

INTRODUCTION

1.0 Preamble

The work presented in this thesis was inspired by the poor prognoses and long-term survival rates of human patients with acute myeloid leukemia (AML) that are largely attributed to a lack of therapeutic options and a poor mechanistic understanding of disease. It was also motivated by the seminal findings by Takahashi et al., which detail the generation of personalized induced pluripotent stem cells (iPSCs) using cellular reprogramming (Takahashi et al., 2007). Accordingly, this dissertation aims to demonstrate that cellular reprogramming of human AML patient somatic cells to iPSCs allows for the generation of personalized sources of normal and dysfunctional blood cells that may form the bases of future cell-based therapies and disease models. As such, this introduction is devised into three major sections that provide an overview of the pioneering and relevant works in the fields of AML, embryonic stem cell (ESC) and pluripotency, and cellular reprogramming and iPSC research that together formed the premise of my research problem, hypothesis and objectives that are subsequently delineated in my summary of intent.

1.1 Acute Myeloid Leukemia

The normal hematopoietic (blood) system is maintained by hematopoietic stem/progenitor cells (HSPCs) capable of self-renewing and differentiating to generate all of the mature lymphoid and myeloid blood cell lineages (Bryder et al., 2006). However,

cancers of the blood system disrupt this normal homeostatic maintenance and lead to dysfunctional hematopoiesis. Rudolf Virchow coined the term "leukemia" in 1856 to describe the excess white blood (myeloid) cells he observed in the blood stream of patients with fever, weakness and enlarged spleens (Tefferi, 2008). Subsequent pathological observations made over the following century began to tease apart the various myeloid neoplasms, with the first cases of AML reported in the 1940s (Tefferi, 2008). We now know AML as a difficult-to-treat and genetically heterogeneous cancer of the hematopoietic system characterized by the inability of immature leukemic cells or "AML blasts" to differentiate into mature cells of the myeloid lineages. The rapid accumulation of these non-functional AML blasts in the bone marrow (BM) and peripheral blood (PB) of patients results in hematopoietic system failure within months, ultimately leading to death (Perl and Carroll, 2007). Although frontline chemotherapeutic treatments achieve high rates of remission, only 20-30% of patients are afforded long-term disease-free survival due to a high rate of disease relapse (Shipley and Butera, 2009; Tallman et al., 2005). Accordingly, increased understanding of underlying mechanisms of AML pathogenesis and the development of novel therapeutic approaches and treatments are required to improve the dismal prognoses currently associated with AML. Here I discuss aspects of AML that are relevant to the purpose of this thesis, including an overview of the enormous genetic heterogeneity observed in AML, its disease relevance, and methods of detection; introduction to epigenetics and the recent advances in the understanding of the AML epigenome; description of the contributions of current *in vitro*

and *in vivo* model systems of human AML and their limitations; and finally, I discuss the current therapeutic management and unmet clinical needs of AML patients.

1.1.1 Heterogeneity of acute myeloid leukemia: cytogenetic and molecular aberrations

The extreme genetic heterogeneity of AML has become progressively more evident over the past 30 years, with a vast number of recurring aberrations discovered both across and within AML patient cases (Dohner et al., 2015; Vardiman et al., 2002; Vardiman et al., 2009). These "leukemia-associated aberrations" are subdivided into large-scale chromosomal (cytogenetic) and gene-specific (molecular) abnormalities, a number of which are summarized in **Tables 1** and **2**. Building on initial morphological, cytochemical, and immunophenotypic diagnostic criteria established in the French-American-British (FAB) 1976 classification of AML (Bennett et al., 1976), the importance of recurring cytogenetic aberrations found in 50-60% of AML patients in subsequent years became increasingly apparent (Grimwade et al., 1998). This led to the development of a new system for disease classification with the publication of "The World Health Organization classification of the myeloid neoplasms" in 2002 (Vardiman et al., 2002; Vardiman et al., 2009). These cytogenetic aberrations serve as diagnostic and prognostic markers to better instruct therapeutic approaches and predict patient outcomes (**Table 1**)(Grimwade et al., 1998; Vardiman et al., 2002; Vardiman et al., 2009). Accordingly, AML patients can be subdivided into favourable [inv(16), t(8;21), t(15;17)], intermediate [normal, +8, +21, +22, del(7q), del(9q), 11q23 abnormalities], and unfavourable [3q abnormalities, -5, -7, del(5q), complex karyotypes] risk categories based

on the cytogenetic abnormalities present at diagnosis (Grimwade et al., 1998). With increased awareness of the genetic component of AML and advances in next-generation sequencing techniques, a number of molecular aberrations have since been described at the resolution of the gene level through sequencing of over 200 AML patient genomes (**Table 2**)(The Cancer Genome Atlas Research Network, 2013; Welch et al., 2012). These findings provide an additional layer of genetic complexity to AML, and will likely result in future revisions of disease classification (Dohner et al., 2015). For instance, the prognostic value of molecular aberrations in genes such as *NPM1*, *FLT3*, and *CEBPA* has already begun to direct clinical practice (Dohner et al., 2010); although, the prognostic values of many other molecular mutations remain to be established due to their recent identification (The Cancer Genome Atlas Research Network, 2013; Welch et al., 2012). Together, these research efforts have led to the identification of a remarkable number of cytogenetic and molecular aberrations associated with AML toward improving disease diagnosis, classification, prognosis and therapeutic approaches. However, with the exception of insights into MLL-AF9 (Krivtsov et al., 2006), the origin and functional contributions of these aberrations to human AML pathogenesis remain largely unknown due to limitations of current model systems of AML and require further investigation.

1.1.2 Molecular techniques for the detection of AML cells

The identification and detection of leukemia-associated aberrations would not have been possible without the use of cytogenetic and molecular techniques. G-banding, spectral karyotyping, array comparative genomic hybridization and fluorescence *in situ* hybridization (FISH) are routinely employed to detect cytogenetic aberrations, while

polymerase chain reaction and DNA sequencing-based approaches are used to identify molecular aberrations in AML samples. The ability to probe for and visualize cytogenetic aberrations on a per cell basis using G-banding and/or FISH serves as a powerful tool for distinguishing AML blasts harbouring distinct leukemia-associated aberrations from normal cells devoid of aberration.

Early works in the 1950s developed techniques for preparing condensed metaphase chromosomes from individual cells on a microscope for visualization toward determining the chromosome number (karyotype) in human cells (Hsu, 1952; Hughes, 1952; Tjio and Levan, 1956). It was not long before this "chromosomal spread" technique was applied to human tumor cells to determine that they possessed abnormal aneuploid or euploid karyotypes (Hsu and Moorhead, 1957), thereby providing an initial glimpse into the power of analyzing chromosomal spreads to distinguish normal and cancerous cells. The subsequent development of G-banding techniques – Giemsa staining of metaphase chromosomes producing distinct dark (AT rich) and light (GC rich) "band" patterns unique to each chromosome – provided further opportunity to detect abnormal variations in seemingly normal diploid (n=46) karyotypes (Bickmore, 2001). For example, G-banding of human Burkitt Lymphoma cells revealed that although 46 chromosomes were present, an extra region or "band" was repeatedly observed in one chromosome 14 per cell (Manolov and Manolova, 1972). Further analyses using G-banding would later reveal that this was attributed to a translocation between chromosomes 8 and 14 [t(8;14)], a characteristic chromosomal aberration common to Burkitt Lymphoma samples (Zech et al., 1976). Together, these studies established the utility of G-banding, a technique that

remains a frontline clinical diagnostic to identify the presence and frequency of cytogenetic aberrations (insertions, deletions, translocations, -ploidy) within AML samples (Dr. Mohammed Almakadi, personal communication). However, this approach requires an expertly trained eye and is therefore not practical for quick adaptation and use in non-clinical settings.

If G-banding is the tortoise of cytogenetic techniques, then FISH is considered the hare: a practical and robust technique that can be performed in a high-throughput manner to detect cytogenetic aberrations (Levsky and Singer, 2003; Speicher and Carter, 2005). Based on the concept of using fluorescently-labeled probes to target and visualize regions of a chromosome (Bauman et al., 1980; Levsky and Singer, 2003), FISH allows users to rapidly identify leukemic cells carrying known leukemia-associated aberrations. For instance, FISH performed using commercially available DNA-based probes that bind specifically to the centromere region of chromosome 8 would establish whether AML blasts harbouring trisomy 8 are present within a BM sample (the presence of three fluorescent probe signals versus the normal two)(**Chapter 2, Figure 1a**). Similarly, chromosomal translocations can be detected when probes that bind to two adjacent genes surrounding a breakpoint region no longer co-localize upon visualization, as is the case with AML-associated translocations involving 11q23 (**Chapter 2, Figure 1a**). As such, FISH holds great utility in the clinical setting where follow-up samples may be probed to evaluate whether therapy has reduced the frequency of AML blasts and in the experimental setting where it can be used to distinguish normal from leukemic cells in an AML BM or PB sample.

1.1.3 Epigenetics and the abnormal AML epigenome

Epigenetics literally means "over the genetics" and was coined by Conrad Waddington in 1942 as a "branch of biology which studies the causal interactions between genes and their products, which bring the phenotype into being" (Goldberg et al., 2007; Waddington, 1942). Through increased understanding of the molecular mechanisms responsible for the actualization of phenotype, epigenetics can now be summarized as the processes that control gene expression by regulating DNA conformation/accessibility and the recruitment of transcriptional machinery without affecting the DNA sequence (Goldberg et al., 2007). These processes are largely mediated by proteins that affect DNA methylation and histone modifications, and microRNA that disrupt mRNA expression (Goldberg et al., 2007). In the context of the developing embryo, epigenetic reprogramming is responsible for coordination of normal development (Rivera and Ross, 2013), while dysfunctional regulation in somatic cell types has been associated with cancerous phenotypes (Dawson and Kouzarides, 2012). Here I aim to provide the reader with a high level summary of DNA methylation and histone modifications, and the relevance of epigenetics to AML. This summary should also provide fundamental insights that place later discussions of pluripotency and cellular reprogramming techniques into context.

DNA methylation is carried out by enzymes known as DNA methyltransferases (DNMT), such as DNMT1, DNMT3A, and DNMT3B, which catalyze the conversion of cytosine to 5-methylcytosine. This chemical modification generally occurs in cytosine/guanine rich regions of DNA known as CpG islands that are often found in gene

promoters (Bird, 2002; Davuluri et al., 2001), and ultimately represses gene expression (Li et al., 1993). Conversely, the TET family of enzymes are responsible for DNA demethylation required for re-activation of genes (Kohli and Zhang, 2013). Another level of transcriptional regulation occurs through the post-translational modification of histones – protein scaffolds that package the DNA. Modifications include citrullination, phosphorylation, ribosylation, sumoylation and ubiquitylation, but acetylation and methylation are the most relevant and best understood. These processes are carried out by histone deacetylases (HDACs), histone acetyl transferases (HATs), histone demethylases (HDMs) and histone methytransferases (HMTs), and serve to activate or repress gene expression based on conformational changes and/or recruitment of transcriptional machinery to the histone (Bernstein et al., 2006).

It has recently become evident that AML epigenomes no longer resemble those of their normal blood cell counterparts. By performing global DNA methylation profiling, Figueroa et al. demonstrated that 344 human AML samples could be subdivided into 16 subclasses based on unique methylation signatures (Figueroa et al., 2010). Interestingly, this work demonstrated that distinct epigenetic states were associated with distinct cytogenetic and molecular aberrations. Later work using next-generation whole exome sequencing and DNA methylation analyses of 200 AML genomes and epigenomes corroborated these findings, and further mapped unique epigenetic profiles to underlying cytogenetic and molecular aberrations (The Cancer Genome Atlas Research Network, 2013). Taken together, these results suggest that specific changes in the epigenome during AML pathogenesis may occur in response to leukemia-associated aberrations.

Accordingly, a number of leukemic mutations affect known regulators of DNA methylation and chromatin modification such as DNMT, TET, and MLL (The Cancer Genome Atlas Research Network, 2013). However, causal links between leukemia-associated aberrations and the abnormal AML epigenome remain poorly resolved, and therefore, the study of epigenetics in the context of AML has become a fast-growing area of research in the field (Oki and Issa, 2010).

1.1.4 *In vitro* and *in vivo* models of human AML: understanding the paradigms, contributions and limitations

It is currently hypothesized that AML is functionally organized in a manner that resembles normal hematopoiesis, whereby disease is initiated and sustained by a rare population of leukemia stem cells (LSCs) that gives rise to dysfunctional AML progenitors incapable of normal differentiation to mature myeloid cells (Bonnet and Dick, 1997; Lapidot et al., 1994). *In vitro* and *in vivo* model systems and assays have been instrumental in developing this current understanding of disease, but they are not without their limitations. Here I overview the model systems of human AML that have brought us to our current understanding of disease, and discuss how novel complementary model systems are required to further progress this understanding.

Clonogenic Progenitor Assay

The clonogenic progenitor or "colony forming unit" (CFU) assay was first developed in 1966 when Ray Bradley and Donald Metcalf discovered a small fraction of healthy murine BM cells were capable of forming clonal colonies when plated in semi-solid growth medium (Bradley and Metcalf, 1966). Over the span of colony formation and

growth they were able to observe single-cell morphological changes associated with the differentiation of primitive BM cells to mature myeloid cells (Bradley and Metcalf, 1966). These primitive BM cells are termed hematopoietic progenitor cells (HPCs), and are experimentally defined by their ability to generate mature hematopoietic colonies when subjected to the CFU assay. Metcalf later adapted this assay to both the human and leukemic systems by measuring the capacity of myelomonocytic leukemia progenitors to form colonies (AML-CFU)(Metcalf et al., 1969). These experiments revealed that AML progenitors were capable of initiating differentiation processes similar to normal HPCs, but were unable to achieve full morphological maturation. The formation of these immature "blast" colonies provided the first *in vitro* demonstration of the AML differentiation blockade (Metcalf et al., 1969). This assay has since been improved to efficiently read out HPCs and AML progenitors through formation of monocytic, granulocytic, erythrocytic, megakaryocytic, and blast colonies (Griffin and Lowenberg, 1986; Wognum et al., 2013), but the principles of the assay remain the same. Together, these works not only established a system in which to model the AML differentiation blockade (Sachs, 1978), but they also established the value of the CFU assay in detecting and quantifying normal and AML progenitors (Moore et al., 1973; Sachs, 1978). The use of the CFU assay, in combination with morphological and cytogenetic assessments, as a means of distinguishing normal and leukemic cells was further developed in this thesis and was invaluable to the conclusions formed.

In vitro stromal co-cultures, suspension cultures and immortalized cell lines

While the CFU assay provided a testing ground to quantify the frequency of AML progenitors and the severity of their differentiation blockade, it was not informative of their functional self-renewal capacity – the ability to maintain their "differentiation" capacity while undergoing cell divisions. Accordingly, stromal co-cultures and hematopoietic cytokine-supplemented suspension cultures were developed in attempt to maintain primitive AML cells in culture (Sutherland et al., 1996). These *in vitro* conditions led to the identification of rare fractions of AML progenitors termed long-term culture initiating cells (LTC-IC) and suspension culture-initiating cells (SC-IC) that were able to persist *in vitro* for up to 8 weeks while maintaining CFU capacity. While these culture conditions shed light on the contribution of the BM microenvironment and extrinsic factors to the maintenance of rare primitive AML cells (Sutherland et al., 1996), they remain ineffective at expanding this population (Montesinos et al., 2006; Sutherland et al., 1996). Alternative strategies using high-throughput drug screens very recently identified small-molecules capable of expanding primitive AML cells in suspension cultures *in vitro* (Pabst et al., 2014), but an incomplete understanding of the global effects of these compounds on AML cells has so far limited their use in studies. Other attempts to capture primitive AML cells *in vitro* through immortalization have allowed for further observations of AML differentiation blockade, growth factor independence/dependence, and immunophenotypic and morphological abnormalities (Koeffler and Golde, 1980). However, the relevance of cell lines has been called into question by findings illustrating that extended periods of culture causes them to lose their molecular resemblance to the

primary disease state (Gillet et al., 2011). Together, these findings illustrate the current landscape of *in vitro* models of AML and their historical contributions to our understanding of disease.

Humanized Mouse AML Xenotransplant

Given the inherent difficulties of maintaining primitive AML cells *in vitro*, work in the lab of John Dick set out to identify and characterize the leukemic cells responsible for disease initiation. Based on their hypothesis that leukemia was organized in a similar fashion to normal hematopoiesis, they reasoned that primitive AML cells could initiate leukemia in a mouse similar to the ability of normal HSPCs to initiate normal hematopoiesis (Kamel-Reid and Dick, 1988; Mosier et al., 1988). They proved this hypothesis by demonstrating that a distinct population of AML cells (1 in 250,000) was able to trigger and recapitulate patient leukemia when transplanted into severe combined immunodeficient (SCID) recipient mice (Lapidot et al., 1994). Subsequent work identified that $CD34^{+}CD38^{-}CD45^{+}$ AML cells – same phenotype as normal HSPCs – were responsible for disease initiation (Bonnet and Dick, 1997). Together, these observations led to the current hypothesis that AML is organized in a functional hierarchy with a rare subset of primitive leukemia stem cells (LSCs) generating and maintaining the tumor through differentiation and self-renewal in a manner similar to their normal HSPC counterparts (Bonnet and Dick, 1997; Hope et al., 2004; Lapidot et al., 1994; Mosier et al., 1988). As such, the LSC assay – the ability of a leukemic cell to initiate and maintain disease in a mouse recipient – is the current gold-standard in the field for assessing the self-renewal and disease initiation capacity of AML cells, and can be used as a pre-

clinical surrogate in which to test the potential therapeutic effects of novel drugs (Sachlos et al., 2012; Wunderlich et al., 2013). However, due to the constraints of *in vitro* systems, the isolation and culture of LSCs for further characterization and studies has remained elusive.

Transgenic mouse models

Transgenic mouse models have been employed in attempt to identify the genetic causalities of AML, and can be created by deriving mice from genetically engineered mouse ESCs (mESCs) carrying an introduced genetic mutation or through transplantation of genetically altered BM. Mutated genes can be introduced at non-endogenous loci where they are under the control of doxycyclin-inducible or designed promoters, or using a "knock-in" approach whereby they are introduced downstream of their endogenous regulatory elements (Cook and Pardee, 2013). In the context of AML, transgenic mouse systems have been used to gain insights into the contributions of leukemia-associated aberrations to disease phenotype and initiation. Mice carrying the t(15;17) PML/RARα mutation not only demonstrated impaired neutrophil maturation, but they also responded to all-trans retinoic acid (ATRA) differentiation treatment similar to human acute promyelocytic leukemia (APL) patients (Brown et al., 1997). Similarly, mice with Mll-involving fusions or Pten knockout develop an aggressive leukemia, while Flt3-ITD knock-ins develop hyper-proliferative disorders, and t(8;21) AML1-ETO mice are susceptible to developing leukemia after administration of carcinogens (Forster et al., 2003; Li et al., 2008; Yu et al., 2010; Yuan et al., 2001). In perhaps the most-relevant scenario to the human system, human HPCs were transformed into engraftable LSCs

through forced expression of MLL-AF9 using retroviral transduction (Krivtsov et al., 2006). However, data from transgenic models can be difficult to interpret as transgene regulation and expression may not accurately reflect physiological levels observed in primary disease, and can lead to inaccurate conclusions about the causal links between mutations and disease phenotype (Brown et al., 1997; Chen et al., 2008; Early et al., 1996; Krivtsov et al., 2006). Although knock-in systems attempt to account for this, the relevance of mouse models to the human system remains in question due to inherent differences in mouse and human biology (Richmond and Su, 2008).

Practical cell-based models are required for mechanistic studies and drug discovery in the human AML system

Although *in vitro* and *in vivo* modeling has been instrumental in progressing our understanding of the functional hierarchy and abnormal differentiation features of human AML, this information has not translated to major improvements in disease treatment over the past 40 years (Burnett et al., 2011). This can be attributed to constraints in our current model systems that limit our ability to confidently delineate the causal/functional contributions of leukemia-associated aberrations and to develop high-throughput drug discovery platforms. AML xenografts and transgenic mouse models remain expensive and laborious vessels that are not conducive to high-throughput studies. Moreover, only a portion of human AML samples contain engraftable LSCs (Ailles et al., 1999), while transgenic mouse models take years to establish and may provide results that are not applicable to the human system (Cook and Pardee, 2013; Richmond and Su, 2008). Therefore, *in vitro* models theoretically represent more practical systems in which to

perform these studies. However, the CFU assay is limited to read-outs of AML progenitors (Griffin and Lowenberg, 1986; Wognum et al., 2013), expansion and maintenance of primitive AML cells *in vitro* remains a challenge (Montesinos et al., 2006), and cell lines vaguely resemble their primary cells of origin (Gillet et al., 2011). Thus, novel complementary model systems that overcome these constraints are required to advance our knowledge of AML pathogenesis and to develop novel therapeutics toward improving patient survival.

1.1.5 Current therapeutic management of AML

Newly-diagnosed AML patients receive multiple stages of intensive therapy designed to sequentially control and eliminate leukemic disease. Despite the functional and genetic heterogeneity observed in AML patients, cytarabine (AraC) chemotherapy forms the backbone of remission induction treatments in the majority of AML patients (Robak and Wierzbowska, 2009). As an anti-metabolite, AraC widely targets proliferating cells (both AML and normal hematopoietic cells) achieving remission rates of 50-80% (Robak and Wierzbowska, 2009). Due to these promising initial responses, AraC has been a mainstay of AML induction therapy for several decades and is unlikely to be replaced, however it does not represent a long-term solution as the 5-year survival rate is only 20-30% due to disease relapse (Shipley and Butera, 2009; Tallman et al., 2005). Following remission induction, there are multiple post-remission consolidation therapies available as preventative measures against relapse, many of which involve additional treatment with high-dose AraC (Roboz, 2012). HSPC transplantation is recognized as the most effective method to prevent leukemic recurrence, however this

option is limited by the availability of safe transplantation sources (Burnett et al., 1998; Ofran and Rowe, 2012; Roboz, 2012). In the absence of successful remission induction, further aggressive intervention is thought to provide minimal benefit, and therefore palliative care is often the strategy of choice for cases of chemo-refractory AML (Song and Lipton, 2005). Taken together, this highlights the urgent clinical need for improved disease therapeutics and safe sources of HSPCs for transplantation to increase long-term survival rates in AML patients.

A more complete mechanistic understanding of the contributions of leukemia-associated aberrations to disease should help facilitate the development of therapies that effectively target disease. This proof of concept is illustrated by a rare success in AML treatment whereby ATRA and arsenic trioxide administration eliminates the aberrant cellular effects mediated by the PML/RARα fusion protein by facilitating its catabolism and inducing differentiation (Ades et al., 2010; Zhou et al., 2007). As such, this therapeutic regimen represents the single example in AML treatment where disease can effectively be "cured". Along these lines, clinical trials are underway to explore the use of compounds that target other leukemia-associated aberrations such as FLT3 and IDH2, with promising results (Wander et al., 2014; Yen et al., 2013). However, earlier trials using 1st generation FLT3 inhibitors failed to show sustainable responses in AML patients due to poor FLT3 specificity; a perhaps unsurprising result given that these inhibitors were developed in other cancers to target FLT3-homologous proteins (Wander et al., 2014). Accordingly, the development of a cell-based model of AML would likely provide

a more relevant and practical platform from which to derive and test selective AML therapeutics prior to conducting expensive pre-clinical and clinical trials.

Performing $CD34^{+}CD45^{+}$ HSPC transplantation to provide long-term reconstitution of the normal hematopoietic system after consolidation therapy has been shown to significantly reduce the rate of disease relapse (Burnett et al., 1998). However, this effective, cell-based therapeutic option is limited by the availability of HSPCs for transplantation. Currently, clinical transplantation of HSPCs relies on three somatic/adult sources: BM, mobilized PB (MPB), and umbilical cord blood (UCB)(Hodby and Pamphilon, 2011). Access to these sources of HSPCs is limited by the number of willing/compatible donors (allogeneic), risk of leukemic reinfusion (autologous) and/or by low numbers of HSPCs that can be obtained from a single harvest (UCB)(Copelan, 2006; Hagenbeek and Martens, 1985; Hodby and Pamphilon, 2011; Li et al., 2009). Efforts over the past two decades to increase the number of purified HSPCs by *ex vivo* expansion have had variable and ultimately limited success (Hodby and Pamphilon, 2011; Kelly et al., 2010). For example, recent clinical trials report conflicting results regarding whether expansion of UCB is even beneficial (Kelly et al., 2010). In light of these shortcomings, alternative sources of HSPCs are in greater demand than ever.

1.2 Embryonic Stem Cells and Pluripotency

The landmark derivation of human embryonic stem cells (hESCs) by Thomson et al. in 1998 ignited the field of human stem cell research. Given their potential for unlimited self-renewal capacity and differentiation into all somatic cell types, a feature known as "pluripotency", hESCs represented an exciting but controversial pluripotent stem cell (PSC) platform from which to potentially develop cell-based therapies, disease models and drug/toxicity screens (Thomson et al., 1998). In this section I briefly overview the defining works for PSC research, discuss the extrinsic and intrinsic regulators of the pluripotent state, and summarize the assays and hallmark features of pluripotency as they pertain to the human system.

1.2.1 Origins and definitions of pluripotent stem cells

Leroy Stevens began his career as a junior researcher at the Jackson Laboratory in 1953 investigating the incidence of cancers in mice following exposure to cigarette ingredients. What he found instead would form the basis of the field of PSC research. Using a variety of mice strains in his initial experiments, Stevens discovered that strain 129 mice had an inheritable susceptibility (~1% of mice) to the spontaneous formation of testicular tumours (Stevens and Little, 1954). Histological investigation revealed that these tumours were comprised of diverse adult tissues including bone, blood, fat, nervous, epithelia, marrow, muscle and glandular, as well as undifferentiated embryonal cells (Stevens and Hummel, 1957; Stevens and Little, 1954). These results were consistent with the formation of teratocarcinomas – malignant tumours containing disorganized compositions of derivatives of the three embryonic germ layers (ectoderm, endoderm, and

mesoderm), as well as primitive undifferentiated embryonal cells – which had never been identified in mouse, but were previously observed in human and horse (Stevens and Hummel, 1957). As such, strain 129 mice represented a tractable model system in which to investigate the origins of these tumours. Stevens' believed that the embryonal cells may be "pluripotent", that is, capable of differentiating to all somatic cell types and sustaining unlimited self-renewal, and therefore, responsible for the tumour formation (Stevens and Hummel, 1957). Accordingly, tumours subcutaneously re-grafted into secondary mice either maintained their size or progressively grew into larger tumours in a manner that correlated with the absence or presence of embryonal cells in the parent tumour, respectively (Stevens, 1958; Stevens and Hummel, 1957). These findings were in line with Stevens' initial hypothesis, leading him to conclude:

> **"**Probably most, if not all, of the tissues in these teratomas originate from a pluripotent stem cell, and differences between sublines reflect differences in the developmental capacities of their stem cells." (Stevens, 1958)

I not only include this statement because Stevens was ultimately correct as I briefly detail below (with the caveat that these cells were of malignant origin), but because I believe that this was the moment at which the field of PSC research was unknowingly born. Although he had yet to generate definitive proof, Stevens' early works loosely established that teratocarcinomas were derived from malignant pluripotent embryonal cells with 1) multilineage differentiation capacity as observed by formation of all three embryonic layers and 2) self-renewal capacity as observed by the ability to maintain progressive tumour growth and undifferentiated cell types after transplantation.

Subsequent works in the field identified that malignant embryonal carcinoma cells (ECCs), neoplastic counterparts of the embryonal cells responsible for normal embryogenesis, were responsible for teratocarcinoma formation (Kleinsmith and Pierce, 1964; Pierce and Dixon, 1959; Stevens, 1960) and could be isolated and cultured *in vitro* (Evans, 1972; Kahan and Ephrussi, 1970; Rosenthal et al., 1970). Although ECCs exhibited features of pluripotency through their ability to contribute to normal mouse development when injected into blastocysts (Brinster, 1974), their true pluripotent nature was questioned given their neoplastic origins, abnormal karyotypes, and variable/limited differentiation potential (Evans, 1972; Kahan and Ephrussi, 1970; Rosenthal et al., 1970; Stevens and Hummel, 1957). This prompted Martin Evans and Matthew Kaufman, and Gail Martin shortly thereafter, to ask whether normal embryonic cells responsible for the development of entire organisms could similarly be isolated during early embryogenesis and cultured *in vitro* toward capturing normal pluripotency in a dish. By plating the inner cell mass (ICM) of the mouse blastocyst on a supportive feeder layer of mouse embryonic fibroblasts (MEFs) these groups were independently able to derive mouse embryonic stem cell (mESC) colonies with normal karyotype and capable of teratoma[1] formation *in vivo* and differentiation *in vitro* (Evans and Kaufman, 1981; Martin, 1981). In the ultimate test of pluripotency, cultured mESCs were later shown to give rise to an entire organism (Nagy et al., 1993). Together, these works provided the initial derivation method and

[1] Teratomas are the benign equivalent of teratocarcinomas: teratocarcinomas are derived from malignant ECCs, while teratomas are derived from normal ESCs. Damjanov, I., and Andrews, P.W. (2007). The terminology of teratocarcinomas and teratomas. Nat Biotechnol *25*, 1212; discussion 1212.

description of ESCs that motivated future studies of pluripotency and development, and the derivation of ESCs from other organisms (Evans, 2011).

Years later, James Thomson adapted these techniques to the primate system allowing for the derivation of ESCs from the ICM of human blastocysts generated through *in vitro* fertilization (Thomson et al., 1998; Thomson et al., 1995). These hESCs grew in colonies and possessed normal karyotype, were capable of indefinite self-renewal while maintaining differentiation capacity *in vitro*, and were capable of generation of all three embryonic germ layers through teratoma formation when injected into mouse testes. This breakthrough in stem cell research provided a platform in which to study pluripotency and development in the human system *in vitro* and carried the promise of generating cells for regenerative medicine and drug discovery. However, their derivation and potential application to regenerative medicine was surrounded by ethical concerns (Lo and Parham, 2009), which were later circumvented through the generation of induced pluripotent stem cells (iPSCs) using cellular reprogramming techniques that are described in section 1.3 of this introduction.

1.2.2 *In vitro* conditions required for maintenance of hPSCs

Due to their pluripotent nature, hPSCs are capable of indefinite self-renewal and growth. However, specific culture conditions are required to maintain pluripotency. Culture on irradiated MEFs (iMEFs) was initially required for maintenance of undifferentiated hPSC colonies *in vitro* (Thomson et al., 1998), but was soon replaced by feeder-free conditions using Matrigel extracellular matrix and MEF-conditioned media (MEF-CM) supplemented with basic fibroblast growth factor (bFGF)(Levenstein et al.,

2006; Xu et al., 2001). In these latter culture conditions, it was discovered that hPSCs were capable of generating an autologous niche of human dermal fibroblasts (HDFs) that supported their growth in feeder- and MEF-CM-free conditions (Wang et al., 2005b). Together, indicating that self-renewal and maintenance of undifferentiated state is regulated in a non-cell-autonomous manner by the stem cell niche and/or its secreted extrinsic factors. Accordingly, in 2007 our lab provided critical insights into the extrinsic role the niche played in maintenance of pluripotency. Bendall et al. found that bFGF's role in the maintenance of hESCs was not through direct activation of hPSCs, but rather its stimulation of the autologous HDFs to secrete insulin-like growth factor II (IGFII) – a soluble factor that alone was sufficient to maintain primitive, undifferentiated hPSCs (Bendall et al., 2007). A better understanding of the role of the stem cell niche and extrinsic factors in regulating the pluripotent state in a non-cell autonomous manner has ultimately contributed to the development of chemically-defined, xeno-free culture conditions for hPSCs toward enabling their use in clinical applications (Chen et al., 2011).

1.2.3 Transcriptional and epigenetic regulation of pluripotency

The intrinsic transcriptional regulation of pluripotency is maintained by the core pluripotency transcription factors (TFs) OCT4, SOX2, and NANOG (OSN)(Boyer et al., 2005), with the extrinsic signals described above ultimately contributing to maintenance of pluripotency through the modulation of this network (Boiani and Scholer, 2005). OCT4, a mammalian pit/oct/unc TF, is required for the formation of the ICM during embryogenesis, and its loss of expression is embryonic lethal in mice (Nichols et al.,

1998). Moreover, knockdown of its expression using RNA interference in hESCs results in their differentiation (Matin et al., 2004). Similarly, the expression of the homeoprotein NANOG in the ICM is required for normal formation and development of the epiblast prior to generation of the three embryonic germ layers, and its loss of expression in ESCs *in vitro* results in their differentiation (Chambers et al., 2003; Mitsui et al., 2003). Finally a deficiency for SOX2, a Sry-related HMG box TF, is embryonic lethal and contributes to abnormal development of the epiblast from the ICM (Avilion et al., 2003). In hESCs, its expression level contributes to maintenance of the pluripotent state as over- and under-expression leads to differentiation (Adachi et al., 2010). Together, these studies provided definitive evidence for the contribution of OSN to the initiation and/or maintenance of the pluripotent state during early embryogenesis and *in vitro* culture. Toward further understanding the regulatory circuitry of pluripotency, Boyer et al. performed chromatin immunoprecipitation combined with DNA microarrays to identify OSN target genes in hESCs (Boyer et al., 2005). OSN co-occupied many target gene promoters of actively expressed self-renewal pathways and TFs including OSN themselves. Interestingly, OSN co-occupation was also observed on a number of transcriptionally inactive genes important for the specification of the three embryonic germ layers, indicating a repressive effect (Boyer et al., 2005). These results delineated that the core regulatory circuitry of pluripotency was maintained by the synergistic roles of OSN to activate their own expression and that of known-self renewal pathways, and to repress TFs required for differentiation from the pluripotent state.

Epigenetic regulation provides an additional layer of control of the pluripotent state through balancing self-renewal and differentiation processes (Bibikova et al., 2006; Xie et al., 2013). hPSCs possess distinct DNA methylation patterns (Bibikova et al., 2006), indicating that patterns of DNA methylation must be carefully regulated and maintained during self-renewal and replication *in vitro*. Accordingly, disruption of DNMT1 in hESCs leads to rapid cell death (Liao et al., 2015) and is embryonic lethal in mice (Li et al., 1992). Moreover, OCT4, NANOG and genes required for cellular function remain highly unmethylated in the pluripotent state until differentiation, while genes associated with mature lineages are highly methylated (Fouse et al., 2008). Similarly, hPSCs have been found to harbour both activating and repressing marks at histones of lineage-specifying gene promoters, termed bivalent chromatin domains, which keeps target genes poised for expression once proper cues have been received to exit the pluripotent state (Bernstein et al., 2006). Together, the essential role of epigenetic regulation in maintenance of pluripotency is unsurprising given that resetting of the epigenome to a pluripotent state is required for normal development (Rivera and Ross, 2013), a concept further discussed in section 1.3.

1.2.4 Defining hPSCs: molecular and functional hallmarks of pluripotency

hPSCs are easily recognizable in *in vitro* cultures given their distinctive growth pattern as flat, rounded colonies consisting of densely-packed cells with large nuclei and minimal cytoplasm (Thomson et al., 1998). In addition to these morphological features, hPSCs are distinguished and characterized by a unique complement of molecular, immunophenotyptic and functional features that have been validated in a multitude of

hPSC lines (Adewumi et al., 2007). The transcriptional and protein-level co-expression of OSN is a requisite and prominent identifier of pluripotency given their role in controlling the pluripotent network (Boyer et al., 2005), while the antigens SSEA3, SSEA4 and TRA-1-60 represent cell-surface markers found uniquely on hPSCs (Adewumi et al., 2007). Although these latter markers do not have essential roles in pluripotency (Brimble et al., 2007), their expression enables experimentally invaluable live-cell staining techniques for quick detection and immunophenotypic sorting of hPSCs. At the functional level, all hPSCs have the capacity to form teratomas consisting of the three embryonic germ layers when injected into mouse testes (Thomson et al., 1998). This "teratoma assay" represents the gold-standard for assessing human pluripotency, as deriving humans to test for pluripotency similar to the mouse system certainly carries heavy ethical implications (Nagy et al., 1993). Moreover, it is reminiscent of Stevens' early work in the 1960s, with the distinction that normal PSCs should undergo full differentiation *in vivo* and are therefore, not capable of forming transplantable tumours like their malignant counterparts (Sachlos et al., 2012; Stevens, 1958). Pluripotent functionality can also be assessed *in vitro* using embryoid body (EB) or co-culture based assays in which PSCs are collected into clumps and cultured in lineage-specifying media conditions toward the generation of derivatives of the three embryonic germ layers including neural (Zhang et al., 2001), lung (McIntyre et al., 2014), and hematopoietic (Chadwick et al., 2003; Kaufman et al., 2001) lineages – a feature that holds great potential for regenerative medicine if properly harnessed, and one that I will discuss further in section 1.3.4: Applications of iPSCs.

1.3 Cellular Reprogramming and Induced Pluripotent Stem Cells

The Nobel Prize-winning work performed by Takahashi et al. under the supervision of Shinya Yamanaka in 2007, in which human fibroblasts were reprogrammed into induced pluripotent stem cells (iPSCs) by expression of pluripotent-associated TFs, ushered in an era of stem cell research carrying the promise of personalized regenerative medicine (**Figure 1**)(Takahashi et al., 2007). iPSCs possessed hallmark features of pluripotency and represented a patient-specific source of pluripotent cells that circumvented the ethical and immune incompatibility concerns previously associated with hESCs. In this section I briefly review the pioneering studies by John Gurdon, Henry Harris and Robert Davis that formed the underlying principles of cell fate alteration, describe Yamanaka's seminal findings and mechanistic insights into the reprogramming process, and finally discuss current and potential applications of iPSCs in regenerative medicine.

1.3.1 Foundational concepts of cellular reprogramming: lessons from somatic cell nuclear transfer, cell fusion, and transcription factor reprogramming studies

Somatic Cell Nuclear Transfer

In the 1950's Robert Briggs and Thomas King set out to investigate whether the complete nuclei present in the fertilized egg (zygote) was conserved in somatic cell types after differentiation. Using a technique termed somatic cell nuclear transfer (SCNT), living nuclei were isolated from the differentiated endoderm of *Rana pipiens* (Northern Leopard Frog) during early development and injected into activated, enucleated females'

eggs. Unlike "cloned" embryos derived from nuclei obtained from early undifferentiated embryonal cells (blastula), endoderm-derived embryos did not mature into larvae (Briggs and King, 1957). They interpreted and attributed this result to a loss of genetic material in mature somatic cell types (Briggs and King, 1957). However, a young graduate student at Oxford University named John Gurdon was subsequently able to clone sexually mature tadpoles and normal adult frogs through nuclear transfer of blastula and differentiated endoderm, including terminally differentiated intestinal epithelium cells (Gurdon, 1960, 1962; Gurdon et al., 1958). In direct contrast to Briggs' and King's findings, whose technically-sound results but incorrect interpretation were later attributed to limitations of the *R. pipiens* system (Gurdon, 2006), the successful cloning of adult frogs using SCNT provided definitive evidence that the entire genome is retained during differentiation processes. More importantly, the ability of stochastic factors within the oocyte cytoplasm to reprogram mature somatic cells back to the pluripotent state provided the initial indications that 1) cell state is not always fixed, that is, seemingly mature somatic cell types can be experimentally reprogrammed to adopt an alternative cell fate, and 2) cytoplasmic factors exert powerful control of gene transcription responsible for cell phenotype and function. These pioneering demonstrations by a determined graduate student who was labelled as having "no chance of doing the work of a specialist" (Gurdon, 2006) formed the underlying principles for epigenetic regulation and cellular reprogramming, and earned John Gurdon a Nobel Prize in Medicine alongside Shinya Yamanaka in 2012.

Cell Fusion: The heterokaryon experimental system

Cell fusion refers to the experimental procedure of combining two or more cells through fusion of their cytoplasms to generate either a synkaryon, in which the resulting cell contains a single nucleus harbouring all parent cell chromosomes, or a heterokaryon, in which the nuclei are maintained in the cytoplasm as individual entities (Ogle et al., 2005). The latter cell hybrid results when fusing cells of different types or species, and was the focus of fusion experiments initiated in the 1960s as a means of investigating whether factors from non-oocyte cell cytoplasm could also alter cell phenotype. Founding work by Henry Harris and John Watkins established the first example of heterokaryon generation through fusing mouse Ehrlich ascites cells and human HeLa cells (Harris and Watkins, 1965). Remarkably, these hybrid cells remained viable, with the continued contribution of RNA expression from mouse and human nuclei leading to a phenotype that was distinct from the contributing parental cells (Harris and Watkins, 1965). The subsequent demonstration that cancerous mouse cell phenotypes could be suppressed following fusion to normal mouse fibroblasts, but restored after loss of normal chromosomes, provided further evidence that cell state is not fixed and can be experimentally manipulated (Harris et al., 1969). Moreover, it suggested that normal fibroblasts were supplying a factor responsible for the suppression of malignancy (Harris et al., 1969). Numerous studies have subsequently utilized this experimental system to gain insights into cellular reprogramming (Blau et al., 1983; Cowan et al., 2005; McBurney et al., 1978; Piccolo et al., 2011; Tada et al., 2001), providing further evidence

that cell fate can be manipulated by cytoplasmic factors that initiate nuclear transcription and remodel the epigenome.

Transcription factor reprogramming

In 1987 Robert Davis, Harold Weintraub and Andrew Lassar attempted for the first time to alter cell fate using defined factors through the transfection and expression of muscle-specific TFs in mouse fibroblasts (Davis et al., 1987). They reasoned that the dominant capability of unknown factors found in the myoblast cytoplasm to initiate transcription of muscle-specific genes in the heterokaryon system (Blau et al., 1983) could be attributed to the presence of proteins that 1) regulate the epigenome allowing for downstream demethylation and activation of muscle genes and/or 2) act as direct activators of muscle-specific gene programs. Building on their previous finding which suggested it was the latter scenario (Lassar et al., 1986), it was therefore not unreasonable to hypothesize that ectopic expression of muscle-specific genes could alter fibroblast fate toward muscle. By screening and transfecting a cDNA library of muscle-specific TFs, it was demonstrated that ectopic expression of the gene MyoD alone was sufficient to convert fibroblasts to myoblasts (Davis et al., 1987). Although this work marked the birth of TF-mediated reprogramming and provided the first example of using defined factors to alter cell fate, this powerful reprogramming method was not widely recognized or adopted in the field until the early 2000's (Graf, 2011).

1.3.2 Induced pluripotent stem cells: cellular reprogramming to pluripotency is achieved through ectopic transcription factor expression

The above studies formed three underlying principles of cellular reprogramming: 1) cell fate can be altered, 2) the pluripotent cytoplasm contains dominant, undefined factors capable of reverting somatic cells back to a state of epigenetic, transcriptional and functional pluripotency, and 3) the ectopic delivery of TFs represents a powerful molecular tool in which to alter cell fate. Kazutoshi Takahashi and Shinya Yamanaka not only recognized these principles, but they also envisioned the potential utility of hPSCs in regenerative medicine if ethical and immune incompatibility concerns associated with hESCs could be overcome. As such, the duo set forth to define factors responsible for the reprogramming of somatic cells to pluripotency and subsequently use them as tools to generate PSCs. Similar to Davis' previous work, a library of 24 candidate genes associated with the pluripotent state were assessed for their ability to generate mPSC colonies from MEFs. Although no single factor was capable of inducing pluripotency within 16 days, the use of all 24 factors was, indicating that generating "pluripotent stem cells induced from MEFs by 24 factors" was possible – cells now referred to as induced pluripotent stem cells (iPSCs). Using a subtractive reprogramming factor approach, it was discovered that Oct4, Sox2, Klf4 and c-Myc (OSKM) were critical for the generation of iPSCs. These iPSCs shared transcriptional, epigenetic, immunophenotypic, and functional hallmarks of pluripotency akin to ESCs, and required the same extrinsic culture conditions. Remarkably, the same results were achieved in the human system and enabled the generation of personalized iPSCs (Takahashi et al., 2007). Together, these seminal

findings demonstrated the ability of TFs to revert somatic cells' epigenetic and transcriptional networks back to a pluripotent state similar to previous approaches using SCNT and heterokaryons (Cowan et al., 2005; Gurdon, 1962; Tada et al., 2001). However, by circumventing technical, ethical and incompatibility issues associated with these previous methods, generation of iPSCs provided a tractable and unprecedented opportunity for personalized regenerative medicine (**Figure 1**). As such, Shinya Yamanaka was awarded the Nobel Prize in Medicine in 2012 for the impact of these works.

1.3.3 Reprogramming to pluripotency requires resetting of somatic cell transcriptomes and epigenomes

Although numerous studies have since demonstrated a variety of reprogramming TF cocktails and methods are capable of achieving pluripotency from a number of cell types, including blood (Lee et al., 2014; Loh et al., 2009; Theunissen and Jaenisch, 2014), much of the reprogramming process remains undefined. Within the first 48 hours OSK activate accessible genes that promote reprogramming, but are unable to access chromatin domains that are inactivated by histone modifications (Soufi et al., 2012). However, through subsequent changes in epigenetic regulation the genome becomes progressively more accessible due to widespread chromatin remodeling, ultimately allowing for further activation of genes required for initiation and maintenance of the pluripotent state (Buganim et al., 2012; Hussein et al., 2014; Koche et al., 2011). Paradoxically, this suggests that gene expression alters the epigenome, which is responsible for altering gene expression and enabling changes in phenotype to manifest. Ultimately, an increased

understanding of the reprogramming process and its effects on the transcriptome and epigenome should better direct the applications and derivation of iPSCs. For example, our group and others have shown that iPSCs can retain an "epigenetic memory" of their starting cell type despite widespread changes in chromatin structure. Specifically, iPSCs derived from UCB are able to differentiate to the hematopoietic lineage more efficiently than fibroblast-derived iPSCs due to incomplete DNA methylation and unique histone marks at hematopoietic-specific genes, suggesting that reprogramming in the human system does not completely revert back to a naïve pluripotent state (Lee et al., 2014). Accordingly, this knowledge may better direct the selection of starting cell types for studies aimed at generating cell-based therapies or modeling epigenetic diseases. Similarly, identification and elimination of barriers to reprogramming may increase reprogramming efficiency toward enabling derivation of difficult to reprogram cell types (Rais et al., 2013).

1.3.4 Applications of induced pluripotent stem cells: autologous therapies and disease modeling

In vitro differentiation of hPSCs

Numerous protocols have been developed to harness the differentiation of PSCs toward the generation of a host of cell types including cardiac (Mummery et al., 2012), hematopoietic (Chadwick et al., 2003), intestinal (Spence et al., 2011), lung (McIntyre et al., 2014), neural (Kim et al., 2014; Zhang et al., 2001), and pancreatic lineages (Rezania et al., 2012); but the ability of hPSCs to give rise to the hematopoietic lineage is further described in this section based on the focus of this thesis. Our group provided first

demonstration of the generation of $CD34^+CD45^+$ HPCs (Chadwick et al., 2003). This was achieved through culture of hESC-derived EBs in media supplemented with bone morphogenetic protein 4 (BMP4) and hematopoietic cytokines: FLT3 ligand (FLT3L), granulocyte-colony stimulating factor (G-CSF), interleukin-3 (IL-3), interleukin-6 (IL-6), and stem cell factor (SCF)(Chadwick et al., 2003). By definition, these HPCs possessed progenitor function *in vitro* through differentiation to myeloid lineages in the CFU assay (Chadwick et al., 2003), however, long-term reconstitution capacity characteristic of HSPCs was not observed following transplantation *in vivo* (Wang et al., 2005a). It was suggested that these findings were attributed to an inability of PSC-derived blood cells to activate molecular programs similar to adult somatic HSPCs (Wang et al., 2005a); a challenge the field has faced for years. However, recent works reporting the derivation of transplantable HSPCs from hPSCs represent incremental advances being made toward achieving robust, long-term hematopoietic reconstitution using hPSC derivatives (Amabile et al., 2013; Doulatov et al., 2013; Suzuki et al., 2013). In addition to these studies, other groups have established protocols to generate transfusion products such as red blood cells (RBCs) and platelets from hPSCs (Feng et al., 2014; Lu et al., 2008; McIntyre et al., 2013; Nakamura et al., 2014). Together, these continued advances within the area of hematopoietic specification from hPSCs represent a subset of the collective efforts of the field to generate clinically-relevant cell types for use in personalized cell-based therapies and disease modelling.

Personalized cell-based therapies

Given their immense self-renewal and differentiation capacities, iPSCs may provide

a renewable and even potentially unlimited source of patient-specific (autologous) human cells for use in cell-based therapies. This could address the clinical obstacles associated with donor (allogeneic) cell sources, such as immune-incompatibility/transplant rejection (Copelan, 2006; Li et al., 2009), and provide cell sources that are not clinically available such as neural cells (Kim et al., 2014; Zhang et al., 2001). One of the compelling examples for the use of human iPSCs with regard to autologous cellular transplantation was shown by Hanna et al., wherein hematopoietic cells derived from iPSCs corrected for a hemoglobin mutation reduced the blood cell defect in a humanized mouse model of sickle cell anemia (Hanna et al., 2007). Similarly, iPSC-derived stroma-like cells have been shown to attenuate limb ischemia in mice (Lian et al., 2010), and contribute to functional bone tissue *in vivo* in non-human primates (Hong et al., 2014). Importantly, autologous but not allogeneic sources of iPSC-derived neurons elicited minimal immune response following transplantation into non-human primate brains (Morizane et al., 2013). Although safety concerns have recently been raised with the use of iPSCs due to the identification of mutations occurring during reprogramming and culture (Ji et al., 2012; Ruiz et al., 2013), the functional and biological significance of these findings remains unclear as cancer/tumour development was not observed in any of the above studies (Hanna et al., 2007; Hong et al., 2014; Lian et al., 2010; Morizane et al., 2013). At the very least, these findings illustrate the need for genetic screening prior to clinical implementation, as has been performed in the recent and promising clinical trials in humans using PSC-derivatives (Kimbrel and Lanza, 2015). Together, these works illustrate the potential impacts, utilities and shortcomings of using cellular

reprogramming to generate personalized cell sources that avoid immune-rejection, and continue to motivate the field toward clinical implementation.

Disease modelling

The advent of patient-specific reprogramming also allows for the generation of iPSCs from diseased patients, offering an unprecedented opportunity to generate *in vitro* disease models that can be used for understanding disease and for enabling drug-screening platforms. Accordingly, disease-specific iPSCs have been generated from a variety of somatic cells derived from patients with inherited and acquired diseases, where underlying genetic components and affected cell types are known (Carette et al., 2010; Ebert et al., 2009; Kim et al., 2013; Kim and Zaret, 2015; Kinnear et al., 2013; Kotini et al., 2015; Kumano et al., 2012; Marchetto et al., 2010; Nagai et al., 2010; Stricker et al., 2013). In most cases, differentiation of iPSCs to relevant cell types exhibited disease features, providing examples that disease modeling using iPSC technology is feasible. For instance, fibroblast-derived iPSCs generated from human patients with inherited spinal muscular atrophy, a fatal neurological disease caused by mutations in the gene *SMN1*, gave rise to degenerative motor neurons consistent with disease phenotype. Subsequent experiments using these dysfunctional motor neurons showed early evidence that drug intervention may be useful in improving the disease (Ebert et al., 2009). In a similar approach, Stricker et al. demonstrated that cancerous neural stem cells derived from human glioblastoma tumours, an acquired somatic cancer of the brain characterized by multiple genomic aberrations and an aberrant epigenome, could be reprogrammed to iPSCs. Notably, a majority of glioblastoma-related aberrant epigenomic marks were reset

upon reprogramming to pluripotency. Despite this epigenetic resetting, subsequent redifferentiation back to the neural lineage resulted in a disease phenotype. This result indicated that reprogramming can still be used to develop model systems in cancers that harbour an abnormal epigenetic component, and suggested that the genomic aberrations may be responsible for the aberrant epigenome. Interestingly, epigenetic marks were slowly restored at some gene loci following redifferentiation, however, it was not investigated further whether the aberrant epigenome was re-established over time (Stricker et al., 2013).

Despite these proof of principle studies, disease modelling using iPSCs may not be best-suited for complex/poorly understood diseases and may be difficult to achieve because of difficulties in reverting diseased cells back to the pluripotent state. For instance, derivation of iPSCs from schizophrenia patient fibroblasts and subsequent differentiation to the neural lineage generated defective neurons that exhibited decreased connectivity and synaptic proteins (Brennand et al., 2011). Although this may have provided initial insights into disease, schizophrenia is a complex neurological disorder suggested to affect multiple cell types due to unknown and complex underlying genetic component(s) (Javitt et al., 2008; Sullivan et al., 2003; Wong and Van Tol, 2003). As such, it is difficult to ascertain which cell types to generate and whether fibroblasts harbour the genetic components responsible for disease phenotype, highlighting that a basic understanding of disease is required before the relevance of iPSC models can be clearly established. Developing efficient iPSC models can be similarly hindered by the inability to generate iPSCs directly from diseased cells. A number of studies have

recently demonstrated that reprogramming gastrointestinal, chronic myeloid leukemia (CML), glioblastoma, myelodysplastic syndrome, and pancreatic ductal adenocarcinoma cancer cells to pluripotency is possible and can allow for disease modeling (Carette et al., 2010; Kim et al., 2013; Kim and Zaret, 2015; Kotini et al., 2015; Kumano et al., 2012; Nagai et al., 2010; Stricker et al., 2013). However, reprogramming was highly inefficient, with marginal success often observed across primary patient samples. The failure of cancer cells to robustly reprogram was neither explicitly discussed or addressed in these papers, and represents a potential roadblock that could limit the widespread utility and applicability of cellular reprogramming in developing disease models for human cancers.

1.4 Summary of Intent

The past 40 years has seen major advances in the understanding of AML heterogeneity leading to improved disease classification and diagnosis (Burnett et al., 2011; Grimwade et al., 1998; Vardiman et al., 2002; Vardiman et al., 2009). However, these findings have not yet translated to improved therapeutic outcomes, as relapse and dismal rates of disease-free survival remain predominant prognoses for the majority of AML patients (Dohner et al., 2015). Transplantation therapies that have proven effective at reducing disease relapse remain limited by the rare availability of safe sources of HSPCs (Burnett et al., 1998; Hodby and Pamphilon, 2011). Moreover, elucidation of mechanisms underlying human AML pathogenesis and high-throughput discovery of novel therapeutics is hindered by the lack of practical human cell-based models of disease. Accordingly, the requisite development of novel cell-based therapies and disease models should expedite advances in the therapeutic management of AML.

The advent of personalized cellular reprogramming techniques through the generation of human iPSCs (Takahashi et al., 2007) provides a potential opportunity to generate autologous hematopoietic cell sources to address these unmet needs. However, whether reprogramming of human AML patient somatic cells to pluripotency and subsequently to normal and dysfunctional hematopoietic cells is even possible must first be addressed, and forms the basis of this thesis.

Therefore, I have posed two key questions with regards to cellular reprogramming of human AML patient somatic cells:

1) *Can AML patient fibroblasts (AML Fibs) be reprogrammed to iPSCs, and would this enable generation of a novel source of normal autologous HPCs?*

2) *Are AML patient leukemic cells amenable to reprogramming to pluripotency, and does this allow for derivation of HPCs that model features of primary disease?*

Investigating these questions will provide key proof of concepts and initial insights toward future development of personalized cell-based therapies and *in vitro* models of disease for AML patients.

Based upon our current knowledge, I hypothesize that ***cellular reprogramming of human AML patient somatic cells to iPSCs is possible and will enable derivation of autologous sources of normal and dysfunctional HPCs.***

Accordingly, I defined two objectives to test this hypothesis:

1) Investigate if reprogramming AML Fibs to pluripotency will provide a novel, patient-specific source of autologous HPCs that are capable of normal differentiation *in vitro* and devoid of leukemia-associated aberration (**Figure 2**)

2) Evaluate whether generation of iPSCs from AML patient leukemic cells harbouring known leukemia-associated aberrations is possible, and if this will allow for derivation of HPCs that exhibit differentiation blockade similar to primary leukemic cells. (**Figure 3**)

We first investigated whether personalized sources of normal hematopoietic cells could be generated for AML patients whose leukemic cells harboured known leukemia-

associated aberrations (**Chapter 2**). Through expression of reprogramming TFs in dermal AML Fibs we demonstrated that AML patient-specific iPSC generation was possible, and allowed for derivation of $CD34^{+}CD45^{+}$ HPCs. Using the CFU assay, in combination with morphological and FISH analyses, we demonstrated that these HPCs were capable of normal differentiation to mature myeloid lineages *in vitro* in contrast to the patients' matched leukemic cells. These findings provided the proof of principle that cellular reprogramming allows for the generation of normal autologous hematopoietic progenitors from AML patients, suggesting that iPSCs may represent a suitable source of autologous cells for future transplantation therapies.

During the tenure of the above experiments we also discovered that OCT4 TF delivery combined with culture in pluripotent-supportive media was minimally sufficient to induce pluripotency in AML and normal Fibs (**Chapter 3)**. OCT4-derived iPSCs ($iPSC^{OCT4}$) possessed morphological, molecular, immunophenotypic and functional hallmarks of bona fide iPSCs and were capable of hematopoietic differentiation *in vitro.* These findings redefined conventional pluripotency reprogramming methods (Takahashi et al., 2007; Takahashi and Yamanaka, 2006), and in combination with previous findings from our lab (Mitchell et al., 2014a; Mitchell et al., 2014b), indicated that OCT4 is a powerful tool for cell fate reprogramming.

Toward capturing and modeling the diverse dysfunctional features of human AML, we next asked whether cellular reprogramming to pluripotency could be applied to AML patient leukemic cells (**Chapter 4**). We first assessed whether conventional reprogramming conditions were sufficient to induce pluripotency in leukemic cells from

12 independent AML patients. Surprisingly, AML iPSCs could not be generated using these conditions despite efficient delivery of reprogramming TFs. However, we found that modified reprogramming conditions allowed for the reproducible generation of AML iPSCs from one AML sample harbouring the MLL-AF9 leukemia-associated aberration. *In vitro* differentiation assays coupled with molecular, immunophenotypic and morphological analyses revealed that these AML iPSCs and their derivative HPCs expressed MLL-AF9 and possessed dysfunctional differentiation features similar to primary leukemic cells. These results indicated that reprogramming of AML is highly inefficient, but possible. Moreover, they demonstrated that AML iPSCs and their derivative HPCs exhibited limited differentiation capacity, suggesting that AML iPSCs may provide a model system in which to explore disease mechanisms and screen for novel therapeutics.

By demonstrating that personalized sources of normal and dysfunctional HPCs can be generated from AML patient somatic cells this dissertation has advanced our understanding of the potential applications of cellular reprogramming technologies in human AML research. These initial findings should motivate further studies aimed at developing personalized iPSC-based therapies and disease models toward improving AML patient quality of life and long-term survival rates.

Figure 1. Cellular reprogramming to pluripotency provides an unprecedented opportunity for generation of patient-specific tissues. Through expression of four defined pluripotent transcription factors (OCT4, SOX2, KLF4, cMYC), Takahashi and Yamanaka were able to reprogram human fibroblasts to an epigenetic, transcriptional and functional state of pluripotency (Takahashi et al., 2007). The ability to generate patient-specific induced pluripotent stem cells (iPSCs) provides an opportunity to harness their inherent differentiation potential toward generation of autologous cell sources for cell-based therapies and disease modeling.

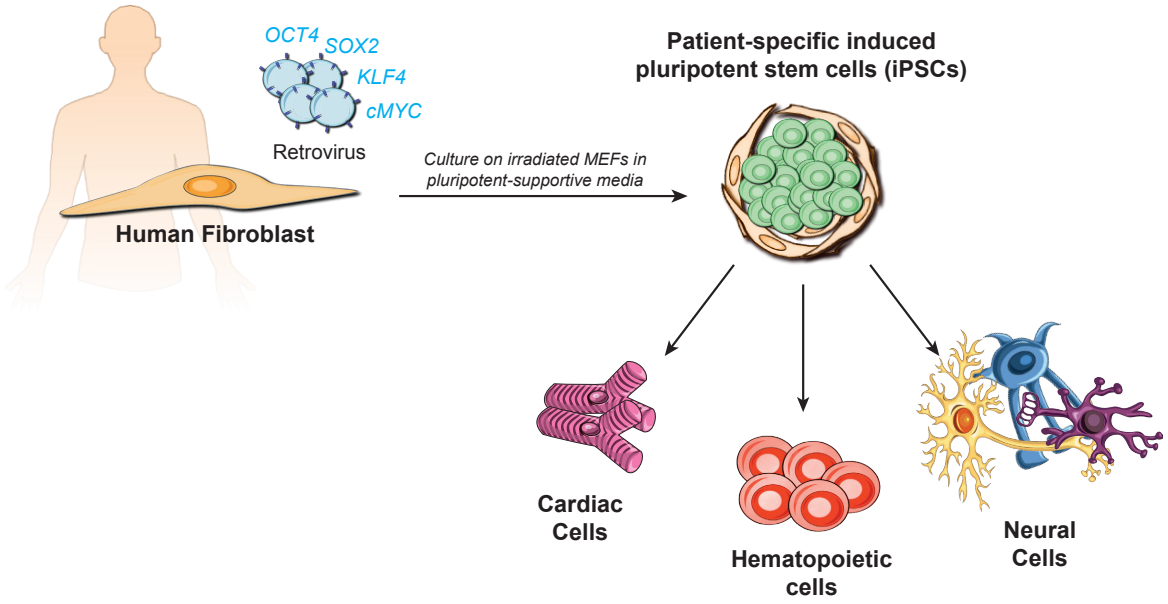
OCT4
SOX2
KLF4
cMYC
Retrovirus
Human Fibroblast
Culture on irradiated MEFs in pluripotent-supportive media
Patient-specific induced pluripotent stem cells (iPSCs)
Cardiac Cells
Hematopoietic cells
Neural Cells

Figure 2. Objective 1. Conventional reprogramming methods will be used to investigate if human AML patient fibroblasts can be reprogrammed to iPSCs and subsequently derived into HPCs that are capable of normal differentiation *in vitro*. FISH probes will be used to investigate whether autologous HPCs are devoid of the leukemia-associated aberration found in the patients' matched leukemic cells.

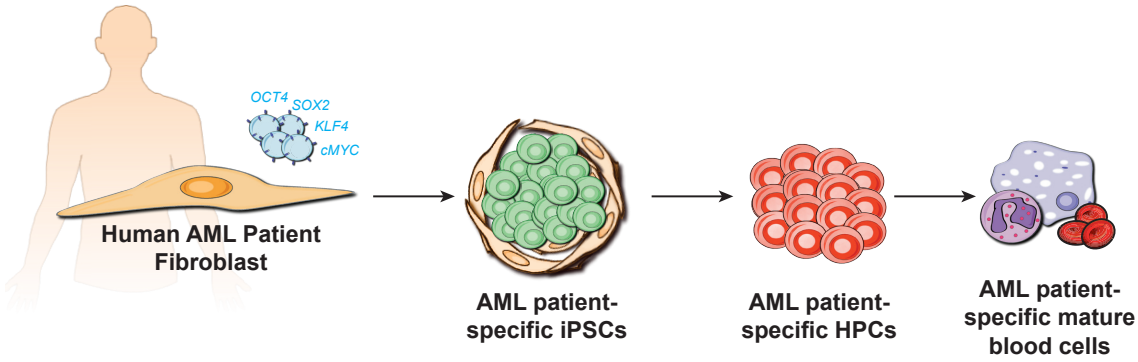
OCT4
SOX2
KLF4
cMYC
Human AML Patient Fibroblast
AML patient-specific iPSCs
AML patient-specific HPCs
AML patient-specific mature blood cells

Figure 3. Objective 2. Conventional reprogramming methods will be used to investigate if human AML patient leukemic cells can be reprogrammed to iPSCs and subsequently derived into dysfunctional HPCs that are incapable of normal differentiation *in vitro* similar to primary leukemic cells. FISH will be performed in iPSCs to confirm their leukemic cell origin based on presence of the leukemia-associated aberration.

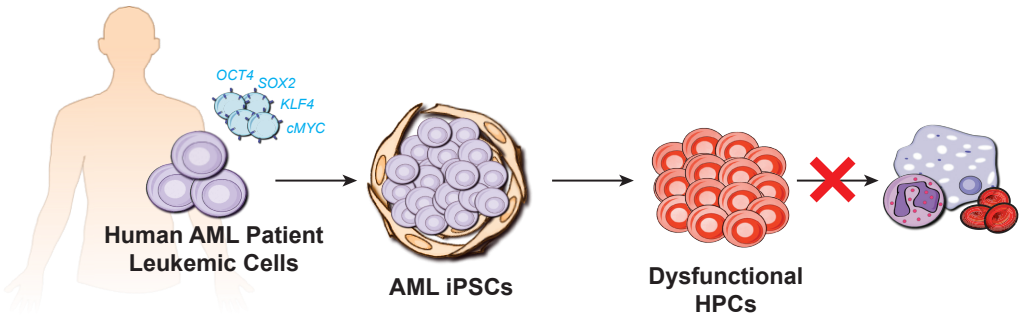
OCT4
SOX2
KLF4
cMYC
Human AML Patient
Leukemic Cells
AML iPSCs
Dysfunctional
HPCs

Table 1. Common recurring leukemia-associated cytogenetic aberrations

Gene Names	Cytogenetic Aberration
CBFß/MYH11	Inversion mutation: inv(16)(p13q22), 5'*CBFß* – 3'*MYH11* fusion gene
DEK/NUP214	Translocation mutation: t(6;9)(p23;q34), 5'*DEK* – 3'*NUP214* fusion gene
MECOM/RUNX1	Translocation mutation: t(3;21)(q26;q22), 5'MECOM – 3'RUNX1 fusion gene
MLL/CREBBP	Translocation mutation: t(11;16)(q23,p13), 5'*MLL* - 3'*CREBBP* fusion gene
MLL/AF9	Translocation mutation: t(9;11)(p22;q23), 5'*MLL* – 3'*AF9* fusion gene
PML/RARα	Translocation mutation: t(15;17)(q24.1;21.1), 5'*PML* – 3'*RARα* fusion gene
RBM15/MKL1	Translocation mutation: t(1;22)(p13;q13), 5'RBM15 – 3'MKL1 fusion gene
RPN1/EVI1	Inversion or translocation mutation: inv(3)/t(3;3)(q21;q26.2), fusion of *RPN1* enhancer upstream of *EVI1* leads to *EVI1* overexpression
RPN1/MEL1	Translocation mutation: t(1;3)(p36;q21), fusion of *RPN1* enhancer upstream of *MEL1* leads to *MEL1* overexpression
RUNX1/RUNX1T1	Translocation mutation: t(8;21)(q22;q22), 5'RUNX1 – 3'RUNX1T1 fusion gene
Chromosomal Deletions	del(5q), del(7q), del(9q), -5, -7
Chromosomal Gains	+8, +21, +22

(Grimwade et al., 1998; Hsiao et al., 2005; Krivtsov et al., 2006; Kundu and Liu, 2001; Mochizuki et al., 2000; Rubin et al., 1990; Sanden et al., 2013; Vardiman et al., 2009; Zhang et al., 2004)

Table 2. Common recurring leukemia-associated molecular aberrations

Gene Name	Mutation(s)
CDC42	Missense mutations (F28L, G12V)
CEBPA	Nonsense mutation (Q87X), insertion mutation (R306-K313 duplication)
DIS3	Missense mutations (D488N, R514K, M667V)
DNAH9	Missense mutation, L1016R
DNMT3A	Missense mutation, R882C
FLT3	Internal tandem duplication (ITD, exon 14), Tyrosine kinase domain (TKD) missense mutation (D835Y, D835V)
IDH1	Missense mutation (R132C)
IDH2	Missense mutation (R172K)
JAK2	Missense mutation (V617F)
KIT	Missense mutation (D816Y)
KRAS	Missense mutation (G12D, G13D)
NPM1	Insertion (956-959), TCTG duplication (exon 12)
NRAS	Missense mutation (G13R, Q61K)
PHF6	Nonsense mutation (R319X), frameshift insertion (P200), missense mutation (H329L)
PTPN11	Missense mutation (G226A, G1508C)
RUNX1	Missense mutation (K83E), Nonsense mutation (Y260X), Frameshift insertion (R135fsX177)
SMC1A	Missense mutation (A078V, R816S)
SMC3	Missense mutation (Y136N, R381Q, K795E)
STAG2	Frameshift insertion (R617), Missense mutation (A733T), nonsense mutation (R1033)
TET2	Missense mutation (N119S, Y592H, I1025L), nonsense mutation (E121X)
WT1	Nonsense mutation (exon 1, 7, 9)

**indicated gene mutations represent most prevalent variant(s)*

(Abdel-Wahab et al., 2009; Beghini et al., 1998; Bos et al., 1987; Ding et al., 2012; Falini et al., 2005; Gaidzik et al., 2012; Ho et al., 2009; Hou et al., 2008; Kandoth et al., 2013; Kayser et al., 2009; King-Underwood et al., 1996; Levine et al., 2005; Ley et al., 2010; Michaud et al., 2002; Patel et al., 2011; Schnittger et al., 2011; Thol et al., 2014; Van Vlierberghe et al., 2011; Vanni et al., 2005; Vardiman et al., 2009; Welch et al., 2012; Yamamoto et al., 2001)

CHAPTER 2

Cellular reprogramming allows generation of autologous hematopoietic progenitors from AML patients that are devoid of patient-specific genomic aberrations

PREAMBLE

This Chapter is an original published article. It is presented in its published format.

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**Co-first authors*

I designed the study, assembled, analyzed and interpreted the data and wrote the manuscript with input from Dr. Jong Hee Lee and my supervisor Dr. Mickie Bhatia. I performed BM, Fib and iPSC culture and immunophenotypic characterization, FISH, CFU assays, blood morphology analyses, and a subset of reprogramming and EB differentiation experiments. Dr. Jong Hee Lee performed reprogramming, iPSC culture and EB differentiation experiments. Sarah Laronde provided technical assistance with iPSC culture and characterization. Steve Dingwall and Rahul Kushwah provided technical assistance with FISH. Aline Fiebig-Comyn performed cell injections for teratoma assays and technical assistance with histological preparations. Drs. Leber and Foley provided AML patient BM samples and provided intellectual contributions to study design. Dr. Arianna Dal Cin provided AML patient skin biopsies. Dr. Mickie Bhatia oversaw the entire study, assisted in manuscript preparation and finalized the manuscript.

This body of work was inspired by the fact that transplantation therapies that have proven effective at reducing disease relapse remain limited by the rare availability of safe sources of HSPCs (Burnett et al., 1998; Hodby and Pamphilon, 2011). Prior to these experiments, the precedent for using iPSCs in autologous cell based therapies had been set (Hanna et al., 2007; Lian et al., 2010). However, it remained to be evaluated whether cellular reprogramming techniques could be used to generate a healthy autologous source of blood cells for AML patients toward addressing the clinical shortage.

Cellular reprogramming allows generation of autologous hematopoietic progenitors from AML patients that are devoid of patient-specific genomic aberrations

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ABSTRACT

Current treatments that utilize hematopoietic progenitor cell (HPC) transplantation in AML patients substantially reduce the risk of relapse, but are limited by the availability of immune compatible healthy HPCs. Although cellular reprogramming has the potential to provide a novel autologous source of HPCs for transplantation, the applicability of this technology toward the derivation of healthy autologous hematopoietic cells devoid of patient-specific leukemic aberrations from AML patients must first be evaluated. Here we report the generation of human AML patient-specific hematopoietic progenitors that are capable of normal *in vitro* differentiation to myeloid lineages and are devoid of leukemia-associated aberration found in matched patient bone marrow. Skin fibroblasts were obtained from AML patients whose leukemic cells possessed a distinct, leukemia-associated aberration, and used to create AML patient-specific induced pluripotent stem cells (iPSCs). Through hematopoietic differentiation of AML patient iPSCs, coupled with cytogenetic interrogation, we reveal that AML patient-specific HPCs possess normal progenitor capacity and are devoid of leukemia-associated mutations. Importantly, in rare patient skin samples that give rise to mosaic fibroblast cultures that continue to carry leukemia-associated mutations; healthy hematopoietic progenitors can also be generated via reprogramming selection. Our findings provide the proof of principle that cellular reprogramming can be applied on a personalized basis to generate healthy HPCs from AML patients, and should further motivate advances toward creating transplantable hematopoietic stem cells for autologous AML therapy.

KEYWORDS

Acute myeloid leukemia, chromosome aberrations, human induced pluripotent stem cells, hematopoietic progenitor cells, reprogramming

ABBREVIATIONS

AML: acute myeloid leukemia; BM: bone marrow; CFU: colony forming unit; FISH: fluorescence *in situ* hybridization; iPSC: induced pluripotent stem cell; AML BM: AML patient-derived bone marrow; AML Fib: AML patient-derived fibroblast

INTRODUCTION

Acute myeloid leukemia (AML) is characterized by the rapid growth of non-functional immature myeloid cells (AML blasts) in the bone marrow (BM) and peripheral blood (PB) of patients, leading to anemia, bleeding, increased risk of infection and ultimately death [1, 2]. Accumulated clinical data has identified recurrent leukemia-associated genomic aberrations in 50-60% of AML patients [3-5], and these mutations are utilized as informative diagnostic and prognostic markers that are useful in managing patient therapy. Current treatments achieve high rates of remission, but subsequent relapse contributes to a reduction to 20-30% of patients who attain disease-free survival [6, 7].

Although hematopoietic progenitor cell (HPC) transplantation during consolidation therapy significantly reduces relapse [8], safe autologous sources of HPCs required for normal hematopoietic recovery are limited, and include concerns of reinfusion of leukemic cells with genomic abnormalities. Unfortunately, current graft purging methods [9] do not alleviate the risk of leukemic cell reinfusion and relapse in autologous BM transplantation settings [10-12]. Alternatively, use of allogeneic blood sources to avoid leukemic abnormalities (BM, mobilized PB, and cord blood (CB))[13] for transplantation in AML patients is restricted by the availability of matched donors, and the long-term complications associated with an inability to separate graft-versus-host disease (GVHD) from the beneficial graft-versus-leukemia (GVL) effect [6, 14, 15]. Furthermore, alternative efforts over the past decades to increase the low numbers of HPCs that can be obtained for the management of a single patient [16] by *ex vivo* expansion have had variable success [13, 17], where recent clinical trials question the benefits of expanded

HPCs [17]. As such, the generation of novel autologous sources of HPCs to circumvent limited availability and complications associated with current transplant sources could benefit patient survival, and thus deserves deeper investigation.

The ability to generate induced pluripotent stem cell (iPSCs) that share phenotypic, molecular, and functional hallmarks with human embryonic stem cells (hESCs) [18-22], provides an opportunity to develop renewable sources of immune-compatible cells. In the context of AML, generation of AML patient-specific HPCs that are devoid of the leukemic aberration(s) that affect the patient's hematopoietic tissue would provide a transformative approach in establishing a healthy autologous blood source for transplantation during AML therapy. Although robust long-term engraftment of PSC-derived HPCs in murine xenografts has not been fully demonstrated [23, 24], incremental advances have been made [25-27]. However, multiple studies have delineated protocols to differentiate human PSCs to HPCs that possess *in vitro* multipotent functionality [28-31]. Independent of advancements required for the generation of transplantable long-term HPCs from hPSCs, the potential of using reprogramming to generate healthy blood cells from an AML patient has yet to be explored and it remains unclear whether generation of AML patient HPCs is even possible. To this end, we obtained dermal fibroblasts from human AML patients whose leukemic cells possessed known leukemia-associated genomic aberration, and used reprogramming technology to generate HPCs. By probing for the absence of this aberration, in conjunction with immunophenotypical, functional, and morphological *in vitro* assessments as compared to the patients' AML blasts, we

provide evidence that derivation of healthy autologous sources of blood using cellular reprogramming is possible.

MATERIALS AND METHODS

Human patient samples

Individual disease cases were assessed to determine patient eligibility based on the following criteria: 1) disease was clinically classified as AML; 2) AML blasts possessed a recurrent leukemia-associated genomic aberration; 3) AML blasts were obtained by BM aspiration; and 4) patient consented to provide one dermal fibroblast skin biopsy. Informed consent was obtained from all sample donors in accordance with Research Ethics Board-approved protocols at McMaster University. Bone marrow aspirates were obtained from consenting leukemic patients at the Juravinski Cancer Center (Hamilton, Canada) as available, and from healthy patients (Lonza, Basel, Switzerland. http://www.lonza.com). Primary bone marrow mononuclear cells were prepared using density gradient centrifugation (20 min, 1500 rpm) in Ficoll-Paque Premium (GE Healthcare Life Sciences, Piscataway, NJ. http://www.gelifesciences.com), and ammonium chloride treatment (Sigma-Aldrich, St. Louis, MO. http://www.sigmaaldrich.com) for 5 min at 4°C. Samples were assessed by flow cytometry for cell surface hematopoietic markers. Dermal skin biopsies (5mm x 5mm) were obtained from the forearm of consenting patients at the Juravinski Cancer Center. Primary human fibroblast cultures were established as described [32], and assessed by flow cytometry.

Human cell culture

Human dermal adult forearm fibroblasts were cultured in Fib media [DMEM with 10% v/v fetal bovine serum (Neonatal Bovine Serum, HyClone, Logan, UT.

http://www.hyclone.com), 1% v/v non-essential amino acid (Gibco, Grand Island, NY. http://www.invitrogen.com), and 1 mM L-glutamine (Gibco)]. Patient-specific iPSCs were derived and cultured on irradiated mouse embryonic fibroblasts (iMEFs) in F12 iPSC media [DMEM/F12 (Gibco) with 20% Knockout Serum Replacement (Gibco), 100 μM β-mercaptoethanol, 100 μM nonessential amino acid (Gibco), 1 mM L-glutamine (Gibco)] supplemented with 10 ng/ml basic fibroblast growth factor (bFGF); F12 iPSC media was not supplemented with antibiotics. iPSC-derived embryoid bodies (EBs) were cultured in hematopoietic differentiation media [KO-DMEM (Gibco) with 20% Knockout Serum Replacement (Gibco), 100 μM β-mercaptoethanol, 100 μM nonessential amino acid (Gibco), 1 mM L-glutamine (Gibco)] supplemented with 50 ng/ml granulocyte colony stimulating factor (Amgen Inc., Thousand Oaks, CA. http://www.amgen.com), 300 ng/ml stem cell factor (Amgen Inc.), 10 ng/ml interleukin-3 (IL-3; R&D systems), 10 ng/ml interleukin-6 (IL-6; R&D systems, Minneapolis, MN. http://www.rndsystems.com), 25 ng/ml bone morphogenetic protein 4 (BMP4; R&D systems), and 300 ng/ml Flt-3 ligand (Flt-3L; R&D systems)].

Patient-specific iPSC generation

Plasmids pSIN4-EF2-O2S and pSIN-EF2-K2M developed by James A. Thomson (University of Madison-Wisconsin) were obtained from Addgene (Cambridge, MA. http://www.addgene.org). Virus containing plasmid was produced from HEK 293FT Cells with 2nd generation pMD2.G and psPAX2 packaging plasmids. Viral supernatants were harvested 72h after transfection and concentrated by ultracentrifugation. Human adult dermal fibroblasts (10^5) were incubated with concentrated lentiviral vectors in Fib

media supplemented with 8 μg/ml polybrene (Sigma-Aldrich) for 48 h, then washed and fed fresh Fib media. 96h after initial lentiviral transduction, Fibs were dissociated and seeded on 150,000 iMEFs and maintained in F12 iPSC media conditions. iPSC colonies emerged between day 16 and 25 post-transduction, and were individually isolated, expanded on iMEFs, and verified for TRA-1-60 expression through live staining using TRA-1-60 DyLight 488 (Stemgent, Cambridge, MA. http://www.stemgent.com). For immunocytochemistry, iPSCs were fixed in 4% paraformaldehyde, permeabilized using BD permeabilization buffer (if required), stained with TRA-1-60, OCT4, SOX2, or NANOG antibodies (BD Biosciences, San Jose, CA. http://www.bdbiosciences.com), and counterstained with 4',6-diamidino-2-phenylindole (DAPI).

Teratoma assay

The developmental potential of human AML Fib iPSCs *in vivo* was assessed by teratoma assay. Briefly, confluent undifferentiated iPSC cultures were treated with 200 U/mL collagenase IV (Invitrogen, Carlsbad, CA. http://www.invitrogen.com) for 2 min at 37°C, scraped into clumps using a 5 mL pipette, collected and centrifuged at 1000 rpm for 10 s, resuspended in 30 uL of media, and injected into the testicle of NOD/SCID mice. One well of a 6 well plate (equivalent to 700,000-900,000 cells, as determined by cell count) was injected per mouse. Teratomas were harvested after 8-10 weeks, sectioned, and stained by hematoxylin and eosin. Images were acquired using ScanScope CS digital slide scanner with Aperio Image Scope software.

Hematopoietic differentiation of iPSCs

EBs were generated by suspension culture as previously described [33]. Briefly, confluent undifferentiated iPSC cultures were treated with 200 U/mL collagenase IV (Invitrogen) for 2 min at 37°C, scraped into clumps using a 5 mL pipette, and transferred to 6- or 12-well ultralow attachment plates (Corning Inc., Corning, NY. http://www.corning.com) to form EBs. EBs were cultured for 15 days in hematopoietic differentiation media with medium changes every 4-5 days, and dissociated into single cell suspensions by 0.4 U/mL collagenase B (Roche Life Science, Indianapolis, IN. http://www.lifescience.roche.com) treatment for 2 h at 37°C. Total single cell suspensions were collected for flow cytometric analysis or CFU plating.

Clonogenic CFU Assay

Clonogenic colony-forming capacities of healthy BM and mobilized PB (10,000-30,000 cells), AML BM mononuclear cells (10,000-30,000 cells), and total dissociated EB cell suspensions (20,000-30,000 cells) plated in Methocult H4434 medium (Stem Cell Technologies, Vancouver, Canada. http://www.stemcell.com) were monitored between days 7-16, and colonies were quantified based on morphology between 14-16 days. Individual colonies were isolated and assessed for single-cell morphology, and full wells were collected for FISH analysis. Depending on number of colonies generated in CFU assay, single-cell morphologies of at least three colonies were analyzed to confirm colony quantification criteria and evaluate the maturity of colonies. Briefly, colonies were isolated and resuspended in 100 uL PBS and spun onto microscope slides using the Shandon Cytospin 3 (Block Scientific, Inc., Bellport, NY.

http://www.blockscientific.com). Morphological features were visualized by Giemsa-Wright staining performed using Shandon Kwik-Diff Stain Kit (Thermo Scientific, Waltham, MA. http://www.thermoscientific.com). Images were acquired using ScanScope CS digital slide scanner with Aperio Image Scope software.

Fluorescence *in situ* hybridization

t(9;11)(p22;q23), del(5)(q13q33), +4, del(16)(q22), and +8 leukemic aberrations were investigated using commercially available, validated FISH probes (Abbott Molecular, Abbott Park, IL. http://www.abbott.com). Cells incubated in 0.075M KCl (37°C, 15 min) were fixed in Carnoy's Solution. Slide preparations and probes were denatured (73°C, 5 min), followed by overnight hybridization in humid 37°C [Locus-specific identifier (LSI) probes] or 42°C [Chromosome enumeration probes (CEP)] incubators. Post-hybridization washes were performed in 0.4x SSC/0.3% NP40, pH 7.0 (73°C, 2 min), followed by 2x SSC/0.1% NP40, pH 7.0 (RT, 1 min), and mounted with DAPI II counterstain (Abbott Molecular). Visualization and analysis was performed using a fluorescence microscope equipped with appropriate filters using MetaMorph software (Molecular Devices, Sunnyvale, CA. http://www.moleculardevices.com). To confirm absence of aberration, $\geq$ 500 nuclei were analyzed. False positive events, detected below threshold of aberration detection established in normal samples, are not depicted in scoring plots.

Flow cytometry

Single cell suspensions were stained using combinations of the following antibodies: CD13-FITC, CD33-PE, CD34-PE, CD45-APC (Miltenyi Biotech, Bergisch Gladbach,

Germany. http://www.miltenyibiotec.com) for hematopoietic phenotoyping; SSEA3-PE and TRA1-60-Alexa Fluor 647 (BD Pharmingen) for live extracellular pluripotent phenotyping; and OCT4-Alexa Fluor 488, SOX2-Alexa Fluor 647 and NANOG-PE (BD Biosciences) for intracellular pluripotent phenotyping of cells fixed and permeabilized using the BD Cytofix/Cytoperm kit. Flow cytometry was performed using the LSRII Flow Cytometer with FACSDiva software (Becton-Dickinson, Franklin Lakes, NJ. http://www.bd.com) and analyzed by FlowJo software (Tree Star, Inc., Ashland, OR. http://www.treestar.com).

Statistical Analysis

Data are presented as mean ± standard error of mean (SEM). Prism software (version 5.0a; GraphPad, La Jolla, CA. http://www.graphpad.com) was used for all statistical analyses, and the criterion for statistical significance was $p < 0.05$.

RESULTS

The majority of AML patient-derived fibroblasts do not share leukemia-associated aberration(s) detected in patient bone marrow

Although a number of known leukemia-associated genomic abnormalities are not shared in non-hematopoietic bone marrow cells derived from leukemic patients [34], this has not been established in dermal skin-derived fibroblasts in culture. To examine this further, dermal skin biopsies were obtained from four human leukemic patients diagnosed with AML carrying the t(9;11)(p22;q23) [35], del(5)(q13q33) [36], trisomy 4 (+4) [37] and del(16)(q22) [38], or trisomy 8 (+8) [39] leukemia-associated aberration(s), respectively (Table 1 and supplemental Fig. 1). These detectable genetic markers enabled us to investigate whether non-hematopoietic dermal skin cells possessed leukemia-associated aberration. Accordingly, AML patient skin fibroblast cultures (AML Fibs) were established from patient skin biopsies [32], with the lack of $CD45^+$ cells indicating the absence of leukemic skin infiltrates (supplemental Fig. 2A-C)[40]. AML Fibs possessed bipolar, elongated morphologies similar to healthy patient-derived Fibs (supplemental Fig. 2B,D-E). Next, we utilized diagnostic fluorescence *in situ* hybridization (FISH) probes (Fig. 1A) to assess early passage AML Fibs in comparison to bone marrow mononuclear cells isolated from each patient (AML BM, supplemental Fig. 2F-G). Analysis of 500 cell nuclei per AML Fib culture revealed that AML Fibs derived from Patient #1 to 3 were completely devoid of the aberration(s) that was readily detected in matched AML BM (Fig. 1B-D). In contrast, the leukemia-associated aberration detected in Patient #4 AML BM (+8) was shared in 8.4% of their AML Fibs (Fig. 1E), indicating

that a genetically mosaic AML Fib culture had been established from the skin biopsy. Taken together, these data indicate that although the majority of human AML Fibs are devoid of AML specific aberration(s), it is possible for them to share aberrations found in the AML BM. Our findings here using human AML Fibs provide further evidence that leukemia-associated aberration can be harbored in non-hematopoietic cells [34].

AML patient-specific iPSCs exhibit functional pluripotency and lack leukemia-associated aberration

It has been previously demonstrated that healthy patient skin-derived Fibs can be reprogrammed to the pluripotent state [18, 19, 22], but this remained to be demonstrated in human AML patients. Toward establishing and characterizing patient-specific iPSC platforms for derivation of hematopoietic cells, we generated iPSCs from AML Patient #1 to 4 Fib cultures using well established reprogramming methods [18, 41]. AML Fib iPSC cultures consisted of flat colonies of densely-packed single cells with large nuclei and scant cytoplasm (Fig. 2A), and were indistinguishable from healthy Fib iPSCs (supplemental Fig. 3A). To assess if AML Fib iPSCs possessed biomolecular hallmarks of pluripotency similar to healthy Fib iPSCs, we performed immunocytochemistry and flow cytometric analyses. Like healthy Fib iPSCs [18], proteins that regulate the core intracellular pluripotency network OCT4, SOX2, and NANOG [42], and extracellular pluripotency markers SSEA3 and TRA1-60 [43] were expressed and localized to AML Fib iPSC colonies (Fig. 2B and supplemental Fig. 3B-C). Next, we subjected AML Fib iPSCs to *in vivo* teratoma formation assays to assess their functional pluripotency capacity. Following intratesticular injection, AML Fib iPSCs demonstrated *in vivo*

pluripotent potential [44] by generating teratomas that possessed early tissue derivatives of the three embryonic germ layers (ectoderm, endoderm, and mesoderm), as evaluated by morphological assessment of hematoxylin and eosin stained tumor sections (Fig. 2C). Together, these results indicate that AML Fibs can be reprogrammed to functional iPSCs that are morphologically, molecularly, and functionally indistinguishable from healthy Fib iPSCs based on standard criteria of human pluripotency [18, 19, 22].

To probe AML Fib iPSCs for patient-specific, leukemia-associated aberration(s), we performed FISH and analyzed 500 nuclei per iPSC line. All patient-specific AML Fib iPSCs were devoid of the abnormality (Fig. 2D) that was detected in matched AML BM (Fig. 1B-E). Interestingly, the +8 aberration harbored in a subpopulation of AML Patient #4 Fibs (Fig. 1E) was not detected in the 500 iPSC nuclei analyzed by FISH (Fig. 2D), suggesting that the aberration was lost during reprogramming [45] or that the reprogramming process favors iPSC generation from genetically normal cells. Together, these data indicate that functional iPSCs devoid of the patient-specific leukemia-associated aberration(s) can be generated from AML Fibs. Moreover, these results demonstrate that the presence of genetic mosaicism in a starting cell population does not affect derivation of genomically normal iPSCs, and is consistent with previous results where reprogramming selects for normal cells [46].

AML Fib iPSC-derived hematopoietic progenitors are devoid of leukemia-associated aberration and exhibit normal differentiation capacity

Since AML Fib iPSCs did not possess leukemia-associated aberration, they represented potential cellular platforms from which to derive healthy hematopoietic cells. Towards

establishing whether AML patient-specific iPSCs possessed the capacity to give rise to normal HPCs, characterized by $CD34^{+}CD45^{+}$ co-expression and functional *in vitro* colony forming unit capacity, we subjected AML Fib iPSCs to an embryoid body (EB) based *in vitro* hematopoietic differentiation assay [31]. Three-dimensional EBs derived from AML Fib iPSC aggregates gave rise to cells co-expressing $CD34^{+}CD45^{+}$ (Fig. 3A-B and supplemental Fig. 4A), similar to healthy Fib iPSC-derived EBs [41]. These results suggest that AML Fib iPSCs possess normal differentiation capacity toward the hematopoietic lineage and, based on $CD34^{+}CD45^{+}$ co-expression, are able to generate putative HPCs. Next, we performed FISH in EB-derived cells to probe for AML patient-specific aberration. Positive events were not detected in 500 cell nuclei analyzed per EB-cell derived population (Fig. 3C), which contrasted that of the patients' AML BM (Fig. 1B-E) and indicated that AML patient-specific putative HPCs did not harbour leukemia-associated aberration. To further evaluate if generation of putative HPCs devoid of leukemia-associated aberration was possible from multiple iPSC lines derived from a single patient, we performed flow cytometric and FISH analyses on EBs derived from additional Patient #4 AML Fib iPSC lines. The use of these iPSC lines, derived from the genetically mosaic AML Fib culture (Fig. 1E), also provided further biological replicates from which to assess if chromosomal abnormalities are lost during the reprogramming process. Consistent with our initial findings, EBs derived from Patient #4 AML Fib iPSC lines gave rise to putative HPCs expressing $CD34^{+}CD45^{+}$ (supplemental Fig. 4B-C), indicating that hematopoietic differentiation potential was not limited to a single iPSC line. Furthermore, the AML BM-specific +8 aberration was not detected (supplemental

Fig. 4D), providing further evidence that genetically normal cells could be derived from a genetically mosaic AML Fib culture. Taken together these data demonstrate that putative HPCs devoid of leukemia-associated aberration can be generated from AML patient-specific iPSCs.

We next assessed and compared the functional capacity of putative HPCs to that of matched AML BM using the *in vitro* colony forming unit (CFU) assay (Fig. 4A) to evaluate if they had normal or leukemic features. First, we characterized the CFU capacity of AML BM to establish baseline criteria for identification of leukemic cells. Despite the inherent diversity and heterogeneity of AML samples [3], Patient #1 to 4 AML BM possessed at least one of the following dysfunctional features suggestive of a leukemic phenotype: impaired CFU capacity characterized by an inability to generate the granulocytic lineage and the presence of persisting single cells [47], presence of cells with immature blast morphology as assessed by clinical standard Giemsa-Wright staining, and/or presence of leukemia-associated aberration as detected by FISH (Table 2, supplemental Fig. 5B,D-F). Importantly, a small population of AML blast progenitors (Patient #4, 1.7%, Fig. 1E) could be detected by CFU and FISH assays (supplemental Fig. 5F), illustrating the sensitivity of leukemic cell detection in the CFU assay. Together these results established criteria for detecting leukemic cells in the CFU assay. On this basis, AML patient-specific putative HPCs were subjected to CFU assay and evaluated for normal versus leukemic capacity. Consistent with healthy Fib iPSC-derived HPCs, all AML patient-specific HPCs exhibited functional capacity to generate multiple myeloid lineages as evidenced by formation of erythroid, granulocytic, and monocytic colonies

(Fig. 4B-C and supplemental Fig. 6A-C). Although frequency of colony types showed variability between patient-specific HPCs, this result is similar to that observed in healthy BM and mobilized PB samples (supplemental Fig. 5A). Next, we assessed individual colonies for normal hematopoietic maturation according to clinically established morphological criteria [48]. Accordingly, Giemsa-Wright staining performed on individual colonies revealed that AML patient-specific HPCs had the capacity to differentiate to mature cells (Fig. 4D and supplemental Fig. 6C), while immature blasts were not detected. Together, these results directly contrasted the dysfunctional differentiation capacity of AML BM and were consistent with results obtained using healthy BM and mobilized PB (Table 2 and supplemental Fig. 5), suggesting that AML patient-specific HPCs possessed normal functional capacity. Next, we performed FISH on total mature hematopoietic colonies to probe for leukemia-associated aberration. Scoring of 500 nuclei per AML patient-specific HPC line revealed that mature hematopoietic colonies derived from HPCs did not possess leukemia-associated aberration(s) that was detected in AML BM CFU (Fig. 4E, Table 2 and supplemental Fig. 5F and 6D). Based on the demonstrated high sensitivity of FISH to detect a small percentage of AML progenitors harboring leukemic aberration (supplemental Fig. 5F), coupled with the rigor of our scoring analyses that exceeded clinical requirements and excluded a 1% chance of genetic mosaicism with a 99% confidence level [49], these results provide substantial evidence that functional, AML patient-specific HPCs are completely devoid of leukemia-associated aberration carried in the patients' own blood cells. Taken together, our data establishes that AML Fib iPSC-derived, functional HPCs ($CD34^{+}CD45^{+}$ co-expression

and the capacity to generate mature cells of multiple myeloid lineages) are devoid of leukemia-associated aberration; directly contrasting features of patients' original leukemic cells.

DISCUSSION

Our current study reveals that cellular reprogramming allows for generation of human AML patient-specific hematopoietic progenitors that are devoid of leukemia-associated aberration and are capable of normal *in vitro* clonogenic differentiation, in direct contrast to matched patient leukemic cells. Given that current sources of healthy blood used for hematopoietic recovery during AML therapy are limited [6, 10-12, 14, 15], we provide initial proof of principle toward generation of novel iPSC-derived, autologous blood sources devoid of leukemia-associated aberration that should enable more AML patients to receive safe transplantations during consolidation therapy and thereby increase the rate of disease-free survival (Fig. 5).

Based on previous work in the human system, the limited capacity of hPSC-derived HPCs to have transplantable hematopoietic stem cell (HSC) properties may be attributed to an inability to activate [24] or downregulate [23] regulatory somatic HSC molecular programs during differentiation [50]. As such, the development of novel differentiation strategies that better specify the hematopoietic lineage from PSCs has been the focus of recent studies aimed at the generation of clinically transplantable HSCs. For instance, temporal inhibition of the early hematopoietic-regulating Hedgehog pathway during *in vitro* differentiation initiates adult hematopoietic gene expression programs [51]. Similarly, *in vivo* differentiation conditions better mimic bone marrow physiology and enable the generation of hematopoietic cells with multilineage reconstitution capacity *in vivo*, perhaps by providing cell extrinsic signals that regulate HSC molecular programs [25, 26]. Finally, forced exogenous expression of HSC-regulating transcription factors

endow PSC-derived $CD34^{+}CD45^{+}$ hematopoietic cells with *in vivo* myeloid lineage reconstitution capacity [27]. Together these recent efforts illustrate incremental advances toward the generation of PSC-derived bonafide HSCs [52]. Our current findings suggest that reprogramming approaches could be utilized to generate healthy, transplantable sources of AML patient-specific HPCs that are capable of restoring normal, short-term myelopoiesis in AML patients to combat anemia, bleeding, and infection due to disease [1, 2] and/or treatment related myeloablation [53]. Moreover, our present study establishes an approach that motivates further effort for the derivation of clinically transplantable HSCs from iPSCs toward circumventing limitations of current hematopoietic sources and enabling long-term hematopoietic recovery in AML patients following therapy.

Given the diversity of AML-associated germline [54, 55] and in utero/adult-acquired somatic mutations [3-5, 56], and the rare presence of these mutations in non-hematopoietic tissues as demonstrated here and previously by Menendez *et al.* [34], it is possible that cases whereby AML Fibs share or independently acquire leukemia-associated aberration will be encountered when our strategy is applied to larger AML patient populations. Recent results indicate that large-scale chromosomal aberrations carried by Fib cultures derived from Miller Dieker Syndrome patients are lost and replaced by wild type duplication during the reprogramming process [45]. Potentially attributed to a similar loss of chromosomal aberration phenomenon during reprogramming, our current findings demonstrate that normal iPSCs can be generated from genetically mosaic AML Fib cultures. This result may also be due to the genetically

normal AML Fib subpopulation preferentially reprogramming to the iPSC state. Independent of which hypothesis is true, our findings suggests that the generation of normal blood progenitors may still be feasible in cases whereby AML Fibs possess large-scale aberrations or heterogeneously harbour leukemia-associated aberration. Furthermore, cellular reprogramming may be a feasible technique toward purging chromosomal abnormalities and generating healthy cell types from cancer or disease patients that carry germline mutations, including AML patients.

It has been postulated that preleukemic mutations may predispose cells to genomic instability and increase their susceptibility to acquiring disease-specific secondary mutations [57, 58] due to enhanced cell survival properties [59]. Recent utilization of next-generation sequencing technologies has enabled identification of preleukemic mutations in HSCs [60, 61], and forms the basis for future delineation of early genetic events that contribute to leukemogenesis. Pending further identification and annotation of these events, deep sequencing should provide further insight into the genomic integrity of patient-specific iPSCs and HPCs. By tracking the absence of leukemia-associated aberration in iPSCs and hematopoietic progenitors/mature cell derivatives, we demonstrate that the AML specific-aberration did not arise at any stage of hematopoietic specification or maturation. This suggests that in addition to being devoid of the AML-specific aberration, AML Fib iPSCs may also be free of preleukemic mutations that predispose them to genetic instability upon *in vitro* hematopoietic differentiation; although, this does not preclude the possibility of long-term genetic instability. We envision that advances in genetic screening and PSC differentiation technologies that

enable next-generation genetic characterization of AML patient-specific HPCs following long-term *in vivo* engraftment in preclinical mouse xenograft models will facilitate further efforts aimed at investigating the long-term safety and genetic stability of these cells prior to clinical application.

Given the potential of PSCs to generate multiple human tissues, cellular reprogramming may also allow for the generation of healthy cell types from other cancer or disease patients requiring transplantation. To date, we are unaware of studies that have utilized aberrations specific to patients' cancer or disease as a marker to interrogate cell populations throughout the reprogramming process toward deriving and characterizing healthy cell types for transplantation purposes, as we have demonstrated here in the context of AML. As such, our study provides the proof of principle to formulate strategies toward developing healthy autologous cellular sources for AML patients, and also for other leukemia or cancer patients whereby distinct aberrations are harbored in the cancerous tissue.

SUMMARY

Generation of AML patient-specific Fib iPSCs establishes a cellular platform from which to derive healthy HPCs that are devoid of leukemia-associated aberration detected in the patients' bone marrow. These autologous HPCs also possess normal *in vitro* differentiation capacity to multiple myeloid lineages as compared to the patients' dysfunctional AML blasts. Our work provides proof of principle that derivation of healthy autologous sources of blood using cellular reprogramming is possible, and should enable more AML patients to receive safe transplantations during therapy toward increasing the rate of disease-free survival.

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AUTHOR CONTRIBUTIONS

K.R.S.: conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing; J.-H.L.: conception and design, collection and assembly of data, and performing reprogramming; S.L., S.D., R.K., and A.F.-C.: collection of data; B.L. and R.F.: conception, provision of study material; A.D.C.: provision of study material; M.B.: conception and design, financial support, data analysis and interpretation, manuscript writing, final approval of manuscript. K.R.S. and J-.H.L contributed equally to this work.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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Figure 1. The majority of AML Fibs are devoid of leukemia-associated aberration. (A) Schematics illustrating patient-specific leukemic aberration(s) identified in AML blast nuclei. FISH probe hybridization regions are indicated (green/red) on affected chromosomes. (B-E) FISH performed in AML patient-derived (i) Fibs and (ii) BM mononuclear cells (n = 1 per AML patient). Aberrations were detected in each patient AML BM, and a population of Patient #4 AML Fibs. Red arrows denote probe separation associated with translocation in Patient #1 AML BM. Adjacent plots depict the frequency of detection of patient-specific, leukemia-associated aberration; blue circles represent number of nuclei analyzed. Blue circles with either one red dot or three green dots represent del(16)(q22) and +4 events in Patient #3 AML BM, respectively; aberrations were never detected in the same nuclei. 500 nuclei were analyzed to exclude 1% genetic mosaicism in AML Patient #1-3 Fibs with 99% confidence [49].

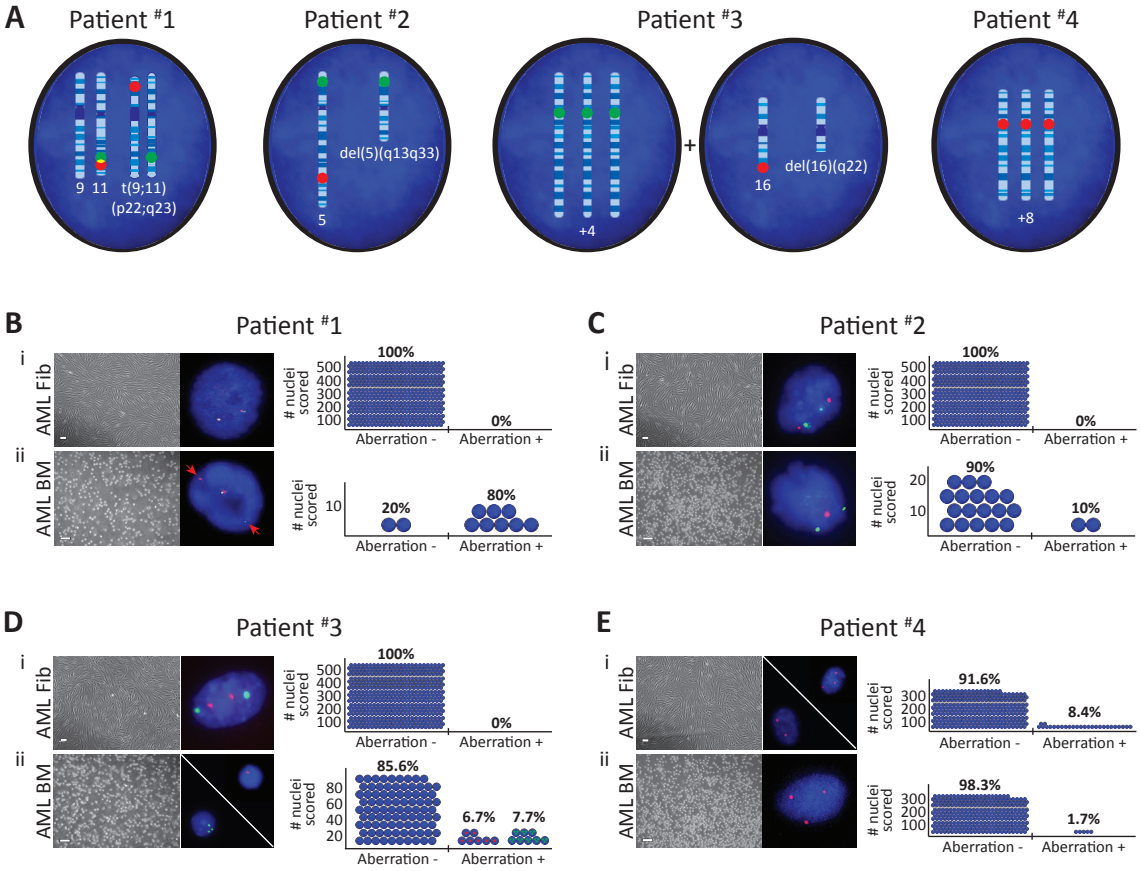

A
Patient #1
Patient #2
Patient #3
Patient #4
9 11 t(9;11) (p22;q23)
del(5)(q13q33)
5
+4
+
del(16)(q22)
16
+8
B
Patient #1
C
Patient #2
D
Patient #3
E
Patient #4
i
ii
AML Fib
AML BM
nuclei scored
Aberration -
Aberration +
100%
0%
20%
80%
90%
10%
85.6%
6.7%
7.7%
91.6%
8.4%
98.3%
1.7%

Figure 2. Characterization of AML patient-specific Fib iPSCs. (A) Representative images of iPSC colonies generated from AML patient Fibs. Highlighted areas are displayed at higher magnification in the right, adjacent images. Scale bars represent 100 μm. (B) Representative immunofluorescence staining of pluripotency markers OCT4, SOX2, NANOG, and TRA-1-60 expressed in AML Fib iPSCs. All pluripotent markers were assessed in 12 total iPSC lines (six iPSC lines from each of Patient #1 and #2), and TRA-1-60 expression was confirmed in at least three iPSC lines derived from each of AML Patient #3 and #4. Scale bar represents 100 μm. (C) Representative teratoma-forming capacity of AML Fib iPSCs. Two independent iPSC lines (one from each of Patient #1 and #2) were subjected to teratoma assay, each in triplicate. AML Fib iPSC-derived teratoma 10-weeks post IT injection (top left). Hematoxylin and eosin stained sections of teratoma sections displaying early tissue derivatives of ectoderm (skin cells), endoderm (gut-like goblet cells) and mesoderm (cartilage). Arrows indicate denoted cell types. (D) FISH performed in patient-specific AML Fib iPSCs (n = 1 iPSC line per AML patient). Aberration identified in matched patient AML BM was not detected. Adjacent plots depict the number of nuclei (blue circle) scored; 500 nuclei were analyzed to exclude 1% genetic mosaicism with 99% confidence [49].

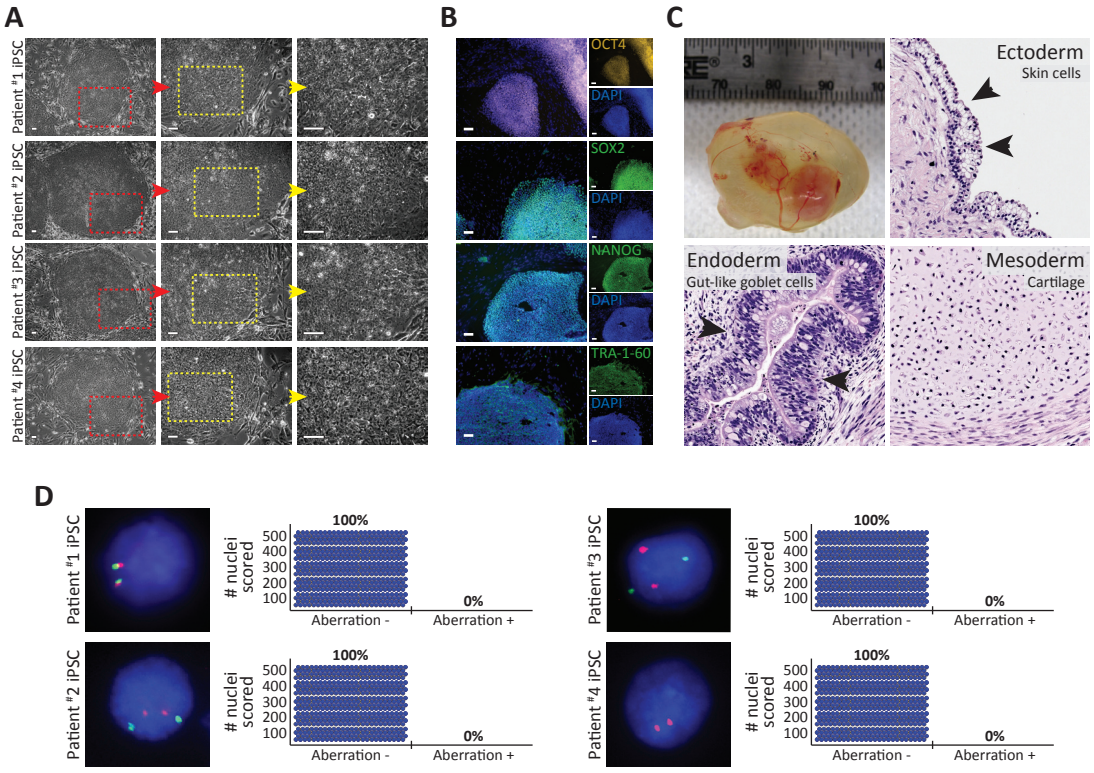

A
Patient #1 iPSC
Patient #2 iPSC
Patient #3 iPSC
Patient #4 iPSC
B
OCT4
DAPI
SOX2
DAPI
NANOG
DAPI
TRA-1-60
DAPI
C
Ectoderm
Skin cells
Endoderm
Gut-like goblet cells
Mesoderm
Cartilage
D
Patient #1 iPSC
Patient #2 iPSC
Patient #3 iPSC
Patient #4 iPSC
nuclei scored
500
400
300
200
100
100%
0%
Aberration -
Aberration +

Figure 3. AML patient-specific putative HPCs are devoid of leukemia-associated aberration. (A) Representative EBs derived from AML patient Fib iPSCs, in hematopoietic differentiation conditions. Scale bar represents 100 μm. (B) Representative plots of flow cytometric analyses used to detect the generation and presence of $CD34^{+}CD45^{+}$ putative hematopoietic progenitors. Flow cytometric analysis for $CD34^{+}CD45^{+}$ expression was performed on a minimum of three independent hematopoietic differentiation experiments for each indicated iPSC line. Percentages represent frequency of total live cells with indicated cell surface phenotype. (C) FISH performed in patient-specific, EB-derived cells from one iPSC line per AML patient. Aberration identified in matched patient AML BM was not detected. Adjacent plots depict the number of nuclei (blue circle) scored; 500 nuclei were analyzed to exclude 1% genetic mosaicism with 99% confidence [49].

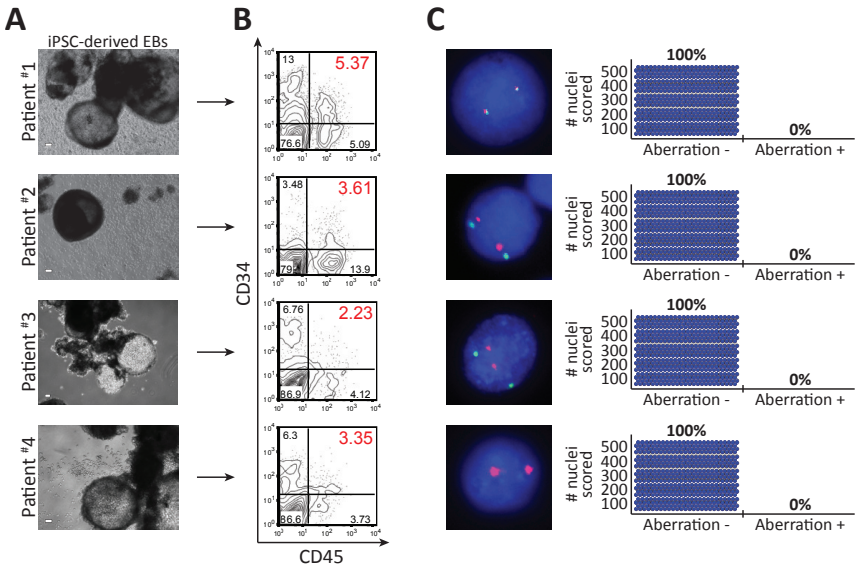

A
iPSC-derived EBs
Patient #1
Patient #2
Patient #3
Patient #4
B
CD34
CD45
13
5.37
76.6
5.09
3.48
3.61
79.4
13.9
6.76
2.23
86.9
4.12
6.3
3.35
86.6
3.73
C
nuclei scored
100%
500
400
300
200
100
0%
Aberration -
Aberration +

Figure 4. AML patient-specific HPCs are capable of normal *in vitro* differentiation to mature blood cells and are devoid of leukemia-associated aberration. (A) Experimental strategy used to characterize patient-specific, putative HPCs *in vitro*. Methodologies used to assess normal hematopoietic functional capacity are indicated in red. (B) Putative HPC functionality assessed by multilineage differentiation capacity in *in vitro* CFU assay. Bars represent mean frequencies of mature hematopoietic colonies generated + SEM (n=3 independent experiments per patient-specific HPC line). AML patient Fib iPSC-derived HPCs generate all mature lineages, consistent with healthy patient Fib iPSC-derived HPCs. (C) Representative mature hematopoietic colonies derived from patient-specific HPCs. Scale bars represent 100 μm. (D) Representative single-cell morphologies following Giemsa-Wright staining performed on individual hematopoietic colonies ($n \geq 3$ colonies analyzed per patient-specific HPC line). Scale bars represent 10 μm. (E) FISH performed in total mature hematopoietic colonies derived from patient-specific HPCs. Aberration identified in matched patient AML BM was not detected. Adjacent plots depict the number of nuclei (blue circle) scored; 500 nuclei were analyzed to exclude 1% genetic mosaicism with 99% confidence [49].

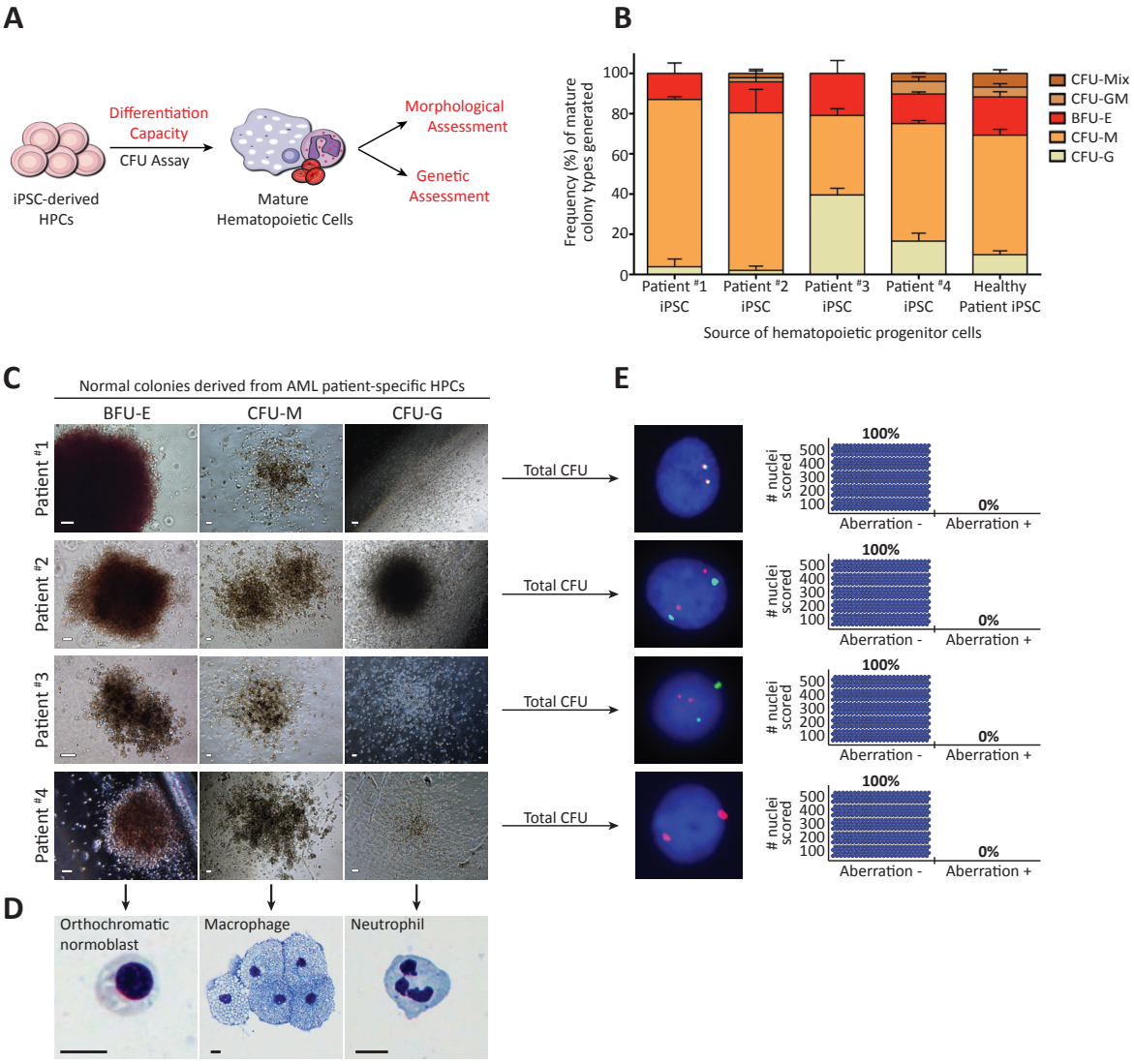

A
Differentiation Capacity
CFU Assay
iPSC-derived HPCs
Mature Hematopoietic Cells
Morphological Assessment
Genetic Assessment
B
Frequency (%) of mature colony types generated
CFU-Mix
CFU-GM
BFU-E
CFU-M
CFU-G
Patient #1 iPSC
Patient #2 iPSC
Patient #3 iPSC
Patient #4 iPSC
Healthy Patient iPSC
Source of hematopoietic progenitor cells
C
Normal colonies derived from AML patient-specific HPCs
BFU-E
CFU-M
CFU-G
Patient #1
Patient #2
Patient #3
Patient #4
D
Orthochromatic normoblast
Macrophage
Neutrophil
Total CFU
E
nuclei scored
100%
0%
Aberration -
Aberration +

Figure 5. Utilization of AML-specific genetic markers to interrogate cell populations throughout reprogramming towards generation of healthy blood cells for transplantation. (A) Experimental strategy developed here to generate and characterize AML patient-specific HPCs that are capable of normal *in vitro* differentiation to the myeloid lineage and are devoid of leukemia-associated aberration found in matched patient BM. As represented by dashed arrow, technological advances in cellular reprogramming may provide novel autologous blood sources for transplantation that circumvent limitations associated with current transplantation options used during AML therapy (denoted in red font). FISH results associated with presence (+) vs. absence (-) of leukemia-associated aberration are indicated above cell populations.

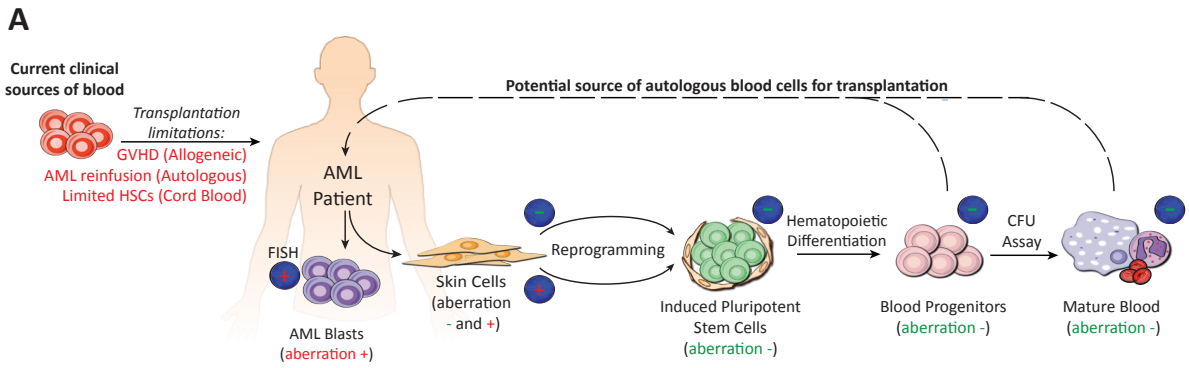

A
Current clinical sources of blood
Transplantation limitations:
GVHD (Allogeneic)
AML reinfusion (Autologous)
Limited HSCs (Cord Blood)
Potential source of autologous blood cells for transplantation
AML Patient
FISH
AML Blasts (aberration +)
Skin Cells (aberration - and +)
Reprogramming
Induced Pluripotent Stem Cells (aberration -)
Hematopoietic Differentiation
Blood Progenitors (aberration -)
CFU Assay
Mature Blood (aberration -)

Table 1. Clinical disease classification of enrolled patients and leukemia-associated aberration(s) detected in their AML blast cells

	Disease Classification	Aberration
Patient 1	AML M5, Monocytic	t(9;11)(p22;q23)
Patient 2	AML, NOS	del(5)(q13q33)
Patient 3	AML M4, Myelomonocytic	+4 and del(16)(q22)*
Patient 4	MDS ⟶ AML	+8

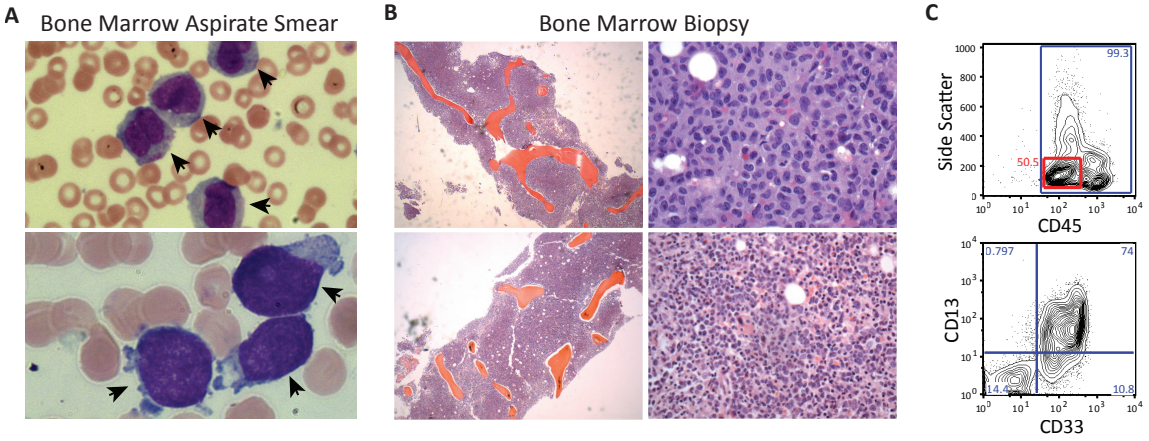
Abbreviations: NOS, not otherwise specified; MDS --> AML, MDS progressed to AML
**Two distinct aberrations detected, but not observed in the same nuclei*

Table 2. Characterizations of CFU assays performed using indicated source of blood cells

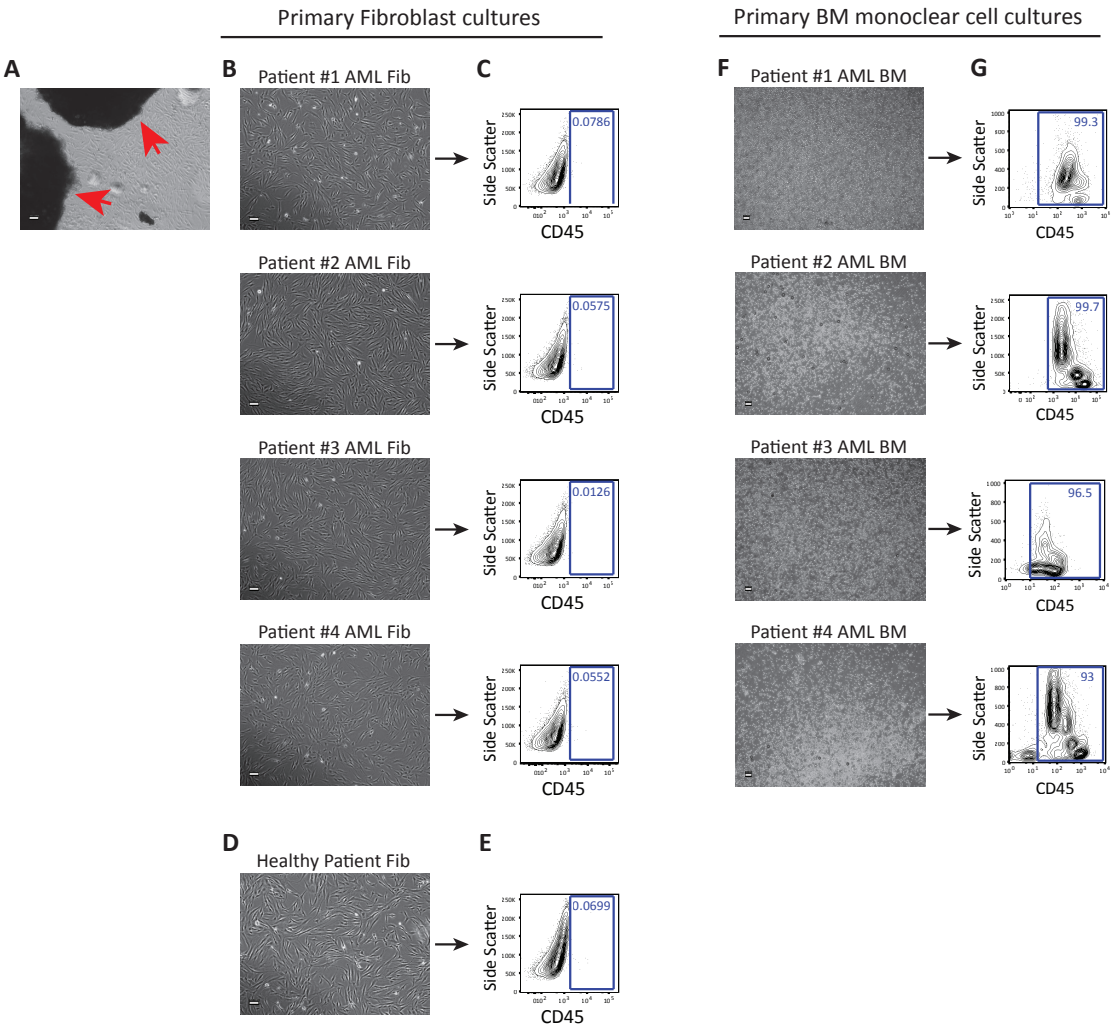
	Healthy Patient		Patient #1		Patient #2		Patient #3		Patient #4	
	BM	HPC	BM	HPC	BM	HPC	BM	HPC	BM	HPC
Impaired differentiation capacity?	No	No	Yes	No	No	No	Yes	No	No	No
Immature single cell morphology detected?	No	N/T	*	No	Yes	No	No	No	No	No
Leukemia-associated aberration detected?	N/A	N/A	*	No	Yes	No	Yes	No	Yes	No

Abbreviations: HPC, iPSC-derived hematopoietic progenitor cells; N/A, not applicable; N/T, not tested
Findings that contrast healthy BM output and are consistent with a leukemic phenotype are indicated in red
**CFU capacity insufficient for further analysis*

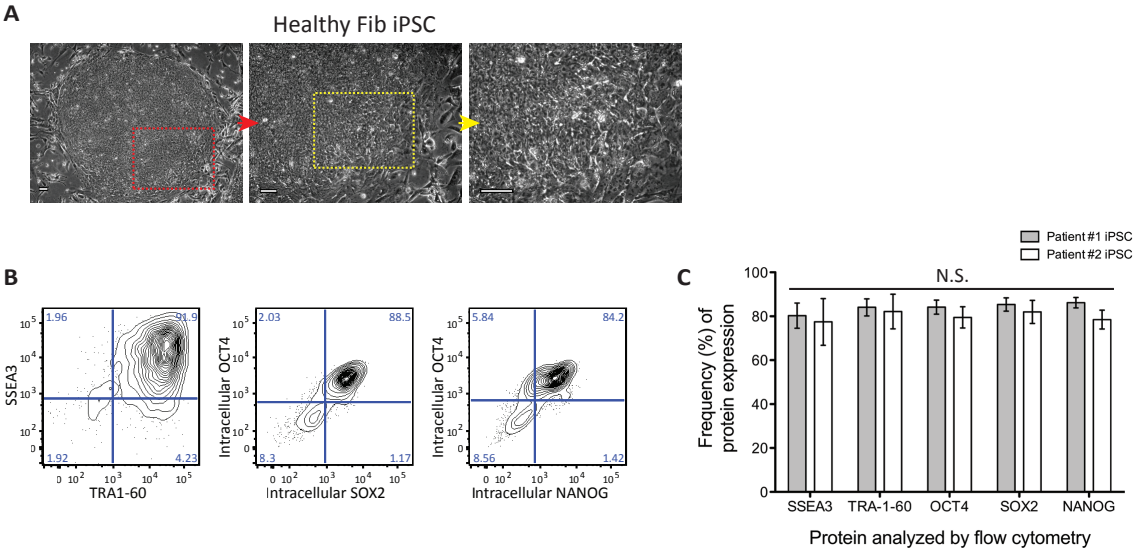
Supplemental Figure 1. Clinical diagnosis of patients with AML possessing a distinct leukemia-associated aberration. (A) Representative AML patient BM aspirate smears performed at clinical diagnosis reveal the presence of immature AML blasts (black arrows). (B) Representative AML patient BM biopsies reveal abnormal hypercellularity of the bone marrow compartment. (C) Representative flow cytometric analysis of an AML patient's diagnostic BM depicts an abnormally large population of immature blasts characterized by low CD45/low SSC (red rectangle), a subset of the $CD45^+$ hematopoietic BM population; and a high frequency of $CD13^+CD33^+$ myeloid cells.


A
Bone Marrow Aspirate Smear
B
Bone Marrow Biopsy
C
Side Scatter
CD45
99.3
50.5
CD13
CD33
0.797
74
14.4
10.8

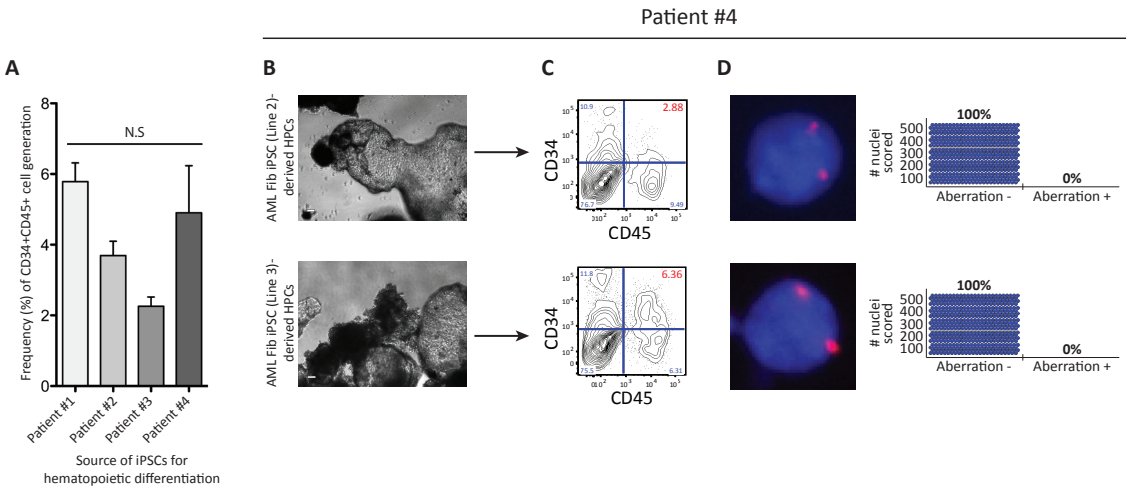
Supplemental Figure 2. Establishing primary cultures. (A) Establishing fibroblast cultures from AML patient skin biopsies. Representative image of two sections of a 5mm x 5mm AML patient skin biopsy (denoted by red arrows) in Fib media giving rise to adherent fibroblasts. Scale bar represents 100 μm. (B) Representative images of AML Fib cultures established from each patient's skin biopsy. Scale bars represent 100 μm. (C) Flow cytometric analyses of AML Fib cultures confirm the absence of $CD45^+$ hematopoietic cells. (D) Fibroblast culture established from healthy patient skin biopsy. Scale bar represents 100 μm. (E) Flow cytometric analysis of healthy Fib culture confirms the absence of $CD45^+$ hematopoietic cells. (F) Mononuclear cells prepared from each AML patient BM aspirate. Scale bars represent 100 μm. (G) Flow cytometric analyses of AML BM assessing expression of pan-hematopoietic marker CD45. Percentages represent frequency of total live cells with indicated cell surface phenotype.


Primary Fibroblast cultures
Primary BM monoclear cell cultures
A
B
C
D
E
F
G
Patient #1 AML Fib
Patient #2 AML Fib
Patient #3 AML Fib
Patient #4 AML Fib
Healthy Patient Fib
Patient #1 AML BM
Patient #2 AML BM
Patient #3 AML BM
Patient #4 AML BM
Side Scatter
CD45
0.0786
0.0575
0.0126
0.0552
0.0699
99.3
99.7
96.5
93

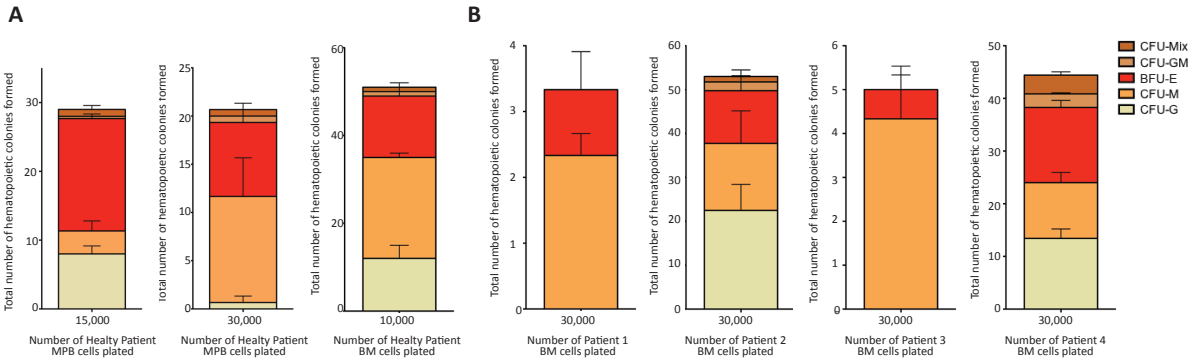
Supplemental Figure 3. Additional iPSC characterization. (A) Representative images of iPSCs generated from healthy patient-derived fibroblasts. Highlighted areas are displayed at a higher magnification in the right adjacent image. Scale bars represent 100 μm. (B) Representative flow cytometric plots assessing pluripotency marker expression on a population of generated AML Fib iPSCs. Percentages represent frequency of total live cells with indicated cell surface phenotype. (C) Frequency of pluripotency marker expression in AML Patient #1 and #2 iPSC cultures as determined by flow cytometric analyses. Flow cytometric analysis was performed in 12 independent iPSC lines (six iPSC lines from each of Patient #1 and Patient #2). Bars represent mean $\pm$ SEM, n=6. Variation between pluripotent markers across all iPSC lines is not significant as analyzed by two-way ANOVA.

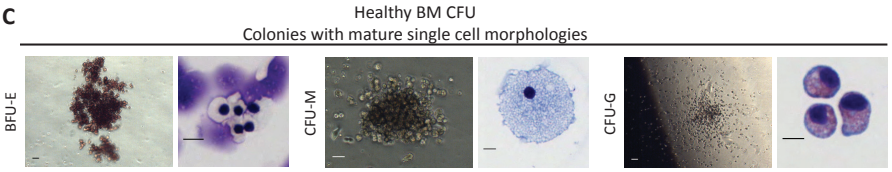

A
Healthy Fib iPSC
B
SSEA3
TRA1-60
1.96
21.9
1.92
4.23
Intracellular OCT4
Intracellular SOX2
2.03
88.5
8.3
1.17
Intracellular OCT4
Intracellular NANOG
5.84
84.2
8.56
1.42
C
Patient #1 iPSC
Patient #2 iPSC
N.S.
Frequency (%) of protein expression
100
80
60
40
20
0
SSEA3
TRA-1-60
OCT4
SOX2
NANOG
Protein analyzed by flow cytometry

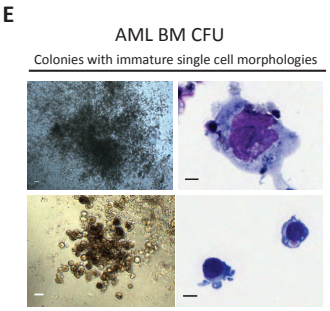
Supplemental Figure 4. Multiple lines of Patient #4 AML Fib iPSCs give rise to HPCs devoid of leukemic aberration. (A) Bar graph quantifying frequency of $CD34^+CD45^+$ derivation from indicated AML patient iPSC lines. Bars represent mean of $\geq$ 3 independent differentiation experiments $\pm$ SEM. Variance is not significant as analyzed by one-way ANOVA. (B) Representative EBs derived from additional AML Patient #4 Fib iPSC lines, in hematopoietic differentiation conditions. Scale bar represents 100 μm. (C) Representative examples of flow cytometric analyses used to detect the generation and presence of $CD34^+CD45^+$ hematopoietic cells. Percentages represent frequency of total live cells with indicated cell surface phenotype. Flow cytometric analysis of $CD34^+CD45^+$ expression was performed on EBs derived from the indicated iPSC lines in three independent experiments. (D) FISH performed in indicated Patient #4-specific, EB-derived cells. Aberration identified in matched patient AML BM was not detected. Adjacent plots depict the number of nuclei (blue circle) scored; 500 nuclei were analyzed to exclude 1% genetic mosaicism in AML Patient #1-3 Fibs with 99% confidence [49].

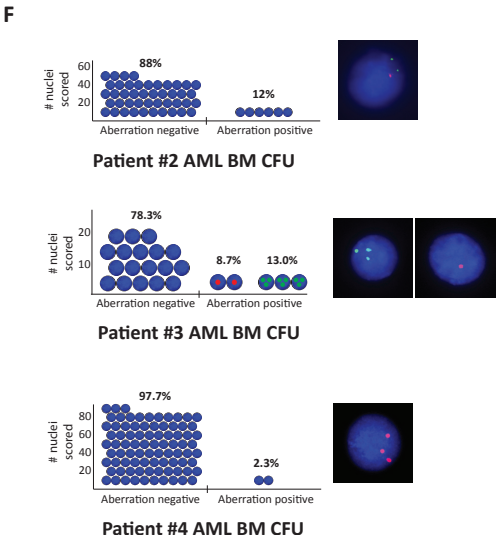

Patient #4
A
B
C
D
N.S
Frequency (%) of CD34+CD45+ cell generation
Patient #1
Patient #2
Patient #3
Patient #4
Source of iPSCs for hematopoietic differentiation
AML Fib iPSC (Line 2)-derived HPCs
AML Fib iPSC (Line 3)-derived HPCs
CD34
CD45
2.88
6.36
nuclei scored
100%
0%
Aberration -
Aberration +

Supplemental Figure 5. Identification of dysfunctional features suggestive of leukemic phenotypes through evaluation of CFU capacity, single-cell morphology, and genetic analysis. Total number of hematopoietic colony types formed from plating (A) healthy BM and mobilized PB, and (B) AML BM. Bars represent mean of three independent experiments + SEM. (C) Representative images of isolated colonies derived from healthy BM, and their associated single-cell morphologies visualized by Giemsa-Wright. Scale bars for colonies represent 100 μm, scale bars for single cell morphologies represent 10 μm. (D) Representative image of persistent single cells observed in Patient #1 and #3 BM CFU. (E) Representative images of isolated colonies derived from AML BM, and their associated immature single-cell morphologies visualized by Giemsa-Wright. Scale bars for colonies represent 100 μm, scale bars for single cell morphologies represent 10 μm. (F) FISH performed in total CFUs derived from Patient #2-4 AML BM. Aberrations were detected in each AML BM CFU. Adjacent plots depict the frequency of detection of patient-specific leukemic aberration; blue circles represent number of nuclei analyzed.

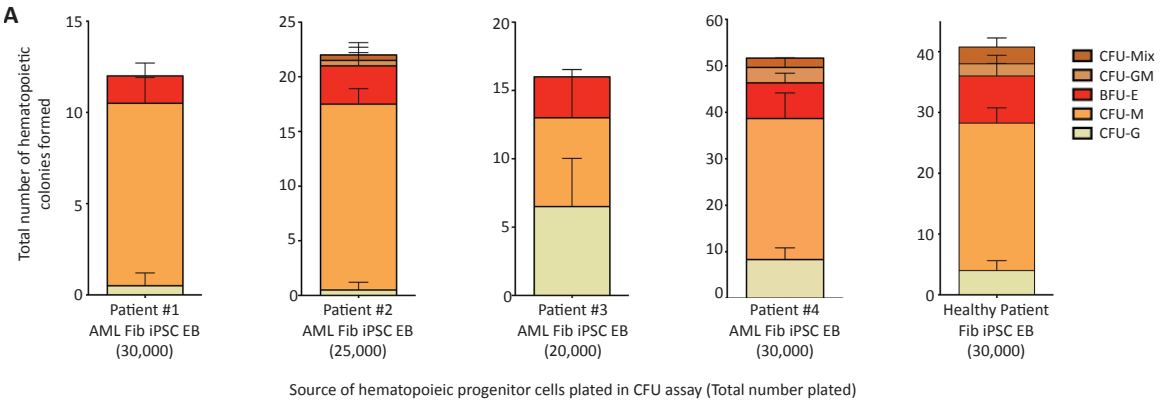

A
B
Total number of hematopoietic colonies formed
Number of Healty Patient MPB cells plated
Number of Healty Patient BM cells plated
Number of Patient 1 BM cells plated
Number of Patient 2 BM cells plated
Number of Patient 3 BM cells plated
Number of Patient 4 BM cells plated
CFU-Mix
CFU-GM
BFU-E
CFU-M
CFU-G


C
Healthy BM CFU
Colonies with mature single cell morphologies
BFU-E
CFU-M
CFU-G
D

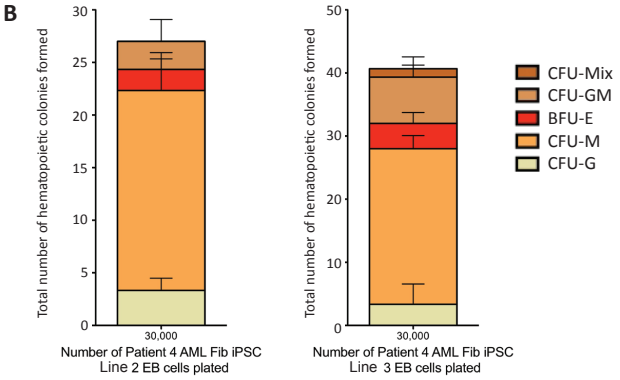

E
AML BM CFU
Colonies with immature single cell morphologies

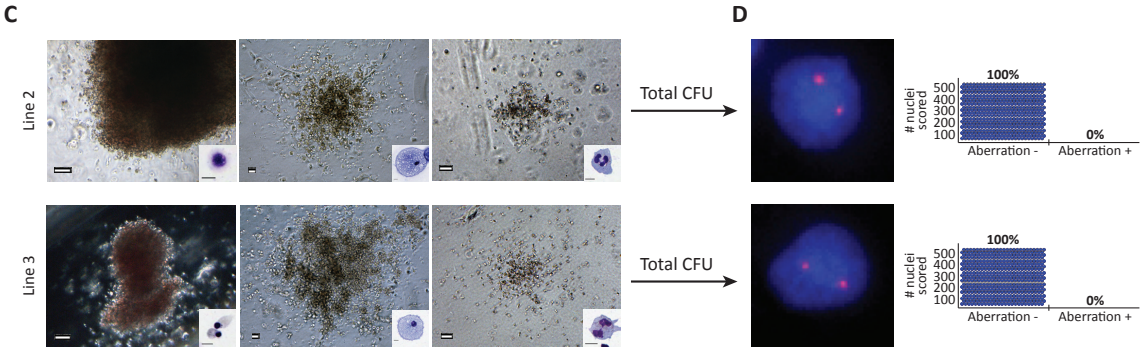

F
nuclei scored
88%
12%
Aberration negative
Aberration positive
Patient #2 AML BM CFU
78.3%
8.7%
13.0%
Patient #3 AML BM CFU
97.7%
2.3%
Patient #4 AML BM CFU

Supplemental Figure 6. Multiple lines of Patient #4 AML Fib iPSC-derived HPCs do not possess leukemic features *in vitro*. (A) Bar graphs depicting total number of hematopoietic colonies generated from plating patient-specific HPCs in clonogenic CFU assay. Bars represent mean number of total mature hematopoietic colonies generated + SEM (n=3 independent experiments per patient-specific HPC line). (B) Evaluation of additional Patient #4 putative HPC functionality by quantification of differentiated colony types in *in vitro* CFU assay. Bars represent mean frequencies of mature hematopoietic colonies generated + SEM, n=3. AML Patient #4 Fib iPSC-derived progenitors generate all mature lineages. (C) Representative mature hematopoietic colonies derived from Patient #4-specific HPCs. Colonies possess normal single cell morphologies (inset). White scale bars represent 100 μm, black scale bars represent 10 μm. (D) FISH analysis performed in total CFUs generated from Patient #4-specific HPCs. FISH analysis reveals lack of leukemia-associated aberration, with adjacent plots depicting the number of nuclei (blue circle) scored.



Patient #4 AML Fib iPSC-derived hematopoietic progenitors evaluated by CFU Assay





CHAPTER 3

Acquisition of pluripotency through continued environmental influence on OCT4-induced plastic human fibroblasts

PREAMBLE

This Chapter is an original published article. It is presented in its published format.

"This research was originally published in Stem Cell Research. Salci KR, Lee JB, Mitchell RR, Orlando O, Fiebig-Comyn A, Shapovalova Z, and Bhatia M. Acquisition of pluripotency through continued environmental influence on OCT4-induced plastic human fibroblasts. 2015 Jul;15(1):221-30. doi: 10.1016/j.scr.2015.06.006. ©2015 Elsevier B.V."

I performed fibroblast and iPSC culture, lentiviral transduction and reprogramming, flow cytometry analyses, EB differentiation, genomic PCR, RT-qPCR and CFU assays. I also analyzed and interpreted data, assembled figures and wrote the manuscript. Dr. JB Lee assisted with initial cell culture and differentiation experiments and performed immunocytochemistry staining. Ryan Mitchell provided intellectual contributions and contributed to the writing of the manuscript. Dr. Luca Orlando performed RT-qPCR experiments for the OCT4 transgene. Aline Fiebig-Comyn provided technical assistance for intra-testicular injections of iPSCs for teratoma assay, harvested tumours and prepared tumor sections for morphological analyses. Zoya Shapovalova provided technical assistance for analyses of aCGH data. Dr. Mick Bhatia oversaw the entire study, provided intellectual contributions and finalized the manuscript.

This body of work was performed in parallel to the works described in **Chapter 2**. At that time my fellow graduate student Ryan Mitchell was working to better understand

the role of OCT4 in facilitating cell fate reprogramming to the hematopoietic and neural lineages without traversing pluripotency (Mitchell et al., 2014b; Szabo et al., 2010). He (correctly) hypothesized that OCT4 expression induced a state of cellular plasticity that allowed cells to respond to environmental cues toward conversion to specified lineages without traversing pluripotency (Mitchell et al., 2014a). Although transcriptional and functional hallmarks of pluripotency were never observed during the time frame required for direct conversion events to take place, I hypothesized that continued exposure of OCT4-plastic fibroblasts to pluripotent-supportive conditions would lead to the acquisition of pluripotency; and that this may represent a less convoluted reprogramming method in which to generate iPSCs from AML Fibs. Ultimately this hypothesis was correct as I observed the emergence of iPSC colonies between 45 and 93 days after initial OCT4 transduction throughout my experiments. Looking back I was fortunate that iPSCs were generated after 46 days of culture during my first experiments, as I would have likely given up on my cultures after two months and concluded that my hypothesis was incorrect. Although these findings represent a less convoluted approach for iPSC generation from normal and AML Fibs, the prolonged time required to generate iPSCs using this method does not currently represent a clinically-feasible approach. However, this study complemented previous findings from our lab (Mitchell et al., 2014a; Mitchell et al., 2014b; Szabo et al., 2010) to further demonstrate that OCT4 is a powerful tool for cell fate reprogramming, and ultimately redefine Yamanaka's conventional methods for pluripotency reprogramming (Takahashi et al., 2007; Takahashi and Yamanaka, 2006).

Acquisition of pluripotency through continued environmental influence on OCT4-induced plastic human fibroblasts

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ABSTRACT

The combination of OCT4 expression and short-term exposure to reprogramming media induces a state of transcriptional plasticity in human fibroblasts, capable of responding to changes in the extracellular environment that facilitate direct cell fate conversion towards lineage specific progenitors. Here we reveal that continued exposure of OCT4-induced plastic human fibroblasts to reprogramming media (RM) is sufficient to induce pluripotency. OCT4-derived induced pluripotent stem cell ($iPSC^{OCT4}$) colonies emerged after prolonged culture in RM, and formed independently of lineage specific progenitors. Human $iPSC^{OCT4}$ are morphologically indistinguishable from conventionally derived iPSCs and express core proteins involved in maintenance of pluripotency. $iPSC^{OCT4}$ display *in vivo* functional pluripotency as measured by teratoma formation consisting of the three germ layers, and are capable of targeted *in vitro* differentiation. Our study indicates that acquisition of pluripotency is one of multiple cell fate choices that can be facilitated through environmental stimulation of OCT4-induced plasticity, and suggests the role of other reprogramming factors to induce pluripotency can be substituted by prolonged culture of plastic fibroblasts.

HIGHLIGHTS

- Environmental conditions influence OCT4-induced plastic Fibs toward pluripotency
- OCT4-derived iPSCs possess biomolecular and functional hallmarks of pluripotency
- OCT4-derived iPSC colonies form independently of lineage specific progenitors
- Acquisition of pluripotency is one of multiple cell fate choices from plastic Fibs

KEYWORDS

Plasticity, reprogramming, OCT4, induced pluripotent stem cell, pluripotency, teratoma

ABBREVIATIONS

CFU: colony forming unit; hFib, human fibroblast; iPSC, induced pluripotent stem cell; MEFCM, mouse embryonic fibroblast conditioned medium; OSKM, OCT4/SOX2/KLF4/c-MYC; OSNL, OCT4/SOX2/NANOG/LIN28; RM, reprogramming media

INTRODUCTION

Foundational cell fate reprogramming studies revealed ectopic expression of pluripotency-associated transcription factors (TF) OCT4, SOX2, KLF4, and c-MYC (OSKM) or OCT4, SOX2, NANOG, LIN28 (OSNL) in human fibroblasts (hFibs) cultured in pluripotent supportive conditions was sufficient for generation of induced pluripotent stem cells (iPSCs) possessing phenotypic, molecular, and functional characteristics akin to embryonic stem cells (ESCs) (Takahashi et al., 2007; Yu et al., 2007). A multitude of reprogramming cocktails and methodologies have since been demonstrated to yield iPSCs from a range of adult cell types (Theunissen & Jaenisch, 2014). Early modifications revealed ectopic expression of SKM could be functionally replaced by expression of structural homologs (Nakagawa et al., 2008) or chemical compounds (Y. Li et al., 2011; Zhu et al., 2010), and altogether bypassed if they were endogenously expressed in starting cell types (Eminli, Utikal, Arnold, Jaenisch, & Hochedlinger, 2008; Giorgetti et al., 2009; J. B. Kim et al., 2009). More recently, groups have identified that Nr5a2 (Heng et al., 2010), E-cadherin (Redmer et al., 2011) and Gata3 (Montserrat et al., 2013; Shu et al., 2013) are capable of substituting for Oct4 when expressed with SKM; while others have replaced OS with Sall4 and Esrrb in combination with NL (Buganim et al., 2014). Furthermore, complete replacement of ectopic expression of OSKM has been achieved using miR302/367 expression in combination with Hdac2 suppression (Anokye-Danso et al., 2011), and a combination of seven small-molecule compounds in the mouse system (Hou et al., 2013). However, despite these advances, a substitute that definitively replaces Oct4's functional activity and activates its

gene targets in the absence of other reprogramming factors has not been identified. As such, OCT4's activity is indispensible for induction and maintenance of pluripotency (Orkin et al., 2008; Sterneckert, Hoing, & Scholer, 2012). Further supporting this concept, ectopic expression of high performance Oct4-VP16 transactivation domain fusion protein in mouse embryonic fibroblasts (MEF) is the only example of single factor reprogramming to achieve germline competent iPSCs without the addition of small molecules, miRNA, or ectopic/endogenous support by additional pluripotent factors (Y. Wang et al., 2011).

Using similar cellular reprogramming principles, two paradigms exist toward achieving cell fate conversion. One approach relies on forced expression of lineage-specific TFs to facilitate lineage conversions (Lujan, Chanda, Ahlenius, Sudhof, & Wernig, 2012; Vierbuchen et al., 2010). The alternative focuses on inducing an unstable or plastic cell state, demarcated by activation of multiple lineage specific gene expression programs, that is capable of responding to environmental cues (R. Mitchell et al., 2014; Orkin & Hochedlinger, 2011). Strategies using short-term exposure to OSKM (Efe et al., 2011; J. Kim et al., 2011), OCT4 with small molecule substitutes of SKM (H. Wang et al., 2014; Zhu et al., 2014), and OCT4 alone (R. Mitchell et al., 2014; R. R. Mitchell et al., 2014; Szabo et al., 2010) in combination with reprogramming media (RM) have been employed towards achieving a cell state that responds to environmental cues. However, these approaches are molecularly distinct as the addition of pluripotency factors SKM convolutes plasticity induction by up-regulating early development and pluripotency genes (Maza et al., 2014; R. Mitchell et al., 2014), indicating that OCT4 alone in

combination with short-term exposure to RM is minimally sufficient to induce plasticity (R. Mitchell et al., 2014). Accordingly, OCT4-induced plastic cells are capable of responding to environmental instruction toward derivation of neural and hematopoietic progenitors without traversing pluripotency (R. R. Mitchell et al., 2014; Szabo et al., 2010). Although molecular/functional evidence indicates that transcriptional hallmarks of iPSCs are not observed in OCT4 plastic cells during short-term exposure to RM, whether continued culture in conditions known to support pluripotency is sufficient to up-regulate these programs remains to be elucidated.

Given that OCT4 activity and RM is minimally required to induce plasticity, and that this combination is also indispensible in the multitude of methodologies described above to achieve pluripotency reprogramming, we asked whether OCT4-induced plasticity was sufficient for pluripotency acquisition. To this end, we induced plasticity in human fibroblasts using OCT4 and short-term exposure to RM. Plastic fibroblasts were subjected to continued maintenance in pluripotent-supportive reprogramming media towards further influencing pluripotency acquisition. Using a combination of morphological, molecular, immunophenotypic, and functional assessments, we provide evidence that our deconvoluted OCT4-induced plasticity approach in combination with continued exposure to pluripotent supportive conditions is sufficient for inducing bona fide pluripotency in adult human fibroblasts.

MATERIALS AND METHODS

Human PSC and primary cell culture

Human dermal adult forearm biopsies (5x5 mm) were obtained from consenting donors in accordance with Research Ethics Board-approved protocols at McMaster University; primary cultures were established as described (Villegas & McPhaul, 2005). Primary human fibroblasts derived from breast dermal tissue were obtained from ScienCell Research Laboratories. All hFibs were cultured in hFib media [DMEM with 10% v/v fetal bovine serum (Neonatal Bovine Serum, HyClone), 1% v/v non-essential amino acid (NEAA; Gibco), and 1 mM L-glutamine (Gibco)].

$iPSC^{OCT4}$ were derived on Matrigel (BD Biosciences) in reprogramming media (RM) [DMEM/F12 (Gibco) with 20% Knockout Serum Replacement (Gibco), 100 μM β-mercaptoethanol, 100 μM nonessential amino acid (Gibco), 1 mM L-glutamine (Gibco) supplemented with 16 ng/ml basic fibroblast growth factor (bFGF) and 30 ng/mL insulin-like growth factor (IGFII)]; and adapted to and cultured in mouse embryonic fibroblast conditioned media (MEFCM) supplemented with 10 ng/ml bFGF to stabilize pluripotency and enable colony expansion. $iPSC^{OSNL}$ were cultured on Matrigel (BD Biosciences) in MEFCM supplemented with 10 ng/ml bFGF. Cells were fed with fresh medium daily and confluent cultures were mechanically or enzymatically passaged every 5-7 days using 1 mg/ml Collagenase IV (Invitrogen).

Preparation of lentiviral vectors

pSIN-EF1α-OCT4-Puro plasmid developed by James A. Thomson (University of Madison-Wisconsin) and PL-SIN-EF1α-eGFP-Puro developed by James Ellis (University

of Toronto) were obtained from Addgene. Virus containing plasmid was produced from HEK 293FT Cells with 2nd generation pMD2.G and psPAX2 packaging plasmids. Viral supernatant was harvested 72h after transfection and concentrated by ultracentrifugation.

Lentiviral transduction of primary human fibroblasts

Human adult dermal fibroblasts (10^4 adherent cells per well of a 12-well plate seeded on Matrigel the day before transduction) were incubated for 24h with concentrated OCT4 or eGFP lentiviral vector in 0.5 mL hFib medium in the presence of 8 μg/ml polybrene (Sigma Aldrich). After 24h of incubation, 2 mL of RM was added to the well. Following a further 24h of incubation, lentiviral transduction conditions were replaced entirely with RM. Cells were maintained in this condition for prolonged culture with media changes and removal of areas of overgrown fibroblasts as necessary. iPSC colonies emerged between days 45 and 93, and were mechanically isolated and further expanded in MEFCM conditions.

Teratoma Formation

To assess the development potential of iPSCs *in vivo,* iPSCs were collected by collagenase IV treatment and injected into the left testicle of NOD/SCID mice (approximately one well of a 6 well plate of 80% confluence for each mouse). At eight to ten weeks, teratomas were harvested, dissected and fixed with 4% paraformaldehyde. Samples were embedded in paraffin and processed with hematoxylin and eosin staining.

hEB formation and hematopoietic differentiation

Human embryoid bodies (hEBs) were generated by suspension culture as previously described (Hong, Werbowetski-Ogilvie, Ramos-Mejia, Lee, & Bhatia, 2010). Medium

was changed with the hEB differentiation medium supplemented with hematopoietic growth factors (hGFs) as follows: 50 ng/ml granulocyte colony stimulating factor (G-CSF; Amgen, Inc., Thousand Oaks, CA, USA), 300 ng/ml stem cell factor (SCF; Amgen, Inc.), 10 ng/ml interleukin-3 (IL-3; R&D systems, Minneapolis, MN, USA), 10 ng/ml interleukin-6 (IL-6; R&D systems), 25 ng/ml BMP4 (R&D systems), and 300 ng/ml Flt-3 ligand (Flt-3L: R&D systems). hEBs were cultured for 16 days and medium was changed every three days. hEBs were enzymatically digested using Collagenase B, and single cells were collected. 10,000 cells were plated in Methocult H4434 medium. Colony forming units were identified and quantified on day 16.

Flow cytometry

Human Fibs and iPSCs were dissociated to single cell suspensions using TrypLE Express. Cells were stained using the following antibodies: CD34-PE or -FITC (Miltenyi Biotech, Bergisch Gladbach, Germany), CD45-APC (Miltenyi Biotech) for hematopoietic EB differentiation experiments. For pluripotent analysis, cells were analyzed for the presence of SSEA3-PE and TRA-1-60-Alexa Fluor 647 (BD Pharmingen) for live cell surface markers. For detection of intracellular pluripotent transcription factors cells were fixed and permeabilized using the BD Cytofix/Cytoperm kit. Cells were incubated overnight at 4°C with conjugated antibodies OCT4-Alexa Fluor 488, SOX2-Alexa Fluor 647 and NANOG-PE (BD Pharmingen). Flow cytometric analysis was performed using the BD LSRII Flow Cytometer with BD FACSDiva software and analyzed with FlowJo software (Tree Star Inc). Fluorescence activated cell sorting (FACS) was performed using BD FACSAria II cell sorter.

Real-time quantitative reverse transcription polymerase chain reaction

Total RNA was extracted from Fib, Fib^{OCT4}, whole iPSC cultures and FACS-isolated TRA-1-60^{+} iPSCs using the RNeasy kit (Qiagen), and cDNA was generated using qScript cDNA SuperMix (Quanta BioSciences). Oligonucleotides were designed for the detection of *OCT4* (total, endogenous and exogenous), *SOX2*, *NANOG* and *GAPDH* (**Table S1**). RT-qPCR was performed on the BioRad CFX96 for 40 cycles for all marker genes. Relative gene expression was calculated using the ΔΔCt method. Endogenous *GAPDH* housekeeping gene was used for normalization.

Immunocytochemistry

Cells were washed once with 1xPBS containing Calcium and Magnesium (Lonza) then fixed with 2% paraformaldehyde using the BD Cytofix fixation buffer. Cells were stained with SSEA3 or TRA-1-60 antibodies (BD Biosciences), and counterstained with DAPI.

PCR for provirus integration

Genomic DNA was extracted from 5x10^{5} hFibs and iPSC^{OCT4} using the Qiagen DNeasy Blood & Tissue Kit. To demonstrate the presence or absence of transgenes in iPSC^{OCT4} and parent Fibs, primers specific to each transgene and IRES sequence were used to amplify the provirus. Primer sets were designed specifically to amplify *OCT4, SOX2, LIN28, NANOG, KLF4,* and *cMYC* provirus. pSIN4-EF2-O2S, pSIN4-EF2-N2L, and pSIN-EF2-K2M plasmids were used for positive controls. Primer sequences are indicated in **Table S2**. PCR reactions were carried out using Thermo Maxima Hot Start Taq DNA Pol. Initial denaturation at 95C (4min) was followed by 35 cycles of denaturation at 95C

(30s), annealing at 60C (30s), and elongation at 72C (50s), followed by a final elongation at 72C (5 min).

DNA fingerprinting

To confirm the dermal and breast fibroblast origins of iPSC^{OCT4} lines, restriction fragment length polymorphism (RFLP) analysis was performed in the Centre for Applied Genomics Genetic Analysis Facility at SickKids Hospital (Toronto).

Array comparative genomic hybridization

Array comparative genomic hybridization (aCGH) was performed by the Princess Margaret Genomic Centre (University Health Network, Toronto) using an Agilent human genome CGH 4x44K microarray with CY3/CY5-labelled genomic DNA isolated from TRA-1-60^{+} iPSC^{OCT4} and sex-matched reference DNA. Data was analyzed on Partek Genomics Suite (v6.6) using the genome copy number segmentation algorithm on log2 ratios converted to copy number, with parameters set to: 15 minimum genomic markers, 0.001 P-value threshold, 0.5 signal to noise ratio, and diploid copy number range from 1.5 to 2.5.

RESULTS

Continued exposure of plastic human fibroblasts to pluripotent supportive conditions is sufficient for the generation of iPSCs

Plastic human fibroblasts (hFibs) generated through ectopic expression of OCT4 and short-term exposure to reprogramming media (RM) are capable of responding to lineage-specific culture conditions towards direct conversion to hematopoietic and neural progenitor cells without first initiating or establishing pluripotency (R. R. Mitchell et al., 2014; Szabo et al., 2010). Despite the lack of evidence that OCT4 plastic cells induce early transcriptional hallmarks of iPSCs, we asked whether pluripotency could be acquired through continued culturing of OCT4 plastic cells in pluripotent supportive conditions. Accordingly, hFibs were transduced with OCT4 ($hFib^{OCT4}$) and cultured in RM to induce plasticity (**Figs. S1A and B**) (R. Mitchell et al., 2014). Consistent with our previous findings, $hFib^{OCT4}$ morphologies transitioned from bipolar-elongated to compact-cuboidal, and this transition was dependent on the combination of OCT4 and RM (**Fig. S1C**), suggesting that plasticity had been induced (R. Mitchell et al., 2014). In addition to predictive morphological features, total populations of plastic $hFib^{OCT4}$ have been identified by their acquisition of lineage specific gene expression profiles (R. Mitchell et al., 2014) and by expression of lineage-specific proteins (R. Mitchell et al., 2014; Szabo et al., 2010). For example, expression of the pan-hematopoietic marker CD45 (Woodford-Thomas & Thomas, 1993) identifies a subset of plastic cells in RM that are capable of responding to hematopoietic culture conditions and undergoing direct conversion to the hematopoietic lineage (R. Mitchell et al., 2014; Szabo et al., 2010). To further corroborate

our morphological assessment that plasticity had been achieved in our current study, we performed flow cytometric analyses on total cell cultures at Day 24 (**Fig. 1A**) using CD45 expression as a marker of plasticity induction. Flow cytometric analyses revealed CD45 expression on a subpopulation of cells (**Fig. 1B**), further indicating that plasticity had been achieved. Moreover, we confirmed that plastic $hFib^{OCT4}$ were devoid of pluripotent markers SSEA3 and TRA-1-60 at this time (**Fig. 1B**) (Takahashi *et al*., 2007). Having confirmed that OCT4 plasticity had been induced, we continued to culture Fib^{OCT4} in RM, also known to support pluripotency, and monitored these cultures for the emergence of iPSC-like cells. After continued maintenance in RM ranging from 45 to 93 days, flat dense colonies of cells with large nuclei and scant cytoplasm resembling iPSCs were observed (**Figs. 1C, S1D and E)**. Flow cytometric and immunocytochemistry analyses revealed that these colonies expressed pluripotent markers SSEA3 and TRA-1-60 (**Figs. 1D and E**). In contrast, we did not detect the emergence of TRA-1-60^{+} colonies from mechanically isolated cultures of $CD45^{+}$ plastic fibs cultured in RM for the same period of time (**Figs. S2A and B**), suggesting that $CD45^{+}$ plastic fibs were not a reprogramming intermediate on the path to iPSCs (Szabo et al., 2010). Next, we attempted to expand $SSEA3^{+}$TRA-1-60^{+} colonies through clonal isolation and maintenance in RM. However, these conditions were not conducive to expansion, resulting in cultures that did not meet our morphological standards for PSC cultures (similar to **Fig. 1F**) (Bendall et al., 2007; Lee et al., 2013). In an effort to assess if pluripotency could be stabilized within these cells toward facilitating clonal expansion in the absence of mouse embryonic fibroblasts (MEF), we passed Fib^{OCT4}-derived $SSEA3^{+}$TRA-1-60^{+} colonies evenly into either RM or

MEF conditioned medium (MEFCM) supplemented with bFGF (Bendall et al., 2007). Culture in MEFCM resulted in stabilization of pluripotency and enabled colony expansion within two passages as evidenced by restoration of standard PSC culture morphology and a significant increase in frequency of $SSEA3^+$ and $TRA\text{-}1\text{-}60^+$ cells as compared to the culture concurrently maintained in RM **(Figs. 1F-J**). Given their morphological features and robust expression of pluripotency-associated markers, we termed these cells $iPSC^{OCT4}$.

In an effort to ensure that $iPSC^{OCT4}$ colony generation was attributed to ectopic expression of OCT4 we generated restriction fragment length polymorphism (RFLP) signatures of $iPSC^{OCT4}$ and compared them to that of the starting hFib populations (**Figs. 1K and S1G**) confirming that $iPSC^{OCT4}$ generation was not a result of culture cross contamination with existing PSC lines from our lab. Furthermore, polymerase chain reaction (PCR) analysis performed on genomic DNA isolated from $iPSC^{OCT4}$ colonies revealed the exclusive presence of OCT4 provirus integration (**Fig. 1L**), indicating that $iPSC^{OCT4}$ were derived from $hFib^{OCT4}$. To confirm that the formation of iPSC colonies was directly correlated to ectopic expression of OCT4 alone we used flow cytometric analysis to assess pluripotent marker expression in non-transduced, parent hFibs. These analyses revealed no expression of endogenous OCT4, SOX2, or NANOG (**Fig. 1M**), indicating that $iPSC^{OCT4}$ generation was attributed to OCT4-induced plasticity induction, and not due to OCT4 expression in combination with endogenous pluripotent TF expression as previously demonstrated in neural progenitors (J. B. Kim et al., 2009).

Together, these data confirm that iPSC^{OCT4} are derived from plastic OCT4-expressing adult human fibroblasts.

iPSC^{OCT4} possess biomolecular, immunophenotypic and functional characteristics similar to iPSC^{OSNL}

We next sought to further validate the pluripotent features of iPSC^{OCT4} through comparison to iPSC generated previously in our lab using a multi-factor approach (iPSC^{OSNL}) (Hong, Lee, Lee, Ji, & Bhatia, 2011). To assess the expression of core pluripotency factors OCT4, SOX2, and NANOG (Orkin et al., 2008), cultures of iPSC^{OCT4} and iPSC^{OSNL} with comparable confluence and undifferentiated cell content (**Figs. 2A**) were collected for gene expression and flow cytometric analyses. Endogenous expression of OCT4, as well as activation of endogenous SOX2 and NANOG transcripts, was observed in both iPSC lines (**Fig. 2B**); demonstrating that exogenous OCT4-mediated reprogramming is capable of activating pluripotent markers similar to multi-factor approaches used for pluripotent reprogramming (Chan et al., 2009). Furthermore, in three iPSC^{OCT4} lines derived from three separate human donors, OCT4 transgene expression was decreased relative to Fib^{OCT4} and provided minimal contribution to total OCT4 expression similar to that observed in iPSC^{OSNL} (Yu et al., 2007) and iPSC^{OSKM} (Chan et al., 2009; Takahashi et al., 2007) (**Fig. 2C**). At the protein level, flow cytometric analyses revealed that activated expression of OCT4, SOX2, and NANOG, and expression of SSEA3 and TRA-1-60 cell surface pluripotent markers was similar between iPSC^{OCT4} vs iPSC^{OSNL} cultures maintained at comparable undifferentiated colony densities (**Fig. 2A and D**); this protein-level pluripotent marker expression was also

observed in lower colony-density cultures of additional iPSC^{OCT4} lines derived from independent hFib sources (**Fig. S1F**). Together, these molecular and phenotypic characteristics possessed by three independent iPSC^{OCT4} lines are consistent with "type III" bona fide iPSCs (Chan et al., 2009). Next, we used array comparative genomic hybridization to assess the karyotype of three independent iPSC^{OCT4} lines, at a genomic resolution beyond that obtained using standard cytogenetic procedures (Elliott, Elliott, & Kammesheidt, 2010; Martins-Taylor et al., 2011). One iPSC^{OCT4} line was devoid of copy number variation (**Fig. 2E**), while the others possessed two and five amplifications, the majority of which are found in regions/chromosomes that are recurrently altered in cultured hPSCs (**Fig. S3A**)(Baker et al., 2007; Martins-Taylor et al., 2011; Taapken et al., 2011). While these results indicate that iPSC^{OCT4} reprogramming is not dependent on the presence or acquisition of genetic abnormalities to initiate pluripotency and is similar to OSKM and OSNL pluripotent reprogramming (Martins-Taylor et al., 2011; Takahashi et al., 2007; Yu et al., 2007), it is possible that the incidence of genetic abnormalities may be increased due to prolonged culture required for pluripotency acquisition using OCT4 alone. Together these results demonstrate that iPSC^{OCT4} possess the biomolecular hallmarks of pluripotency similar to iPSCs generated using a multi-factor reprogramming approach.

The most stringent test for evaluating pluripotency of human cells is the *in vivo* teratoma formation assay (Thomson *et al*., 1998). To evaluate whether activation of the core pluripotent network conferred functional pluripotent differentiation capacity in iPSC^{OCT4}, we assessed their ability to generate teratomas consisting of tissue derivatives

from the three germ layers (ectoderm, endoderm, and mesoderm) in immune deficient mice. Cultures of $iPSC^{OCT4}$ and $iPSC^{OSNL}$, with comparable confluence and undifferentiated cell content based on morphological observation and SSEA3 expression (**Figs. S3B and C**), were prepared for intratesticular injection. Consistent with bona fide PSC potential, both $iPSC^{OCT4}$ and $iPSC^{OSNL}$ formed teratomas comprised of all three germ layers (**Figs. 2F and S3D and E**) indicating that $iPSC^{OCT4}$ are functionally pluripotent *in vivo*.

In addition to *in vivo* differentiation potential, we further investigated if $iPSC^{OCT4}$ were capable of responding to targeted *in vitro* differentiation conditions. Based on our established competencies in hematopoietic differentiation of PSCs (Chadwick et al., 2003; Hong et al., 2010), we vigorously assessed the *in vitro* hematopoietic capacity of $iPSC^{OCT4}$. Using an embryoid body (EB) based differentiation assay (**Fig. 2G)**(Chadwick et al., 2003), cultures of $iPSC^{OCT4}$and $iPSC^{OSNL}$ were assessed for their ability to generate $CD34^{+}CD45^{+}$ hematopoietic progenitors. Both $iPSC^{OCT4}$ and $iPSC^{OSNL}$ underwent differentiation towards the hematopoietic lineage, generating similar frequencies of $CD34^{+}CD45^{+}$ putative hematopoietic progenitors **(Figs. 2H and I)**. To further characterize the resulting putative hematopoietic progenitors and evaluate whether they shared similar functional capacity to differentiate into mature hematopoietic cells we subjected them to colony forming unit (CFU) assays. The frequency of $CD34^{+}CD45^{+}$ progenitors with CFU potential was similar between blood progenitors derived from $iPSC^{OCT4}$ and $iPSC^{OSNL}$ (**Fig. 2J**). Moreover, both $iPSC^{OCT4}$ and $iPSC^{OSNL}$ derived progenitors displayed multilineage differentiation into all of the mature hematopoietic cell

types read out by the CFU assay (**Fig. 2K**), which was further confirmed by Giemsa-Wright assessment of single cell morphologies (**Fig. 2L and M**). Taken together these results confirm that like $iPSC^{OSNL}$, $iPSC^{OCT4}$ are capable of responding to targeted differentiation cues resulting in the generation of mature tissue types *in vitro*, further validating this unique source of pluripotent stem cells.

DISCUSSION

Here we show that continued (albeit prolonged) culture of plastic hFib^{OCT4} in RM media is sufficient for induction of pluripotency. Importantly, iPSC^{OCT4} possess morphological, biomolecular, and functional hallmarks of bona fide PSCs. Building on our previous works using OCT4 to induce a state of plasticity required for cell fate conversion (R. Mitchell et al., 2014; R. R. Mitchell et al., 2014; Szabo et al., 2010), our current findings highlight that pluripotency represents another cell fate choice for plastic human fibroblasts (**Fig. 3**) and provides further example that cell fate alteration through plasticity induction can be achieved without additional TFs (Efe et al., 2011; J. Kim et al., 2011; J. Li et al., 2013) or small molecules (H. Wang et al., 2014; Zhu et al., 2014).

Despite the myriad of methodologies to induce pluripotency in human somatic cells (Anokye-Danso et al., 2011; Buganim et al., 2014; Eminli et al., 2008; Giorgetti et al., 2009; Heng et al., 2010; Hou et al., 2013; J. B. Kim et al., 2009; Y. Li et al., 2011; Montserrat et al., 2013; Nakagawa et al., 2008; Redmer et al., 2011; Shu et al., 2013; Takahashi et al., 2007; Theunissen & Jaenisch, 2014; Yu et al., 2007; Zhu et al., 2010), exogenous delivery or endogenous activation of OCT4 is indispensible for reprogramming to pluripotency. Furthermore, we are unaware of any report suggesting that iPSCs can be generated without the use of pluripotent-supportive reprogramming media. As we have demonstrated that pluripotency is another destination of plastic human fibroblasts, we suggest that conventional reprogramming to pluripotency (Takahashi et al., 2007; Yu et al., 2007) relies on the combination of OCT4-induced plasticity and pluripotent media instruction, and is expedited or further specified by the addition of

pluripotent-specifying TFs (SKM, SNL). We also propose that this could explain why pluripotency is transiently acquired during OSKM-mediated "transdifferentiation" (Maza et al., 2014). Given the reduced complexity of our reprogramming system, we feel OCT4-induced plasticity represents an ideal model to elucidate the governing mechanisms that allow for alteration of cell fate upon manipulation of native transcriptional programs towards both lineage specific progenitors and pluripotent stem cells alike.

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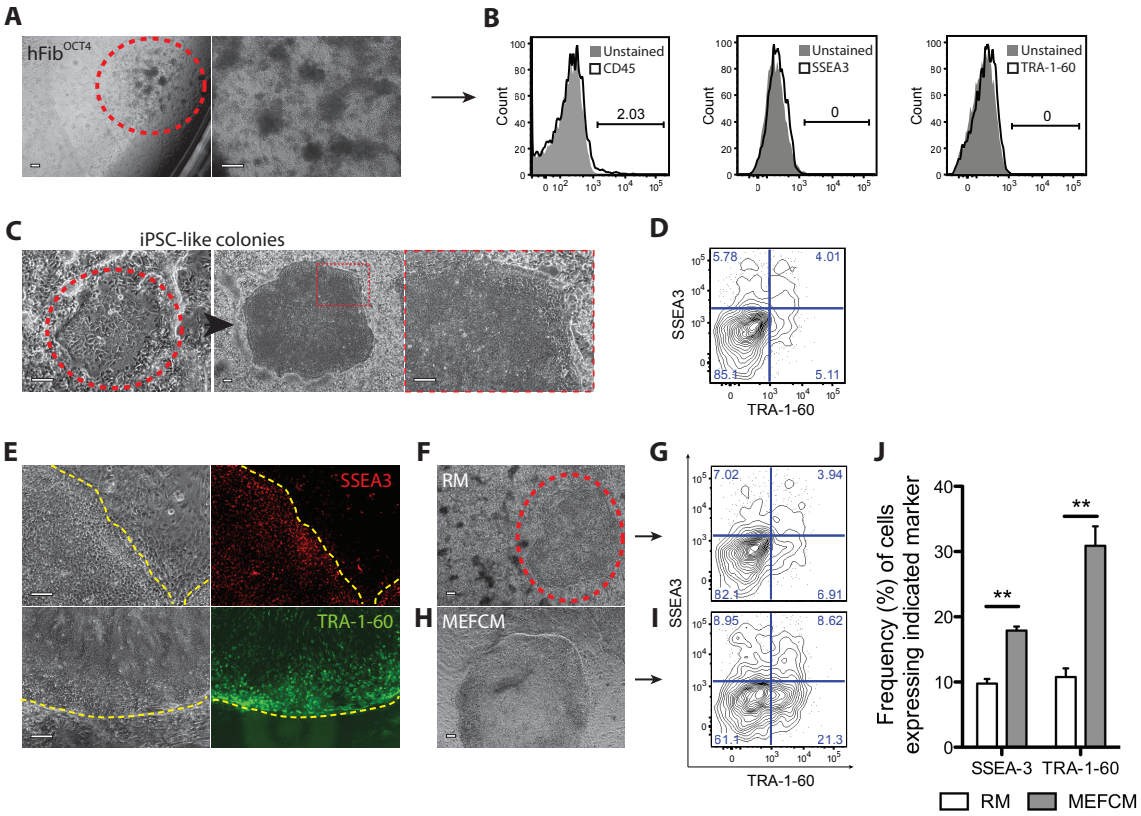
Figure 1. Continued exposure of OCT4 plastic human fibroblasts to pluripotent supportive conditions is sufficient for the generation of iPSCs

(A) Representative images of $hFib^{OCT4}$ culture maintained in RM for 24 days. The presence of regions (red dashed circle and image on right) that resemble plastic hFibs capable of responding to hematopoietic culture conditions and undergoing direct conversion to the hematopoietic lineage suggests that plasticity has been induced. Scale bars represent 100μm. (B) Flow cytometric analysis plots of total dissociated $hFib^{OCT4}$ cultures reveals the presence of $CD45^+$ cells and the absence of $SSEA3^+$ and $TRA\text{-}1\text{-}60^+$ cells. (C) Representative images of an emerging iPSC-like colony (left image, red dashed circle) and its subsequent expansion in its well of origin (center and right image). Enlarged region of highlighted area (center image, red dash rectangle) is depicted in the image on the right. Scale bars represent 100μm. (D) Flow cytometric analysis of $hFib^{OCT4}$ cultures possessing iPSC-like colonies reveals the presence of $SSEA3^+$ and $TRA\text{-}1\text{-}60^+$ cells. (E) SSEA3 and TRA-1-60 staining of iPSC-like colonies. Colony borders are indicated with yellow dashed lines. Scale bars represent 100μm. (F-I) Representative images and flow cytometric analyses of cultures derived from splitting $SSEA3^+TRA\text{-}1\text{-}60^+$-containing cultures maintained in RM (Passage 2, P2) into (F,G) RM or (H,I) MEFCM for two passages (P2-4). iPSC-like colony is indicated with red dashed oval. Scale bars represent 100μm. (J) Comparison of frequencies of $SSEA3^+$ and $TRA\text{-}1\text{-}60^+$ cells in RM versus MEFCM cultures after two passages (P2-4). Bars represent n=3 mean + SEM, $^{**}p < 0.005$. (K) Representative comparison of RFLP signature obtained for parent hFib and associated $iPSC^{OCT4}$. (L) PCR showing exclusive genomic integration of

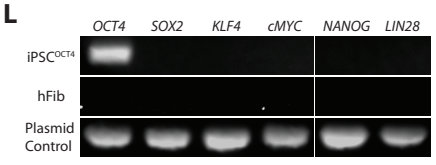
OCT4 in iPSC^{OCT4}. (M) Flow cytometric analysis of non-transduced parent hFibs depicting the absence of endogenous expression of OCT4, SOX2, and NANOG.



K

	Amelogenin		*CSF1PO*		*D13S317*		*D16S539*		*D18S51*		*D19S433*		*D21S11*		*D2S1338*	
Sample	**Allele 1**	**Allele 2**	**Allele 1**	**Allele 2**	**Allele 1**	**Allele 2**	**Allele 1**	**Allele 2**	**Allele 1**	**Allele 2**	**Allele 1**	**Allele 2**	**Allele 1**	**Allele 2**	**Allele 1**	**Allele 2**
Fibroblast 1	X	X	7	10	10	11	9	11	17	21	13	13	28	32.2	19	26
Fibroblast 1 iPSC^{OCT4}	X	X	7	10	10	11	9	11	17	21	13	13	28	32.2	19	26

	D3S1358		*D5S818*		*D7S820*		*D8S1179*		*FGA*		*THO1*		*TPOX*		*vWA*	
Sample	**Allele 1**	**Allele 2**	**Allele 1**	**Allele 2**	**Allele 1**	**Allele 2**	**Allele 1**	**Allele 2**	**Allele 1**	**Allele 2**	**Allele 1**	**Allele 2**	**Allele 1**	**Allele 2**	**Allele 1**	**Allele 2**
Fibroblast 1	15	17	8	11	8	9	14	17	19	23	6	7	6	10	16	17
Fibroblast 1 iPSC^{OCT4}	15	17	8	11	8	9	14	17	19	23	6	7	6	10	16	17



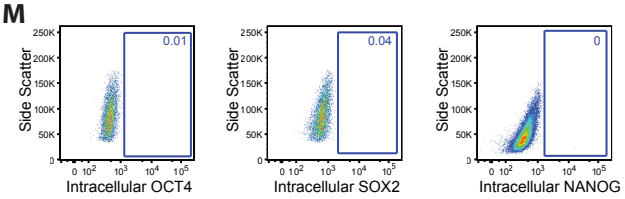


Figure 2. iPSC^{OCT4} possess biomolecular, immunophenotypic and functional characteristics similar to iPSC^{OSNL}

(A) Representative images of established iPSC^{OCT4} and iPSC^{OSNL} cultures prior to molecular and phenotypic characterization. Colony borders are indicated with yellow dashed lines. Scale bars represent 100μm. (B) RT-qPCR data examining endogenous *OCT4, SOX2,* and *NANOG* expression in whole iPSC^{OCT4} (P10) and iPSC^{OSNL} (P52) cultures. Bars represent mean + standard deviation, n=3 technical replicates; gene expression is normalized to GAPDH and compared relative to hFibs. (C) RT-qPCR data examining total and exogenous *OCT4* expression in Fib^{OCT4}, Fib, and TRA-1-60^{+}iPSC^{OCT4} (P12-19). Bars represent mean + standard deviation, n=3 technical replicates; gene expression is normalized to GAPDH and relative to Fib^{OCT4}. (D) Representative flow cytometric analysis plots of pluripotent marker expression in iPSC^{OCT4} and iPSC^{OSNL} cultures. (E) Karyotype of iPSC^{OCT4} (P19), generated by aCGH analysis, depicting the absence of copy number variation. (F) Hematoxylin and eosin staining of teratoma sections depicting cell derivatives of all three embryonic germ layers. Tissue type is denoted, and associated area of interest is highlighted by either red circle or arrow. (G) Representative images of EBs formed *in vitro* from iPSC^{OCT4} and iPSC^{OSNL}, scale bars represent 100 μm. (H) Representative flow cytometric analyses of CD34 and CD45 cell surface marker expression on dissociated, day 16 EBs derived from iPSC^{OCT4} and iPSC^{OSNL}. (I) Frequency of CD34^{+}CD45^{+} cells at day 16 of hematopoietic differentiation, as assessed by flow cytometric analysis. Bars represent n=3 + SEM, results are not significantly different. (J) The frequency of CD34^{+}CD45^{+} cells that give

rise to colony forming units when plated in CFU assay. Bars represent n=3 + SEM, results are not significantly different. (K) Distribution of hematopoietic colony types formed by day 16 of the CFU assay. Bars represent $n \geq 4$ + SEM. (L,M) Representative images of mature hematopoietic colonies (and associated Giemsa-Wright staining) generated from $iPSC^{OCT4}$ and $iPSC^{OSNL}$-derived EBs subjected to CFU assay. White scale bars represent 100 μm, black scale bars represent 10 μm.

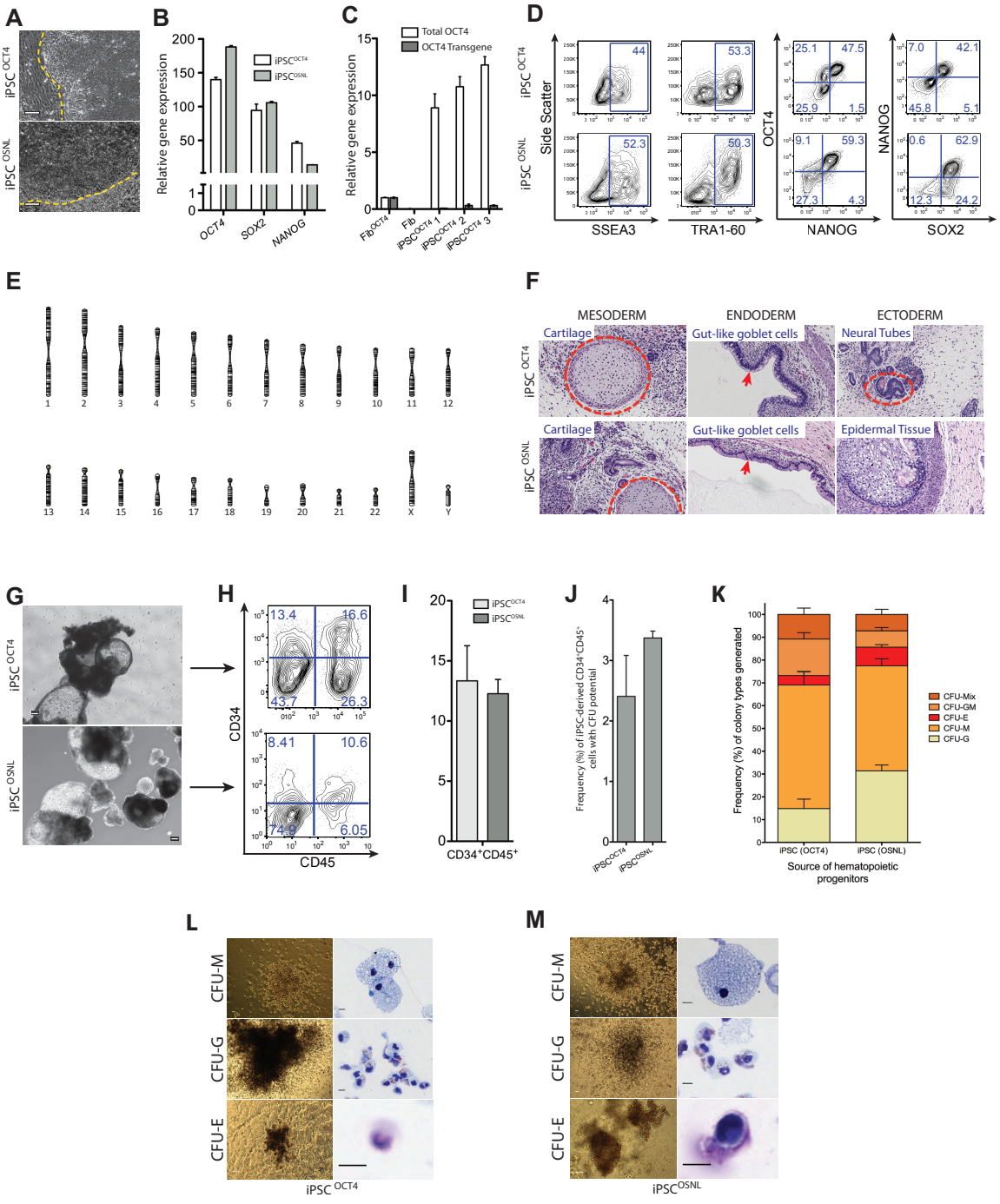
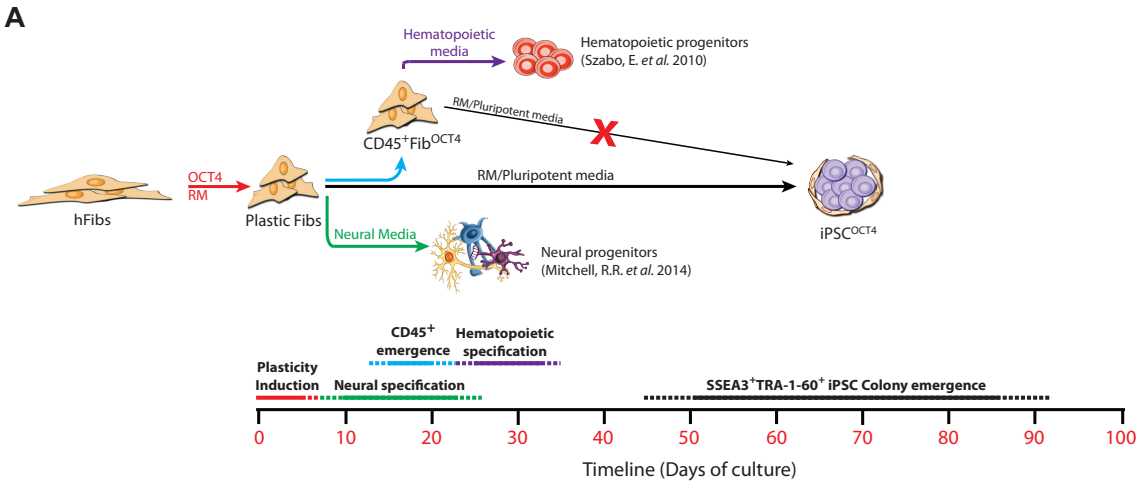

A
iPSC OCT4
iPSC OSNL
B
Relative gene expression
iPSC OCT4
iPSC OSNL
OCT4
SOX2
NANOG
C
Total OCT4
OCT4 Transgene
Relative gene expression
Fib OCT4
Fib
iPSC OCT4 1
iPSC OCT4 2
iPSC OCT4 3
D
iPSC OCT4
iPSC OSNL
Side Scatter
SSEA3
TRA1-60
OCT4
NANOG
NANOG
SOX2
E
F
MESODERM
ENDODERM
ECTODERM
Cartilage
Gut-like goblet cells
Neural Tubes
Cartilage
Gut-like goblet cells
Epidermal Tissue
iPSC OCT4
iPSC OSNL
G
iPSC OCT4
iPSC OSNL
H
CD34
CD45
I
iPSC OCT4
iPSC OSNL
CD34+CD45+
J
Frequency (%) of iPSC-derived CD34+CD45+ cells with CFU potential
iPSC OCT4
iPSC OSNL
K
Frequency (%) of colony types generated
CFU-Mix
CFU-GM
CFU-E
CFU-M
CFU-G
iPSC (OCT4)
iPSC (OSNL)
Source of hematopoietic progenitors
L
CFU-M
CFU-G
CFU-E
iPSC OCT4
M
CFU-M
CFU-G
CFU-E
iPSC OSNL

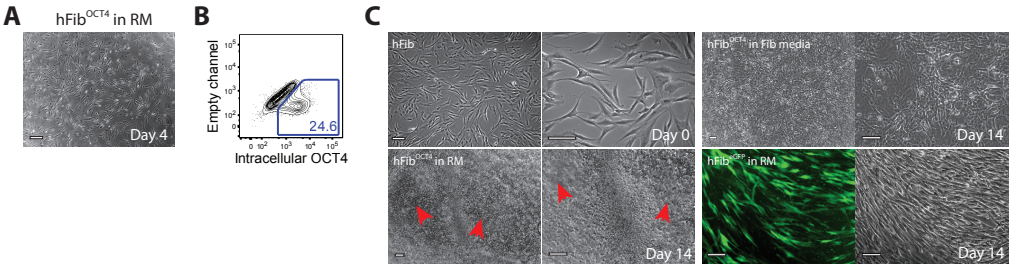
Figure 3. Acquisition of pluripotency is one of multiple cell fate choices that can be facilitated through environmental stimulation of OCT4-induced plasticity

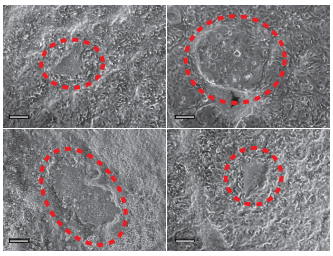
(A) Schematic placing the derivation of $iPSC^{OCT4}$ from plastic cells into context with previous demonstrations of cell fate conversion using OCT4 plastic cells. Given the stochastic nature of cellular fate reprogramming of adult human primary tissues, the range in timing of observed OCT4-induced plasticity events is illustrated by dotted bar lines along the timeline.


A
Hematopoietic media
Hematopoietic progenitors
(Szabo, E. et al. 2010)
RM/Pluripotent media
CD45+FibOCT4
OCT4
RM
hFibs
Plastic Fibs
RM/Pluripotent media
iPSCOCT4
Neural Media
Neural progenitors
(Mitchell, R.R. et al. 2014)
CD45+ emergence
Hematopoietic specification
Plasticity Induction
Neural specification
SSEA3+TRA-1-60+ iPSC Colony emergence
0
10
20
30
40
50
60
70
80
90
100
Timeline (Days of culture)

Supplementary Figure 1. Emergence of iPSC-like colonies from plastic Fibs

(A) Representative image of $hFib^{OCT4}$ cultured in RM four days post-transduction. Scale bar represents 100μm. (B) Flow cytometric analysis of $hFib^{OCT4}$ assessing OCT4 transduction efficiency. (C) Representative images depicting the transition of bipolar-elongated, untransduced hFibs to compact-cuboidal plastic hFibs (denoted by red arrows) that is dependent on the combination of OCT4 transduction and culture in RM. Scale bars represent 100μm. (D) Emerging iPSC-like colonies, indicated by red dashed circles, detected between day 45 and 93. Scale bars represent 100μm. (E) Overviews of the reprogramming experiments performed, timing of iPSC colony emergence, and the number of colonies generated. Source, passage number, and numbers of hFibs transduced in each experiment are indicated. (F) Flow cytometric analyses of SSEA3, TRA-1-60, OCT4, SOX2, and NANOG expression in $iPSC^{OCT4}$ lines (P18 and P17) derived from Fib sources 2 and 3. Lower frequencies of pluripotent markers are solely attributed to the lower density of colonies present in the cultures at the time of analysis; increased colony density was achieved after subsequent passages and confirmed by the presence of $\geq$ 35% $TRA\text{-}1\text{-}60^{+}$ cells (data not shown). (G) Comparison of RFLP signature obtained for parent hFibs and associated $iPSC^{OCT4}$.





E

OCT4 Reprogramming (45-93 Days)

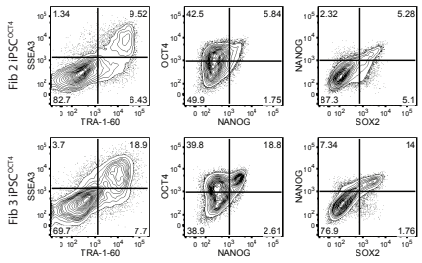
Fib Source 1 (Breast dermal, P4)
Exp't 1: 1 colony (10,000 Fibs); day 46
Exp't 2: 2 colonies (10,000 Fibs); day 45, 52
Exp't 3: 1 colony (10,000 Fibs); day 50
Exp't 4: 2 colonies (10,000 Fibs); day 53, 57
Exp't 5: 4 colonies (10,000 Fibs); days 54-61
Exp't 6: 0 colonies (10,000 Fibs)
Exp't 7: 7 colonies (10,000 Fibs); days 48-56

Fib Source 2 (Forearm dermal, P2)
Exp't 1: 1 colony (10,000 Fibs); day 81

Fib Source 3 (Forearm dermal, P2)
Exp't 1: 1 colony (10,000 Fibs); day 93

Fib Source 4 (Forearm dermal, P2)
Exp't 1: 0 colonies (10,000 Fibs)
Exp't 2: 0 colonies (10,000 Fibs)
Exp't 3: 0 colonies (10,000 Fibs)

Fib Source 5 (Forearm dermal, P2)
Exp't 1: 0 colonies (10,000 Fibs)
Exp't 2: 0 colonies (10,000 Fibs)
Exp't 3: 0 colonies (10,000 Fibs)



G

	Amelogenin		*CSF1PO*		*D13S317*		*D16S539*		*D18S51*		*D19S433*		*D21S11*		*D2S1338*	
Sample	**Allele 1**	**Allele 2**	**Allele 1**	**Allele 2**	**Allele 1**	**Allele 2**	**Allele 1**	**Allele 2**	**Allele 1**	**Allele 2**	**Allele 1**	**Allele 2**	**Allele 1**	**Allele 2**	**Allele 1**	**Allele 2**
Fibroblast 2	X	Y	10	12	10	12	11	12	15	17	14.2	15	28	33.2	20	22
Fibroblast 2 iPSC^{OCT4}	X	Y	10	12	10	12	11	12	15	17	14.2	15	28	33.2	20	22

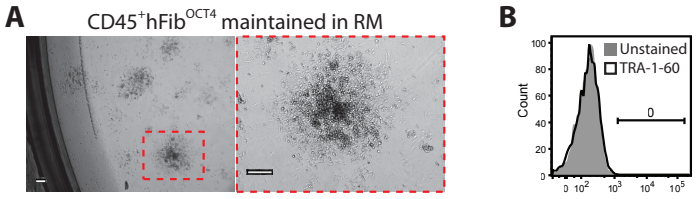
	D3S1358		*D5S818*		*D7S820*		*D8S1179*		*FGA*		*THO1*		*TPOX*		*vWA*	
Sample	**Allele 1**	**Allele 2**	**Allele 1**	**Allele 2**	**Allele 1**	**Allele 2**	**Allele 1**	**Allele 2**	**Allele 1**	**Allele 2**	**Allele 1**	**Allele 2**	**Allele 1**	**Allele 2**	**Allele 1**	**Allele 2**
Fibroblast 2	14	16	11	13	7	11	13	14	21	23	8	9	8	11	16	17
Fibroblast 2 iPSC^{OCT4}	14	16	11	13	7	11	13	14	21	23	8	9	8	11	16	17

	Amelogenin		*CSF1PO*		*D13S317*		*D16S539*		*D18S51*		*D19S433*		*D21S11*		*D2S1338*	
Sample	**Allele 1**	**Allele 2**	**Allele 1**	**Allele 2**	**Allele 1**	**Allele 2**	**Allele 1**	**Allele 2**	**Allele 1**	**Allele 2**	**Allele 1**	**Allele 2**	**Allele 1**	**Allele 2**	**Allele 1**	**Allele 2**
Fibroblast 3	X	X	11	12	8	12	9	12	16	16	14	15	30	33.2	24	25
Fibroblast 3 iPSC^{OCT4}	X	X	11	12	8	12	9	12	16	16	14	15	30	33.2	24	25

	D3S1358		*D5S818*		*D7S820*		*D8S1179*		*FGA*		*THO1*		*TPOX*		*vWA*	
Sample	**Allele 1**	**Allele 2**	**Allele 1**	**Allele 2**	**Allele 1**	**Allele 2**	**Allele 1**	**Allele 2**	**Allele 1**	**Allele 2**	**Allele 1**	**Allele 2**	**Allele 1**	**Allele 2**	**Allele 1**	**Allele 2**
Fibroblast 3	15	17	12	12	9	10	15	16	21	22	7	9.3	8	9	18	19
Fibroblast 3 iPSC^{OCT4}	15	17	12	12	9	10	15	16	21	22	7	9.3	8	9	18	19

Supplementary Figure 2. iPSC^{OCT4} are not derived from CD45^{+}hFib^{OCT4}

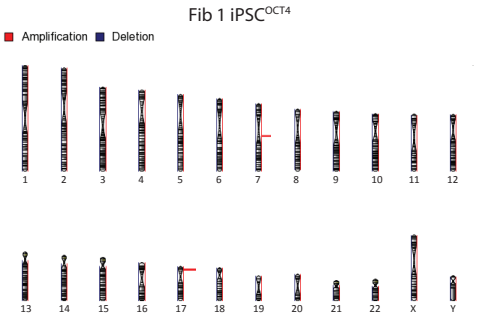
(A) Isolated CD45Fib^{OCT4} colonies maintained in reprogramming media for up to 45 days, without appearance of iPSC-like colonies. Highlighted area is enlarged in the image on the right, scale bars represent 100μm. (B) Flow cytometric analysis plot assessing TRA-1-60 expression in CD45^{+}Fib^{OCT4} cultures maintained in RM for 45 days.


A
CD45+hFibOCT4 maintained in RM
B
Unstained
TRA-1-60
Count
100
80
60
40
20
0
R
0
10^2
10^3
10^4
10^5

Supplementary Figure 3. iPSC^{OCT4} possess *in vivo* pluripotent capability

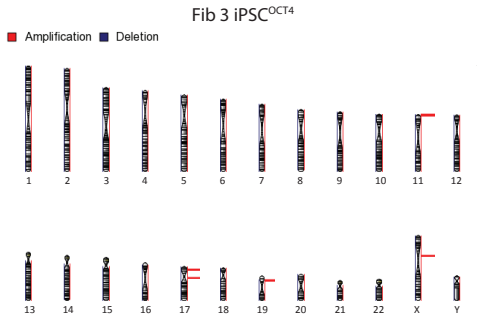
(A) Karyotypes of iPSC^{OCT4} (P14,18), generated by aCGH analysis, depicting the presence of genomic alterations; the majority of which are found in regions/chromosomes that are recurrently altered in cultured PSCs (Baker et al., 2007; Martins-Taylor et al., 2011; Taapken et al., 2011). (B) Representative images of iPSC^{OCT4} and iPSC^{OSNL} cultures prior to intratesticular injection into NOD/SCID mice. Red arrows indicate colony borders, scale bars represent 100μm. (C) Flow cytometric analysis plots depicting the frequency of SSEA3^{+} cells in iPSC^{OCT4} and iPSC^{OSNL} cultures prior to injection. (D) Average frequency of SSEA3^{+} cells collected prior to IT injection, represent $n \geq 5 \pm$ SEM. Teratoma formation frequency represents the number of tumours formed possessing all three germ layers over total number of mice injected. (E) Representative images of teratomas harvested from mice injected with iPSC^{OCT4} or iPSC^{OSNL}.

A



Chromosome	Locus	Length (Mb)	CNV Type
7	7q11.23*	0.58	Amplification
17	17p13.1**	1.37	Amplification

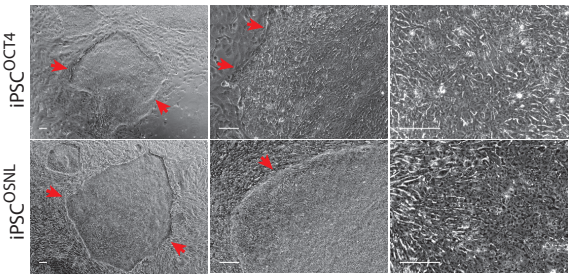
*recurrent CNV found in hPSCs
**associated with chromosome that is recurrently altered in hPSCs



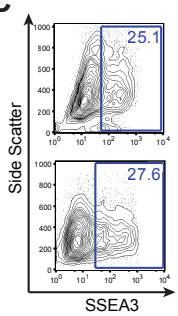
Chromosome	Locus	Length (Mb)	CNV Type
11	11p15.5 - 11p15.4*	3.11	Amplification
17	17p13.1**	1.37	Amplification
17	17q11.2**	0.93	Amplification
19	19p13.2	1.82	Amplification
X	Xp11.23*	0.36	Amplification

*recurrent CNV found in hPSCs
**associated with chromosome that is recurrently altered in hPSCs

B



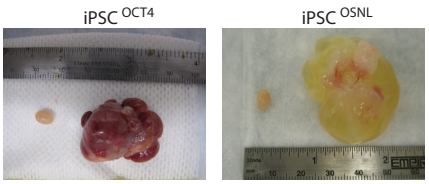
C



D

iPSC Line Injected	SSEA3+ Cells (%)	Teratoma Formation Frequency
iPSC^OCT4	29.17 ± 3.39%	5/5
iPSC^OSNL	27.78 ± 0.74%	6/6

E



Supplementary Table 1. Primer sequences used for RT-qPCR

Primer	Sequences (5'-3')
Endogenous Pluripotent TF Network:	
OCT4-F1	CCCATGCATTCAAACTGAGGTG
OCT4-R1	CCTTTGTGTTCCCAATTCCTTCC
SOX2-F	ATGGAGAAAACCCGGTACGC
SOX2-R	TTTTGCGTGAGTGTGGATGG
NANOG-F	GGACACTGGCTGAATCCTTCC
NANOG-R	CTCGCTGATTAGGCTCCAACC
OCT4 Transgene:	
Total OCT4:	
OCT4-F2	CAGTGCCCGAAACCCACAC
OCT4-R2	GGAGACCCAGCAGCCTCAAA
Exogenous OCT4:	
OCT4-F1	CCCATGCATTCAAACTGAGGTG
IRES-R	AGGGAGAGGGGCGGAATTG
Housekeeping (Normalization):	
GAPDH-F	CCACATCGCTCAGACACCAT
GAPDH-R	GCGCCCAATACGACCAAAT

Supplementary Table 2. Primer sequences used to evaluate genomic integration of provirus

Primer	Sequences (5'-3')
OCT4-F	GCGAACCAGTATCGAGAACC
IRES-R	GAGGAACTGCTTCCTTCACG
IRES-F	GCCAAAAGCCACGTGTATAA
SOX2-R	CATGAGCGTCTTGGTTTTCC
NANOG-F	GAACAATTCAACCTGGAGCA
IRES-R	GAGGAACTGCTTCCTTCACG
IRES-F	GCCAAAAGCCACGTGTATAA
LIN28-R	GCTTCTGCATGCTCTTTCCT
KLF4-F	CCCAATTACCCATCCTTCCT
IRES-R	GAGGAACTGCTTCCTTCACG
IRES-F	GCCAAAAGCCACGTGTATAA
cMYC-R	GGTCGCAGATGAAACTCTGG

CHAPTER 4

Induced pluripotent stem cells and hematopoietic progenitors generated from primary human AML cells harbouring the MLL-AF9 leukemic aberration exhibit dysfunctional differentiation capacity

PREAMBLE

This chapter is an original research manuscript prepared for submission to *Blood*.

**JH Lee and KR Salci contributed equally to this work*

Dr. Jong Hee Lee and I designed experiments with input from my supervisor Dr. Mickie Bhatia. I assembled, analyzed and interpreted data and wrote the manuscript. I performed culture and enrichment of healthy MPB and AML samples prior to reprogramming, culture of iPSCs, flow cytometric analyses, FISH, CFU assays and morphological analyses of mature blood cells. Dr. Jong Hee Lee performed lentiviral transduction, generation and molecular/immunophenotypic characterization of iPSCs, and EB differentiation experiments. Dr. Borko Tanasijevic performed RT-PCR experiments. Zoya Shapovalova provided technical assistance with cell culture, flow cytometry and hematopoietic differentiation. Aline Fiebig-Comyn performed cell injections for teratoma assays and technical assistance with histological preparations. Dr. Mickie Bhatia oversaw the entire study, assisted in manuscript preparation and finalized the manuscript.

Disease modeling proof-of-concepts had been established by reprogramming fibroblasts from patients with inherited neurological diseases (Ebert et al., 2009; Marchetto et al., 2010). However, reprogramming cancer cells to iPSCs had proven more

difficult (Kumano et al., 2012; Stricker et al., 2013) and had not been evaluated using human AML cells. Given the lack of practical cell-based models of AML required for further elucidation of mechanisms underlying AML pathogenesis and high-throughput discovery of novel therapeutics, we were inspired to investigate if we could capture and model AML through generation of iPSCs from primary leukemic cells.

Induced pluripotent stem cells and hematopoietic progenitors generated from primary human AML cells harbouring the MLL-AF9 leukemic aberration exhibit dysfunctional differentiation capacity

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ABSTRACT

A lack of human cell models encompassing the diverse molecular heterogeneity observed in human acute myeloid leukemia (AML) has contributed to a poor understanding of the underlying molecular pathogeneses of disease and has hindered the development of novel therapeutics. Although reprogramming primary human cancer cells to pluripotency has enabled disease modeling of other neoplastic tissues, it remains to be explored in AML. Here we reveal that conventional Yamanaka reprogramming conditions are insufficient to induce pluripotency in 12 primary human AML samples, but demonstrate for the first time that generation of AML iPSCs is possible when reprogramming conditions are modified. Naïve pluripotency-promoting reprogramming conditions [LIF with MEK/GSK3 inhibitors (2i)] improved reprogramming efficiency in normal blood samples and enabled the generation of iPSCs from an AML sample harbouring the MLL-AF9 leukemic aberration. These AML iPSCs exhibited hallmark morphological, molecular and immunophenotypic features of pluripotency, but maintained MLL-AF9 expression and exhibited dysfunctional differentiation features *in vitro* and *in vivo*. Moreover, $CD34^{+}CD45^{+}$ hematopoietic progenitor cells (HPCs) derived from AML iPSCs *in vitro* were incapable of terminal differentiation to mature monocytes, granulocytes and erythrocytes, similar to matched primary leukemic cells. Our study provides proof of principle that disease modeling of AML using iPSCs is possible and foundational insights toward the development of unique reprogramming conditions that should enable efficient iPSC generation from molecularly diverse primary AML samples.

HIGHLIGHTS

- Conventional Yamanaka reprogramming conditions are insufficient to generate iPSCs from primary AML samples
- Naïve (LIF/2i) reprogramming conditions enable derivation of iPSCs from a MLL-AF9^{+} primary AML sample
- AML iPSCs express MLL-AF9 and exhibit dysfunctional differentiation capacity *in vitro* and *in vivo*
- HPCs derived from AML iPSCs are incapable of differentiation into mature myeloid lineages *in vitro* similar to matched primary leukemic sample

KEYWORDS

Acute myeloid leukemia, disease modeling, leukemic aberration, reprogramming

ABBREVIATIONS

AML, acute myeloid leukemia; BM, bone marrow; CFU, colony forming unit; FISH, fluorescence in situ hybridization; HSPC, hematopoietic stem/progenitor cell; iPSC, induced pluripotent stem cell; OSKM, OCT4/SOX2/KLF4/cMYC; PB, peripheral blood; TF, transcription factor

INTRODUCTION

Acute myeloid leukemia (AML) is a complex cancer of the human hematopoietic system that results in the rapid accumulation of immature white blood cells in bone marrow (BM), peripheral blood (PB), and secondary tissues[1]. Disease heterogeneity is characterized by a multitude of epigenetic[2], genetic[2-5], and transcriptional[3] abnormalities that have been identified across clinically diverse AMLs. Although these features carry prognostic importance[1, 6, 7], their functional contributions to the underlying mechanisms of disease pathogenesis remain largely unknown, thereby hindering the development of novel therapeutics and treatment strategies[1]. *In vivo* humanized mouse AML xenotransplant models provide a surrogate of human disease[8], but sample engraftment is not guaranteed[9] and this method is not conducive to high-throughput drug and genetic screens that should be performed *in vitro*. However, *in vitro* primary human AML cell models are limited by the ineffectiveness of hematopoietic cytokine suspension cultures to maintain primitive AML cells[10], the complexities associated with classical long-term stromal co-cultures[11], and potentially convoluted by unknown molecular effects caused by small molecules used for *ex vivo* maintenance[12]. Similarly, immortalized cell lines may provide mechanistic insights[13], but do not faithfully recapitulate disease progression or heterogeneity. As such, *in vitro* cell models for further mechanistic studies of AML and drug development are lacking.

Conventional Yamanaka reprogramming methods using OCT4, SOX2, KLF4 and cMYC (OSKM) pluripotent transcription factor (TF) delivery and pluripotent-supportive culture media supplemented with basic fibroblast growth factor (bFGF) allow for the

derivation of induced pluripotent stem cells (iPSCs) from mature somatic cells[14]. Subsequent application of reprogramming methods to human cancers has enabled modeling of gastrointestinal[15] and chronic myeloid leukemia (CML)[16] cancers from tumour cell lines, and CML[17], glioblastoma[18], myelodysplastic syndrome[19] and pancreatic ductal adenocarcinoma[20] cancers from primary patient-derived cancer cells. However, cancer cell reprogramming is highly inefficient[16, 17, 20, 21], with marginal success often observed across primary patient samples from multiple cancers[17, 18, 20, 21], thereby preventing robust adaptation of this technology to disease modeling and drug screening. Moreover, studies investigating the technical challenges associated with cancer cell reprogramming are lacking. Toward addressing the unmet need for AML cell models and the inefficiency of cancer cell reprogramming, we applied conventional and modified reprogramming techniques to 12 diverse, primary human AML samples and found that MLL-AF9$^+$ iPSCs could be generated that exhibit dysfunctional differentiation features *in vitro* and *in vivo* that are similar to that of primary leukemic disease. Our findings provide proof of principle that modeling of primary AML disease is possible using reprogramming technologies, with initial insights into the cancer cell reprogramming blockade, that together should motivate further studies aimed at achieving robust reprogramming across diverse primary cancer tissues for the purposes of drug discovery and identification of disease mechanisms.

MATERIALS AND METHODS

Human patient samples

BM and PB samples were obtained from consenting human AML patients, and normal MPB from healthy donors, at the Juravinski Cancer Center (Hamilton, Ontario) in accordance with Research Ethics Board-approved protocols at McMaster University. Mononuclear cells were isolated using density gradient centrifugation (20 min, 1500 rpm) in Ficoll-Paque Premium (GE Healthcare Life Sciences), followed by ammonium chloride treatment (Sigma-Aldrich) for 5 min at 4°C. $CD34^+$ enrichment was performed using CD34 magnetic microbead kit with LS columns (Miltenyi Biotec) according to provided protocol.

Human cell culture

AML BM and PB were cultured in hematopoietic media: IMDM with 15% v/v BIT serum substitute (StemCell Technologies), 1% v/v non-essential amino acid (Gibco), and 1% v/v sodium pyruvate (Gibco) supplemented with 100 ng/ml Flt-3 ligand (Flt-3L; R&D systems), 20 ng/ml interleukin-3 (IL-3; R&D systems), 100 ng/ml stem cell factor (SCF; Amgen Inc.), and 100 ng/ml thrombopoieitin (TPO; R&D systems).

During reprogramming AML samples were either cultured on irradiated mouse embryonic fibroblasts (iMEFs) in conventional iPSC media or naïve iPSC media: DMEM/F12 (Gibco) with 20% Knockout Serum Replacement (Gibco), 100 μM β-mercaptoethanol, 100 μM nonessential amino acid (Gibco), 1 mM L-glutamine (Gibco) either supplemented with 10 ng/ml basic human fibroblast growth factor (bFGF) for conventional iPSC media or 20 ng/ml human leukemia inhibitory factor (LIF) with the

two inhibitors (2i) PD0325901 (1 μM, MEK/ERK inhibitor) and CHIR99021 (3 μM, GSK3ß inhibitor) for naïve iPSC media. After derivation, all iPSCs were cultured on iMEFs in conventional iPSC media.

iPSC-derived embryoid bodies (EBs) were cultured in hematopoietic differentiation media: KO-DMEM (Gibco) with 20% Knockout Serum Replacement (Gibco), 100 μM β-mercaptoethanol, 100 μM nonessential amino acid (Gibco), 1 mM L-glutamine (Gibco) supplemented with 25 ng/ml bone morphogenetic protein 4 (BMP4; R&D systems), 300 ng/ml Flt-3L, 50 ng/ml granulocyte colony stimulating factor (G-CSF; Amgen Inc.,), 10 ng/ml IL-3, 10 ng/ml interleukin-6 (IL-6; R&D systems), and 300 ng/ml stem cell factor (SCF; Amgen Inc.).

Cellular Reprogramming

Plasmids pSIN4-EF2-O2S and pSIN-EF2-K2M developed by James A. Thomson (University of Madison-Wisconsin) were obtained from Addgene. Lentivirus produced in HEK 293FT cells using 2nd generation pMD2.G and psPAX2 packaging plasmids was harvested 72h after transfection and concentrated by ultracentrifugation. CD34^{+} primary AML BM and PB, normal MPB and OCI-AML3 cell line (10^5) cultures were exposed to concentrated lentiviral vectors in hematopoietic media supplemented with 8 μg/ml polybrene (Sigma-Aldrich), spun in plates at 1300 rpm at room temperature for 90 m, and incubated at 37°C for 24 h. This procedure was repeated once more. Media was replaced with fresh hematopoietic media 48 h after second incubation (day 4). On day 5, cells were seeded on 2 wells of 200,000 iMEFs and maintained in either conventional or naïve iPSC media. iPSC colonies emerged $\geq$ 20 days post-transduction and were individually isolated

and expanded on iMEFs.

Flow cytometry

AML, normal MPB cells and iPSC-derived HPCs were analyzed with CD34-PE and CD45-APC antibodies (BD Biosciences), AML transduction efficiency with OCT4-Alexa Fluor 647 and SOX2-Alexa Fluor 647 (BD Pharmingen), iPSCs with OCT4-Alexa Fluor 488, SOX2-Alexa Fluor 647, NANOG-PE, SSEA3-PE and TRA1-60-Alexa Fluor 647 (BD Pharmingen), and AML and iPSC-derived CFUs with CD11b-APC, CD14-PE, CD15-PE, and CD45-Pacific Blue antibodies (BD Biosciences and BioLegend). Cells were fixed and permeabilized for intracellular staining using the BD Cytofix/Cytoperm kit. Flow cytometry was performed using the LSRII Flow Cytometer with FACSDiva software (BD) and analyzed by FlowJo software (Tree Star, Inc.). Cells were sorted using the FACSAria II (BD).

Fluorescence *in situ* hybridization

Fluorescence *in situ* hybridization (FISH) was performed on AML BM, iPSC and CFU samples using commercially available FISH probes (Abbott Molecular). Cells were incubated in 0.075M KCl (37°C, 15 min) and fixed in Carnoy's Solution. Slide preparations were denatured in formamide-based solution (73°C, 5 m), mixed with denatured probes (73°C, 5 m in heating block), sealed with Elmer's Rubber Cement, and incubated overnight at humid incubators set to 37°C [Locus-specific identifier (LSI) probes] or 42°C [Chromosome enumeration probes (CEP)]. Post-hybridization washes were performed in 0.4x SSC/0.3% NP40, pH 7.0 (73°C, 2 min), followed by 2x

SSC/0.1% NP40, pH 7.0 (RT, 1 min), and mounted with DAPI II counterstain (Abbott Molecular). Number of nuclei scored are indicated in figure legends.

Teratoma assay

AML iPSCs were collected and injected into NOD/SCID mice testes as previously described[22]. Tumours were harvested after 8-10 weeks, sectioned, and stained by hematoxylin and eosin to assess for formation of derivatives of the three embryonic germ layers.

Hematopoietic differentiation of iPSCs

iPSCs treated with 200 U/mL collagenase IV (Invitrogen) were scraped into clumps and transferred into suspension culture to form embryoid bodies (EBs) in hematopoietic differentiation media as previously described[23]. EBs were collected after 15 days and dissociated into single cell suspensions by 0.4 U/mL collagenase B (Roche Life Science). Total single cell suspensions were analyzed by flow cytometric or CFU analyses.

Clonogenic CFU Assay

AML BM and iPSC-derived EB suspensions were plated in Methocult H4434 medium (StemCell Technologies) to assess clonogenic colony-forming unit (CFU) capacities as previously described[22]. Individual colonies were isolated, resuspended in 100 uL PBS, spun onto microscope slides using the Shandon Cytospin 3 (Block Scientific, Inc.), stained with Giemsa-Wright using Shandon Kwik-Diff Stain Kit (Thermo Scientific) and assessed for single-cell morphology. Full wells were collected for FISH and flow cytometric analyses.

Reverse-transcriptase polymerase chain reaction

Reverse-transcriptase polymerase chain reaction (RT-PCR) was performed on AML BM, iPSCs, and iPSC-derived CFUs to assess MLL-AF9 fusion and GAPDH gene transcripts. Primer sequences (5' to 3') used to detect MLL-AF9 were MLL-exon7-Fwd (GGAAGTCAAGCAAGCAGGTC) and AF9-exon7-Rev (TCGGCTGCCTCCTCTATT TA), and to detect GAPDH were GAPDH-Fwd (CCACATCGCTCAGACACCAT) and GAPDH-Rev (GCGCCCAATACGACCAAAT).

Imaging and Immunocytochemistry

BF and fluorescent images were acquired using an Olympus microscope fitted with a CoolSNAP HQ2 camera (Photometrics Scientific) or the PerkinElmer Operetta High Content Imaging System. Teratoma sections and hematopoietic cytospins were imaged using ScanScope CS digital slide scanner with Aperio Image Scope software.

TRA-1-60 expression was assessed on live iPSCs using TRA-1-60 DyLight 488 (Stemgent). TRA-1-60 staining and 4',6-diamidino-2-phenylindole (DAPI) counterstaining were together performed on iPSCs fixed in 4% paraformaldehyde.

Statistical Analysis

Data are presented as mean ± standard error of mean (SEM) or standard deviation (SD). Prism software (version 5.0a; GraphPad) was used for all statistical analyses, and the criterion for statistical significance was $p < 0.05$.

RESULTS

Primary human AML samples are not amenable to iPSC generation using conventional reprogramming conditions

Conventional reprogramming conditions originally described using human fibroblasts[14] have enabled iPSC derivation from human $CD34^+$ cord blood (CB)[24] and mobilized peripheral blood (MPB)[25] cells. We therefore asked whether these conditions would also allow for the generation of iPSCs when applied to primary human AML samples (**Fig. 1a**). To best reflect the heterogeneity of AML disease observed clinically[3], we curated a diverse library of genetically abnormal primary PB and BM samples for reprogramming experiments (**Tables 1** and **2**). The use of samples with identifiable mutations found uniquely in the leukemic cells would also enable genetic interrogation of downstream iPSCs toward establishing their leukemic cell origin. We first attempted to reprogram six $CD34^+$-enriched AML PB samples (**Table 1** and **Supplementary Fig. 1a**). Given the rare presence of healthy $CD34^+$ hematopoietic stem/progenitor cells (HSPCs) in circulation[26], $CD34^+$ AML PBs represented highly leukemic samples in which to establish whether reprogramming AML was possible. Despite our established competency in reprogramming blood samples to pluripotency[24], we did not observe iPSC colony formation from any of the six PB samples during 60 days of observation post-transduction. As it has been previously demonstrated that paired $CD34^+$ PB and BM cells from AML patients exhibit molecular and functional differences[27], we next attempted to reprogram six primary, $CD34^+$-enriched AML BM samples (**Table 2** and **Supplementary Fig. 1b**) to evaluate whether BM-resident leukemic cells were more amenable to

reprogramming. Dissimilar to AML PB, we observed iPSC colony formation in 2 of the 6 samples attempted (**Table 2**). However, genetic interrogation of ≥ 6 iPSC lines per sample revealed that all colonies were derived from blood cells devoid of leukemic aberration (**Fig. 1b**), demonstrating that BM-residing leukemic cells are equally as refractory to reprogramming as circulating leukemic cells. To investigate whether failed reprogramming was attributed to inefficient pluripotent factor delivery and/or expression in AML cells we assessed OCT4-SOX2 (OS) transduction efficiency and expression in AML versus normal MPB. AML and MPB shared similar cell morphologies, exhibited clumping, and expressed OS following lentiviral transduction similar to GFP control (**Fig. 1c,d**), indicating that AML cells can successfully uptake and express reprogramming factors. Although the frequency of OS^+ cells was lower than that of GFP^+ cells (**Fig. 1e**), this was common and similar between AML and MPB (**Fig. 1f**) and is likely attributed to lower transduction efficiency of OS versus GFP lentivirus. Moreover, leukemic cell viability was not greatly reduced in most samples (**Fig. 1g**), suggesting that cell death following lentiviral transduction was not a limiting factor of reprogramming. We next assessed whether these results were also observed in an AML cell line exposed to reprogramming conditions sufficient for iPSC generation from healthy MPB, and found that similar to primary AML samples, immortalized AML cells are refractory to reprogramming despite efficient TF delivery (**Supplementary Fig. 2a-d**). Together, these results indicate that human AML samples are refractory to reprogramming using conventional conditions despite expression of reprogramming factors, suggesting that a barrier to reprogramming occurs downstream of pluripotent factor delivery and

expression. These findings are corroborated by past attempts using somatic cell nuclear transfer to derive ESCs from acute myeloid leukemia[28], and non-robust reprogramming observed using other malignant cell types[16, 17, 20, 21].

Naïve reprogramming conditions increase reprogramming efficiency and enable the derivation of iPSCs from a primary AML sample harbouring MLL-AF9

We recently determined that naïve pluripotency-supporting conditions, whereby bFGF is substituted for leukemia inhibitory factor (LIF) in combination with MEK/ERK and GSK3 inhibitors (2i), are conducive to iPSC generation from human CB (Lee, JH. et al., under review at *Cell*). Given the increased expression and stability of the pluripotent network under naïve conditions (Lee, JH. et al.), we hypothesized that naïve reprogramming conditions may increase reprogramming efficiency toward enabling AML iPSC generation. We first compared naïve versus conventional reprogramming of normal MPB and found that naïve conditions significantly increased the number of TRA-1-60^{+} iPSC colonies generated (**Fig. 2a**). To assess whether this improved reprogramming efficiency and would facilitate the generation of iPSCs from AML, we compared our unique naive reprogramming approach (**Fig. 2b**) to conventional reprogramming (**Fig. 1a**) using three AML BM samples. We selected two samples (AML 14256 and 15331) that we had generated normal iPSCs (devoid of leukemic mutation) from previously, as this would serve as an inherent control to determine whether reprogramming efficiency increased, and a third sample (AML 14384) which was previously unsuccessful (**Table 2**). Naïve versus conventional reprogramming conditions significantly improved reprogramming efficiency, leading to increases in the number of colonies generated in

two samples and enabling generation of an iPSC line from the third (**Fig. 2c**). Uniquely, all TRA-1-60^{+} iPSC lines derived from AML 15331 BM in naïve, but not conventional, reprogramming conditions expressed core pluripotency factors OCT4, SOX2 and NANOG, SSEA3 and TRA-1-60[29, 30] and harboured the MLL-AF9 aberration found in the leukemic cells[31] (**Fig. 2d,e** and **3a-c** and **Supplementary Fig. 3**); demonstrating that AML iPSC generation is possible using non-conventional reprogramming conditions. However, this effect was not observed in other AML samples, as all iPSCs generated from AML 14256 (trisomy 8) and 14384 (PML/RARα) BM were devoid of the aberration found in the leukemic cells regardless of the reprogramming condition used (**Fig. 2d,e** and **Supplementary Figs. 4,5**), while naïve conditions alone were insufficient to generate AML iPSCs from two additional AML samples harbouring inv(16) and del5q/monosomy 7 aberrations (**Supplementary Fig. 6)**. Taken together, these results suggested that successful AML iPSC generation using naïve conditions may not be attributed to improved reprogramming efficiency alone, but rather a stochastic synergy of culture condition effects and epigenetic, genetic or transcriptional programs unique to AML 15331 leukemic cells. Although further investigation into conditions required to overcome cancer cell reprogramming blockade is required before robust generation of AML iPSCs can be achieved, we demonstrate for the first time that it is possible and proceeded to assess whether these AML iPSCs recapitulated relevant disease features.

AML iPSCs express the MLL-AF9 fusion transcript and exhibit dysfunctional *in vivo* differentiation capacity

We next evaluated the functional ability of AML iPSCs derived from Patient 15331 to generate the three embryonic germ layers *in vivo* using the teratoma assay[33]. Similar to normal hPSCs[14, 33], AML iPSCs injected into mouse testes developed into tumours within 8-10 weeks (**Fig. 3d**). However, morphological analyses of hematoxylin and eosin-stained tumour sections revealed that AML iPSC differentiation potential was limited to ectoderm and infrequently endoderm, with no detection of the mesoderm lineage (**Fig. 3e**). Moreover, each tumour possessed regions of primitive, undifferentiated cell types (**Fig. 3f**). Given that AML iPSCs exhibited morphological, molecular and immunophenotypic features of bonafide iPSCs (**Fig. 3a-c**)[32] and that MLL-AF9 over-expression has a demonstrated driver role in leukemogenesis and differentiation blockade[34], we hypothesized that dysfunctional teratoma generation from AML iPSCs may be attributed to the presence of the leukemic aberration rather than failed acquisition of pluripotency. Accordingly, we performed reverse transcriptase polymerase chain reaction (RT-PCR) to evaluate if the MLL-AF9 genomic aberration was actively expressed in the pluripotent state. Unlike previous work where iPSCs derived from transgenic MLL-AF9-induced mouse leukemia did not express MLL-AF9[35], we found that primary human AML iPSCs harbouring the endogenous MLL-AF9 leukemic aberration expressed the fusion transcript (**Fig. 3g**). Together these results demonstrate that AML iPSCs possess dysfunctional differentiation potential *in vivo*, and suggest that

MLL-AF9 may play a role in this dysfunctional differentiation similar to the impaired differentiation observed in HPCs expressing MLL-AF9[34].

HPCs derived from AML iPSCs recapitulate dysfunctional differentiation features similar to primary leukemic sample

Building on the dysfunctional differentiation features of AML iPSCs *in vivo*, we next asked whether this affected hematopoietic (mesoderm) differentiation capacity *in vitro* using an embryoid-body (EB)-based protocol to generate HPCs[36]. The unique ability to generate isogenic (normal and AML) iPSCs from AML Patient 15331 BM using conventional and naïve reprogramming conditions (**Fig. 2d,e** and **Supplementary Figure 3**) also provided a platform in which to directly compare the functional capacities of normal versus AML iPSC-derived HPCs pending successful generation (**Fig. 4a**). Similar to our *in vivo* experiments, AML iPSC lines exhibited a limited ability to differentiate to $CD34^+CD45^+$ putative HPCs; with an average frequency of 0.15% $CD34^+CD45^+$ cells detected using eight independent lines in 16 differentiation experiments (**Fig. 4b,c**). In contrast, differentiation experiments performed in parallel using six normal iPSCs derived from the same patient led to the efficient generation of $CD34^+CD45^+$ putative HPCs (average frequency of 7.14%) (**Fig. 4b,c**). All normal and AML iPSC-derived cells were subsequently plated in colony forming unit (CFU) assays to assess for clonogenic HPC capacity from $CD34^+CD45^+$ cells through the formation of hematopoietic colonies in semisolid medium[22]. Hematopoietic colony formation was observed from all normal iPSC derivatives, but was limited to 1 of 8 AML iPSC derivatives (**Fig. 4d** and **Table 3**), indicating that AML iPSCs have significantly limited capacity to generate HPCs. These

findings using isogenic iPSCs provide further evidence that AML iPSCs possess impaired differentiation capacity, suggesting that AML iPSCs may provide a platform in which to perform high throughput screens for novel chemical compounds or gene editing strategies that restore normal differentiation features.

To validate whether the impaired differentiation features of AML iPSCs were relevant to the primary leukemic disease, we next compared hematopoietic colonies generated from AML iPSC-derived HPCs and matched patient BM sample. A combination of morphological and flow cytometric analyses revealed that colonies generated from AML iPSC-derived HPCs consisted of promonocytes and promyelocytes that were incapable of maturation to $CD11b^{+}CD14^{+}$ monocytes and $CD15^{+}CD11b^{+}$ granulocytes, with no observed erythrocytic colony formation, similar to the matched AML BM (**Fig. 4d,e**). In stark contrast, normal iPSC-derived HPCs exhibited monocytic, granulocytic and erythrocytic differentiation capacity (**Fig. 4d,e** and **Supplementary Fig. 7**), with full maturation to morphologically and immunophenotypical mature monocytes and granulocytes (**Fig. 4e-g**). FISH performed on total collected colonies confirmed the AML- versus normal- iPSC origins of immature versus mature hematopoietic cells, respectively (**Fig. 4h)**. Moreover, AML iPSC-derived colonies maintained MLL-AF9 expression (**Fig. 4i**), suggesting that MLL-AF9 may play a role in the disruption of these differentiation processes. Together, these results provide the proof of principle that primary human AML-derived iPSCs and downstream HPCs recapitulate leukemia-associated dysfunctional differentiation capacity.

DISCUSSION

Here we've shown for the first time that iPSCs that recapitulate dysfunctional differentiation features similar to primary leukemic disease can be generated from a human AML sample exclusively using naive reprogramming conditions. AML iPSCs harbour and actively express the MLL-AF9 leukemic aberration found in the matched BM and exhibit limited differentiation capacity from both the pluripotent state and in downstream HPCs derived *in vitro*. We propose that our proof of principle findings provide the framework for future studies to further investigate if additional modifications to reprogramming conditions enables robust reprogramming across diverse sets of primary AML samples, and may form a platform from which to use AML iPSCs as surrogates of primary leukemic disease for high-throughput drug screens and mechanistic studies of disease (**Fig. 5**).

The low efficiency of cancer cell reprogramming to pluripotency has yet to be addressed. Similar to previous cancer cell reprogramming works that only achieved iPSC generation from a subset of primary cancer patient samples[17, 18, 20, 21] and a recent demonstration that only normal iPSCs can be generated from t(8;21)$^{+}$ AML[37], we demonstrate that AML cell reprogramming cannot be achieved using conventional reprogramming methods. Our findings provide the initial insights that AML cell reprogramming blockade occurs downstream of pluripotent TF delivery and we hypothesize that this may be attributed to epigenetic and genetic aberrancies inherent to AML samples[2]. Accordingly, the successful generation of iPSCs from MLL-AF9^{+} AML in naive conditions may be a result of an epigenetic state that is more amenable to

reprogramming because of the combined contributions of MLL-AF9 and LIF/2i to epigenetic regulation. MLL-AF9 contributes to gene activation through forming complexes that aberrantly impart activating methylation marks on histone 3[38], and MLL-AF9$^+$ AML cells have extensively demethylated genomes[2]. Similarly, LIF/2i conditions have been associated with a reduction in transcriptionally-repressive DNA methylation as compared to conventional FGF conditions[39], likely due to MEK/ERK and GSK3 inhibitors causing a decrease in DNA methyltransferase (DNMT) expression[40, 41]. Accordingly, AML and other cancers may become more amenable to reprogramming if a "relaxed" epigenetic state that promotes transcriptional activity is induced. This hypothesis is further supported by the finding that generation of iPSCs from primary CML is only possible when valproic acid, a known histone deacteylase inhibitor that leads to decreased DNA methylation[40, 42, 43], is added to the reprogramming cocktail (Dr. Keiki Kumano, personal communication). Therefore, future studies should investigate whether MLL-AF9 expression, LIF/2i and/or epigenetic modifiers improve AML cell reprogramming efficiency toward establishing robust reprogramming conditions for AML that may also extend to other difficult-to-reprogram cancers.

AML iPSCs, but not isogenic normal iPSCs, expressed the leukemia-associated aberration in the pluripotent state, and exhibited dysfunctional differentiation capacities *in vitro* and *in vivo* that mimicked the differentiation blockade observed in primary leukemia. Based on our current results, we cannot conclude that MLL-AF9 is causal in blocking AML iPSC and derivative HPC differentiation as there may be other factors that contribute to differentiation blockade. However, we suggest that AML iPSCs provide a

surrogate system in which to assess if/how MLL-AF9 affects differentiation - similar to previous work using isogenic MDS iPSCs where it was established which genes on chromosome 7 might contribute to disease phenotype[19]. For example, eliminating the expression of MLL-AF9 using short-hairpin RNA[44] or mutation correction using CRISPR technology[45] followed by assessment of iPSC differentiation potential *in vitro* or *in vivo* would provide the first opportunity to determine whether the endogenous mutation is directly responsible for differentiation blockade. This paradigm may also extend to other AML iPSCs once more robust reprogramming conditions are established. Independent of delineating the functional roles of leukemia-associated aberration(s), the dysfunctional differentiation ability of AML iPSCs provides an opportunity to identify small-molecules that promote differentiation. This could serve as an initial screening platform to identify lead drug candidates which could then be validated *in vivo* using more precious primary AML samples. As such, we suggest that AML iPSCs may provide a surrogate platform of leukemic disease from which to investigate the functional contributions of leukemic mutations and to develop drug screening platforms.

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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Figure 1: Conventional reprogramming conditions are insufficient to generate AML-iPSCs from primary AML patient PB and BM. (**a**) Schematic illustrating the reprogramming strategy employed in attempt to generate iPSCs from primary human AML PB and BM samples harbouring identifiable genetic mutations. $CD34^{+}$ enriched primary AML samples were transduced with lentiviral vectors carrying the Yamanaka reprogramming factors. Following transduction, cells were transferred to an iMEF monolayer and maintained in conventional reprogramming conditions (iPSC media supplemented with bFGF). iPSC colony emergence was monitored daily; emerging colonies were isolated, expanded and assessed for the presence of the leukemia-specific mutation. (**b**) FISH performed in BM and matched iPSC colonies revealing the absence of the patient's unique leukemic aberration in iPSCs generated using conventional reprogramming conditions. Percentages indicate frequency of specified mutations. $\geq$ 50 nuclei were scored per BM and iPSC line analyzed; 11 AML 14256 iPSC lines and six AML 15331 iPSC lines were analyzed by FISH. (**c-d**) AML and normal MPB cell morphologies and transgene expression 5 days following indicated lentiviral transduction. Primary AML samples possessed cell morphologies and gene expression patterns similar to normal MPB following lentiviral transduction. Scale bars represent 100 μm. (**e**) Frequency of cells in indicated samples expressing OCT4-SOX2 or GFP protein following transduction. (**f**) OS transduction efficiency (based on SOX2 expression) is lower across all samples relative to GFP, but this lower efficiency is common to both normal and AML samples. (**g**) Bar graph depicting normal MPB and AML cell viability post-transduction. Viability prior to transduction is indicated.

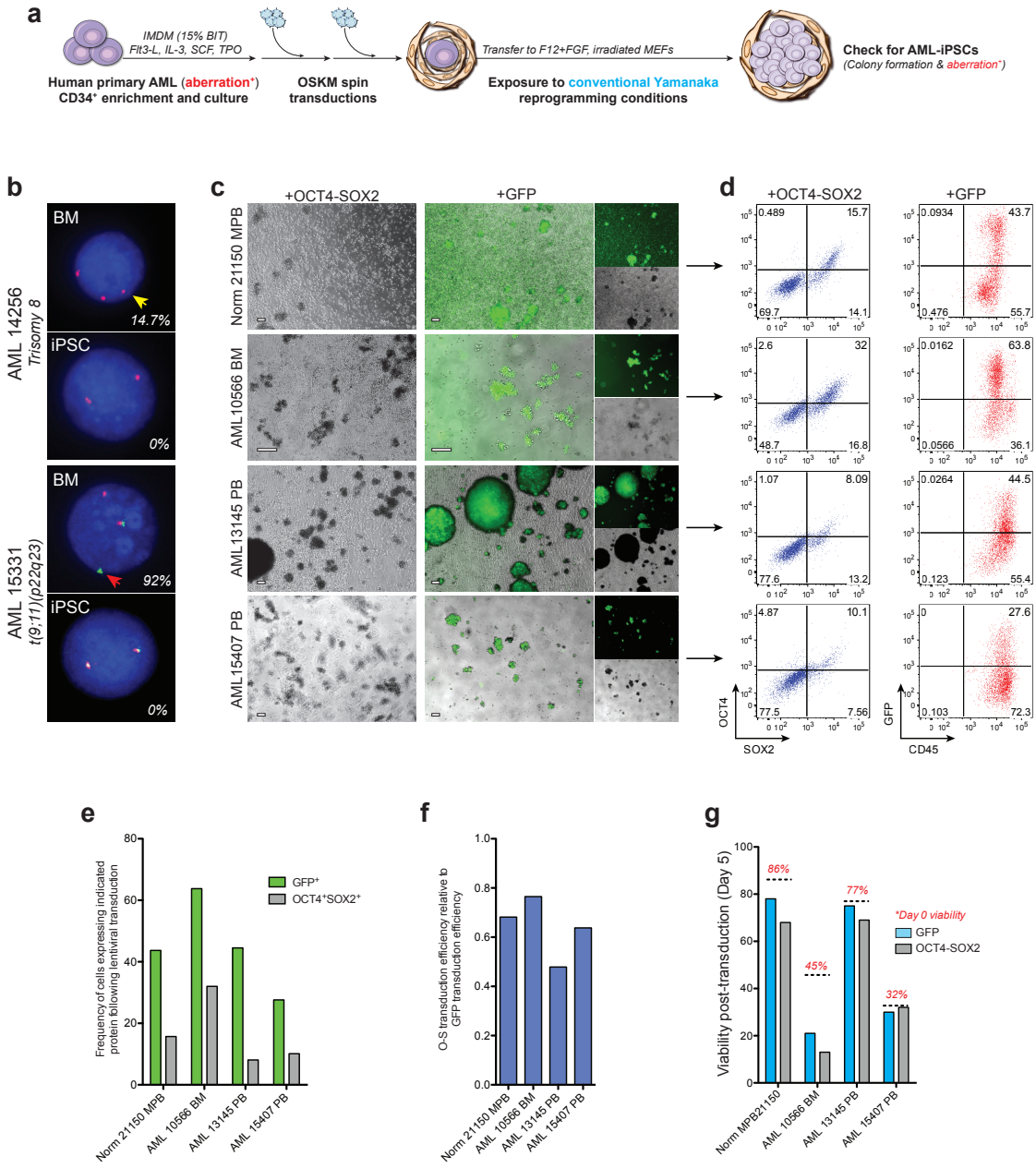

a
IMDM (15% BIT)
Flt3-L, IL-3, SCF, TPO
Human primary AML (aberration*)
CD34+ enrichment and culture
OSKM spin transductions
Transfer to F12+FGF, irradiated MEFs
Exposure to conventional Yamanaka reprogramming conditions
Check for AML-iPSCs
(Colony formation & aberration*)
b
AML 14256
Trisomy 8
BM
14.7%
iPSC
0%
AML 15331
t(9;11)(p22q23)
BM
92%
iPSC
0%
c
+OCT4-SOX2
+GFP
Norm 21150 MPB
AML10566 BM
AML13145 PB
AML15407 PB
d
+OCT4-SOX2
+GFP
0.489
15.7
69.7
14.1
0.0934
43.7
0.476
55.7
2.6
32
48.7
16.8
0.0162
63.8
0.0566
36.1
1.07
8.09
77.6
13.2
0.0264
44.5
0.123
55.4
4.87
10.1
77.5
7.56
0
27.6
0.103
72.3
OCT4
SOX2
GFP
CD45
e
Frequency of cells expressing indicated protein following lentiviral transduction
GFP+
OCT4+SOX2+
Norm 21150 MPB
AML 10566 BM
AML 13145 PB
AML 15407 PB
f
O-S transduction efficiency relative to GFP transduction efficiency
Norm 21150 MPB
AML 10566 BM
AML 13145 PB
AML 15407 PB
g
Viability post-transduction (Day 5)
86%
45%
77%
32%
*Day 0 viability
GFP
OCT4-SOX2
Norm MPB21150
AML 10566 BM
AML 13145 PB
AML 15407 PB

Figure 2: Naïve reprogramming conditions increase reprogramming efficiency and selectively enable the derivation of iPSCs from a primary AML sample harbouring MLL-AF9. (**a**) Bar graphs depicting the number of TRA-1-60^{+} colonies generated from normal MPB using conventional (bFGF) or naïve (LIF/2i) reprogramming conditions. Bars represent mean $\pm$ SD of three technical replicates; *$p<0.05$. (**b**) Schematic illustrating naïve reprogramming conditions used. As compared to conventional reprogramming conditions, LIF/2i was used in place of bFGF in the reprogramming media. (**c**) Bar graphs depicting the number of iPSC colonies generated from indicated AML samples using conventional or naïve reprogramming conditions. Bars represent mean $\pm$ SD of three technical replicates; *$p<0.05$. (**d**) FISH performed in iPSC colonies derived from indicated AML samples using conventional or naïve reprogramming conditions. AML 15331 iPSCs derived in naïve conditions were the only iPSCs that possessed the aberration detected in the patients' matched BM. $\geq$ 50 nuclei were scored per iPSC line. (**e**) Diagram detailing the frequency of the leukemic aberration in each AML BM and the percentage of derivative iPSC lines which carried the same aberration. The number of iPSC lines analyzed is indicated within each pie chart.

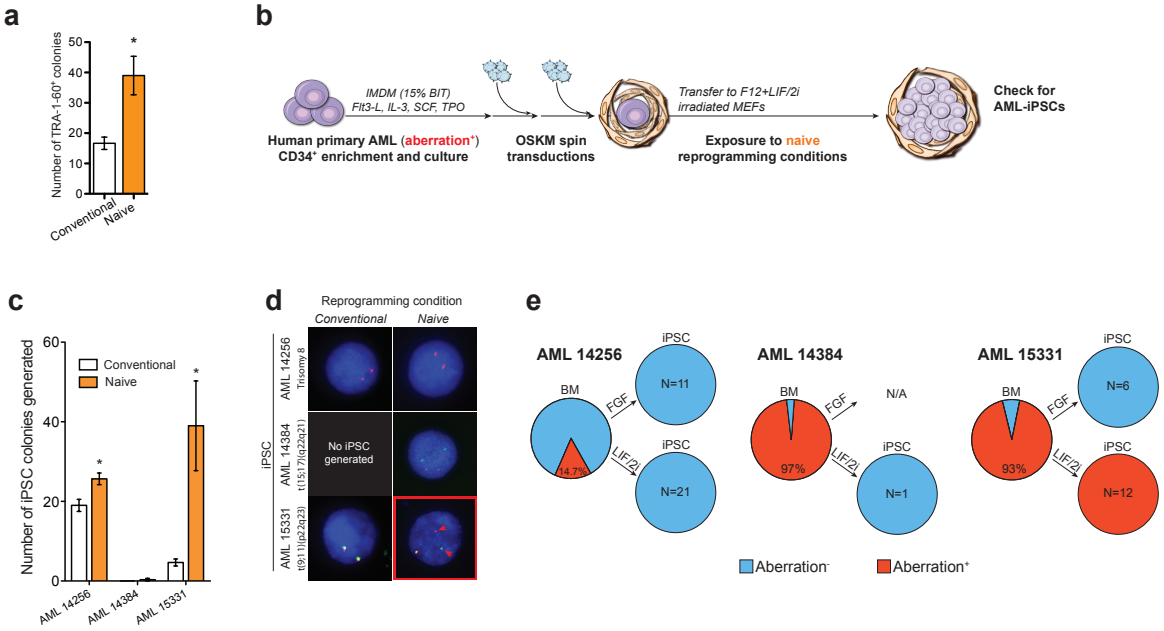

a
Number of TRA-1-60+ colonies
50
40
30
20
10
0
*
Conventional
Naive
b
IMDM (15% BIT)
Flt3-L, IL-3, SCF, TPO
Human primary AML (aberration+)
CD34+ enrichment and culture
OSKM spin
transductions
Transfer to F12+LIF/2i
irradiated MEFs
Exposure to naive
reprogramming conditions
Check for
AML-iPSCs
c
Number of iPSC colonies generated
60
40
20
0
Conventional
Naive
*
*
AML 14256
AML 14384
AML 15331
d
Reprogramming condition
Conventional
Naive
iPSC
AML 14256
Trisomy 8
AML 14384
t(15;17)(q22q21)
No iPSC
generated
AML 15331
t(9;11)(p22;q23)
e
AML 14256
BM
FGF
LIF/2i
iPSC
N=11
iPSC
N=21
14.7%
AML 14384
BM
FGF
N/A
LIF/2i
iPSC
N=1
97%
AML 15331
BM
FGF
iPSC
N=6
LIF/2i
iPSC
N=12
93%
Aberration-
Aberration+

Figure 3: AML iPSCs express the MLL-AF9 fusion transcript and exhibit dysfunctional *in vivo* differentiation capacity. (**a-b**) BF and ICC images depicting three independent, TRA-1-60^{+} AML iPSC lines derived from AML 15331 BM using naïve reprogramming conditions. Scale bars represent 100 μm. (**c**) Flow cytometric plots demonstrating that AML iPSCs express pluripotent markers TRA-1-60, SSEA3, OCT4, SOX2 and NANOG. (**d**) Representative image of a harvested tumour at 8 weeks post-injection of AML iPSCs. (**e**) Hematoxylin and eosin staining demonstrating the generation of ectoderm and endoderm lineages *in vivo*. Endoderm was infrequently observed, while mesoderm lineages were not detected. n=4 mice injected. (**f**) Regions of primitive, undifferentiated cell types were observed in tumours derived from AML iPSCs. (**g**) The MLL-AF9 fusion transcript is expressed in AML iPSCs as detected by RT-PCR.

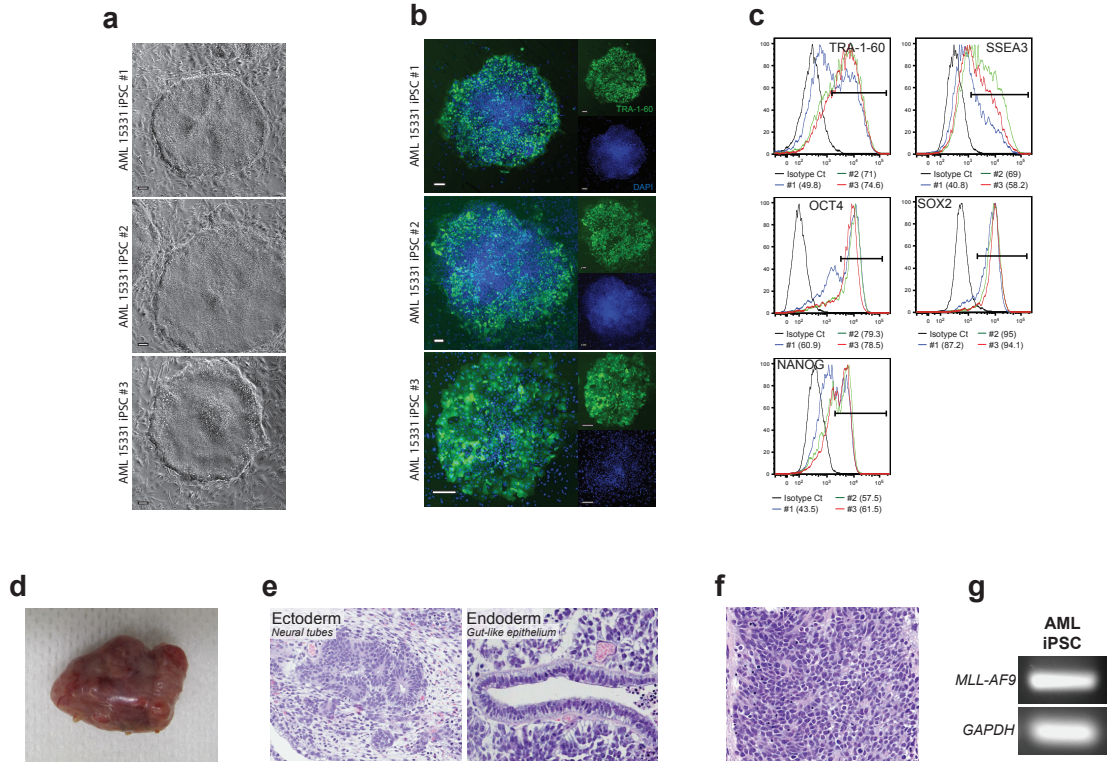

a
AML 15331 iPSC #1
AML 15331 iPSC #2
AML 15331 iPSC #3
b
AML 15331 iPSC #1
AML 15331 iPSC #2
AML 15331 iPSC #3
TRA-1-60
DAPI
c
TRA-1-60
SSEA3
OCT4
SOX2
NANOG
Isotype Ct
#1 (49.8)
#2 (71)
#3 (74.6)
#1 (40.8)
#2 (69)
#3 (58.2)
#1 (60.9)
#2 (79.3)
#3 (78.5)
#1 (87.2)
#2 (95)
#3 (94.1)
#1 (43.5)
#2 (57.5)
#3 (61.5)
d
e
Ectoderm
Neural tubes
Endoderm
Gut-like epithelium
f
g
AML iPSC
MLL-AF9
GAPDH

Figure 4: AML iPSCs recapitulate dysfunctional differentiation features observed in primary leukemic sample. (**a**) Schematic illustrating how the generation of normal blood- and AML-derived isogenic iPSCs generated from AML 15331 BM allows for direct comparison of their hematopoietic differentiation capacities. (**b**) Representative flow cytometric plots analyzing $CD34^{+}CD45^{+}$ expression following hematopoietic differentiation of isogenic normal (top) and AML (bottom) iPSCs. (**c**) Frequency of generation of $CD34^{+}CD45^{+}$ cells from AML iPSCs is significantly lower compared to normal iPSCs derived from the same patient. Lines represent mean $\pm$ SEM; normal iPSC: N=6 iPSC lines, n=1 differentiation attempt per line; AML iPSC: N=8 lines, n=2 differentiation attempts per line. (**d**) Frequency of hematopoietic colony types generated from indicated sources. Bars represent mean $\pm$ SEM; normal iPSC-EBs, N=6; AML iPSC-EBs, N=1. (**e**) Representative images and single cell morphologies of granulocytic and monocytic colonies derived from primary AML BM, AML iPSCs and Normal iPSCs from Patient 15331. Stage of maturation based on morphological assessment is indicated above single cell morphologies. White scale bars represent 100 μm, black scale bars represent 10 μm. (**f**) Flow cytometric analyses depicting the frequencies of maturing granulocytes ($CD11b^{+}$ subset of $CD15^{+}$ population) and mature monocytes ($CD11b^{+}CD14^{+}$) in pooled CFUs from indicated sources. (**g**) Bar graphs depicting the frequency of $CD15^{+}CD11b^{+}$ maturing granulocytes and $CD11b^{+}CD14^{+}$ mature monocytes generated from normal and AML iPSCs. Levels of indicated populations generated from matched primary AML BM sample are demarcated by red dotted lines through the y-axis. Bars represent the mean $\pm$ SD from three total CFU wells. (**h**) FISH

performed in total hematopoietic colonies derived from primary AML, AML iPSCs and Normal iPSCs depicting presence or absence of t(9;11)(p22q23). Translocation involving chromosome 11q23 is denoted by red arrows. 25 nuclei scored per sample. (**i**) RT-PCR using primers specific to the MLL-AF9 fusion mRNA revealing that the cytogenetic aberration detected in the AML BM is expressed in AML iPSC- but not normal iPSC-derived hematopoietic cells.

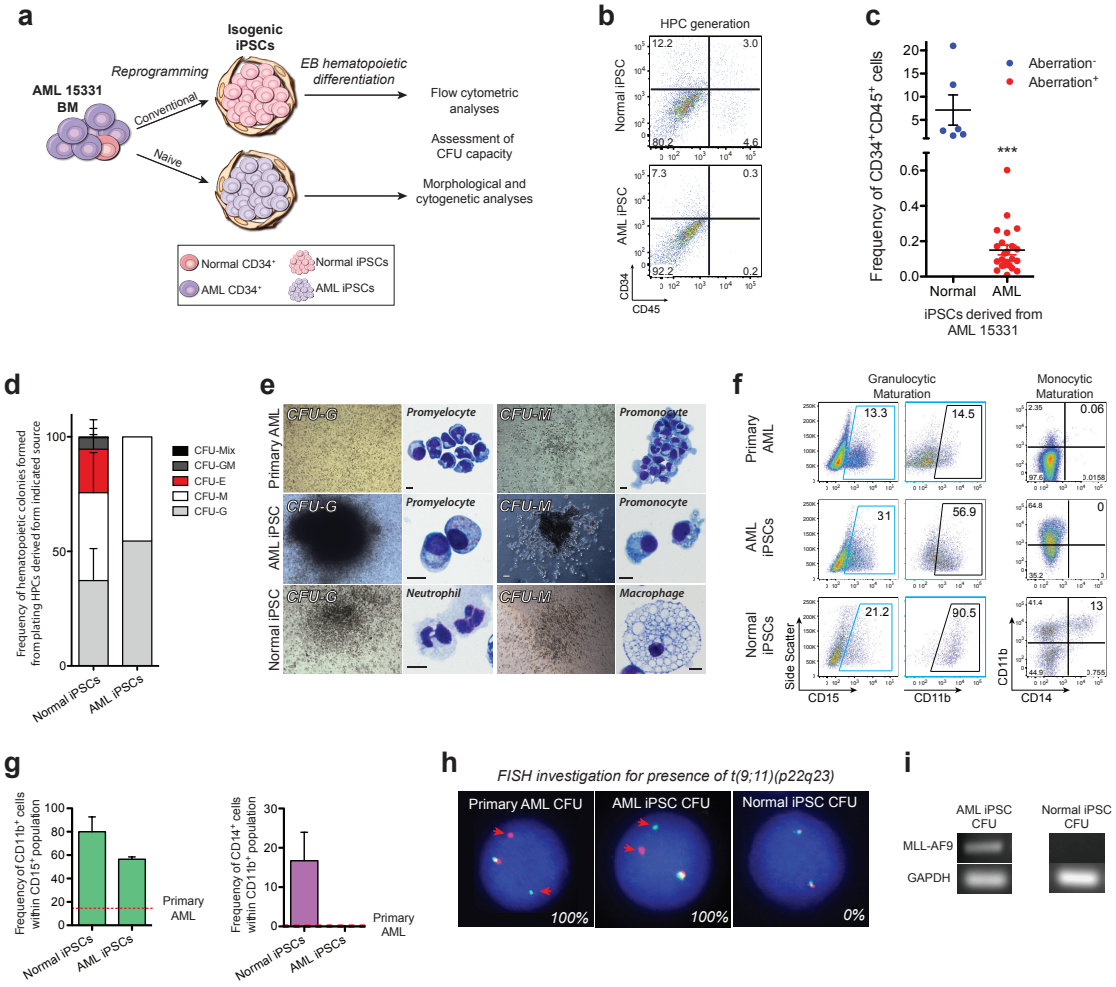

a
Isogenic iPSCs
Reprogramming
AML 15331 BM
Conventional
Naive
EB hematopoietic differentiation
Flow cytometric analyses
Assessment of CFU capacity
Morphological and cytogenetic analyses
Normal CD34+
AML CD34+
Normal iPSCs
AML iPSCs
b
HPC generation
Normal iPSC
AML iPSC
CD34
CD45
12.2
3.0
80.2
4.6
7.3
0.3
92.2
0.2
c
Frequency of CD34+CD45+ cells
Aberration-
Aberration+
***
Normal
AML
iPSCs derived from AML 15331
d
Frequency of hematopoietic colonies formed from plating HPCs derived form indicated source
CFU-Mix
CFU-GM
CFU-E
CFU-M
CFU-G
Normal iPSCs
AML iPSCs
e
Primary AML
AML iPSC
Normal iPSC
CFU-G
Promyelocyte
CFU-M
Promonocyte
Neutrophil
Macrophage
f
Granulocytic Maturation
Monocytic Maturation
Primary AML
AML iPSCs
Normal iPSCs
Side Scatter
CD15
CD11b
CD14
13.3
14.5
2.35
0.06
97.6
2.0158
31
56.9
64.8
0
35.2
21.2
90.5
41.4
13
44.9
7.755
g
Frequency of CD11b+ cells within CD15+ population
Frequency of CD14+ cells within CD11b+ population
Primary AML
Normal iPSCs
AML iPSCs
h
FISH investigation for presence of t(9;11)(p22q23)
Primary AML CFU
AML iPSC CFU
Normal iPSC CFU
100%
100%
0%
i
AML iPSC CFU
Normal iPSC CFU
MLL-AF9
GAPDH

Figure 5: Potential applications and future directions of AML iPSCs. (**a**) Diagram illustrating the proof of principle presented in the current study that AML iPSCs can be generated using modified reprogramming conditions, with future investigations into developing robust cancer cell reprogramming conditions and applications to drug screening indicated. Further understanding how the combination of MLL-AF9 expression and naïve reprogramming conditions enabled the generation of AML iPSCs may allow for the establishment of reprogramming conditions that enable robust reprogramming across primary human AML samples. Moreover, the ability to generate isogenic normal and dysfunctional AML iPSCs and HPCs from the same patient should allow for *in vitro* drug screening studies and identification of underlying mechanisms of disease.

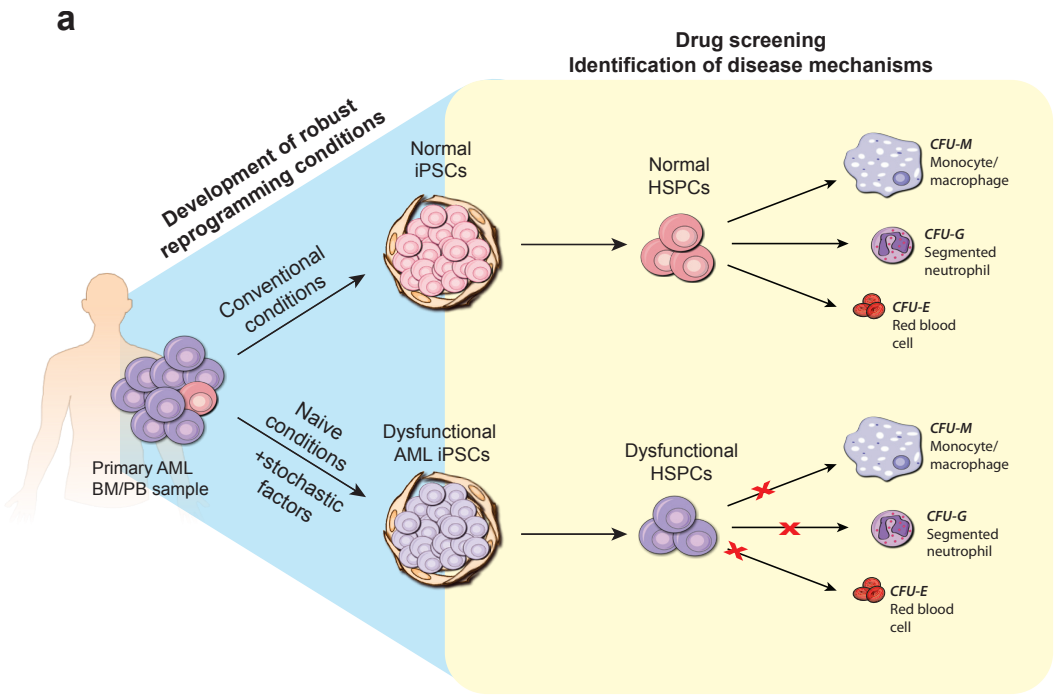

a
Drug screening
Identification of disease mechanisms
Development of robust reprogramming conditions
Normal iPSCs
Conventional conditions
Normal HSPCs
CFU-M
Monocyte/ macrophage
CFU-G
Segmented neutrophil
CFU-E
Red blood cell
Primary AML BM/PB sample
Naive conditions +stochastic factors
Dysfunctional AML iPSCs
Dysfunctional HSPCs
CFU-M
Monocyte/ macrophage
CFU-G
Segmented neutrophil
CFU-E
Red blood cell

Table 1. Primary AML peripheral blood samples used in conventional reprogramming experiments.

Patient Sample	Disease Status	Source	Blast Frequency	Leukemic Aberration(s)
A137	Diagnosis	PB	46%	del(7)(p11.2p15)
A151.1	Diagnosis	PB	60%	inv(16)(p13.1q22)/sl,+8
13145	Diagnosis	PB	94%	N/A
15179.2	Relapse	PB	94%	NPM, FLT3-ITD
15328.1	Diagnosis	PB	23%	t(X;11)(q28;q12), -2, del(3)(q11.2), -5, add(7)(p11.2), -17, +3~5mar; cp
15407	Diagnosis	PB	85%	del(7)(q32), -8, -9, +11, +12, +14, -22; cp

Abbreviations: cp, composite karyotype; N/A, not available; PB, peripheral blood; sl, stem line

Table 2. Primary AML bone marrow samples used in conventional reprogramming experiments.

Patient Sample	Disease Status	Source	Blast Frequency	Leukemic Aberration(s)
10566.1	Diagnosis	BM	> 70%	FLT3-ITD
11555	PMF to AML	BM	35%	JAK2
14256	Diagnosis	BM	> 90%	+8, +13, FLT3-ITD
14384	Diagnosis	BM	69%	t(15;17)(q22;q21)
15331	Diagnosis	BM	> 90%	t(9;11)(p22q23)
19403	Diagnosis	BM	98%	+4, del(16)(q22)

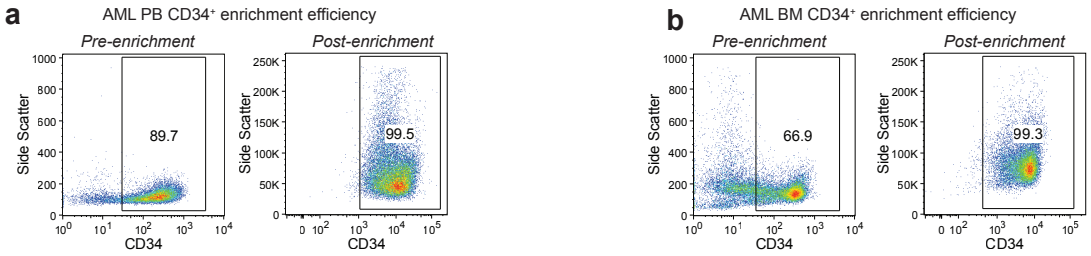
Abbreviations: AML, acute myeloid leukemia; BM, bone marrow; PMF, primary myelofibrosis
Rows highlighted in grey indicate that iPSCs were generated from these samples using conventional reprogramming methods. However, these iPSCs were only derived from normal blood cells as they were devoid of the specified leukemic aberration found in AML blasts.

Table 3. Observations of clonogenic HPC capacity of isogenic iPSC derivatives following differentiation to the hematopoietic lineage *in vitro*.

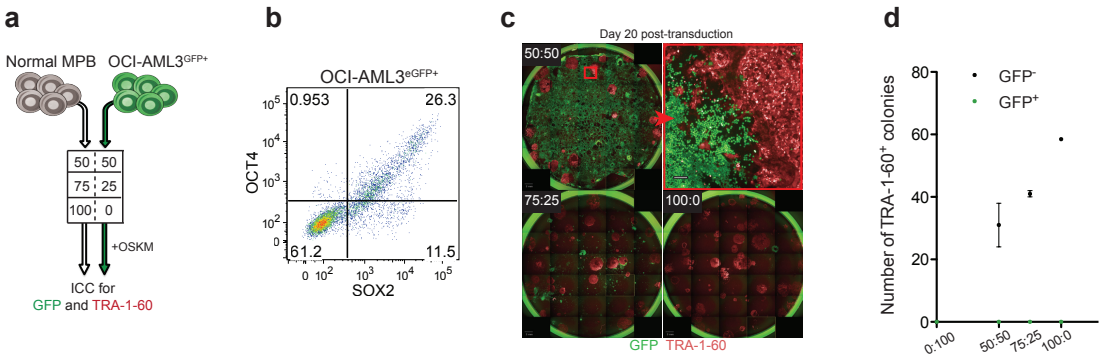
Normal iPSC$^{MLL\text{-}AF9-}$	CFU capacity?	AML iPSC$^{MLL\text{-}AF9+}$	CFU capacity?
N1	Yes	N1	No
N2	Yes	N2	No
N3	Yes	N3	No
N4	Yes	N4	Yes*
N5	Yes	N5	No
N6	Yes	N6	No
		N7	No
		N8	No

n=2 independent differentiation experiments per AML iPSC line, n=1 per normal iPSC line
*indicates that CFU capacity was observed in 1 differentiation experiment

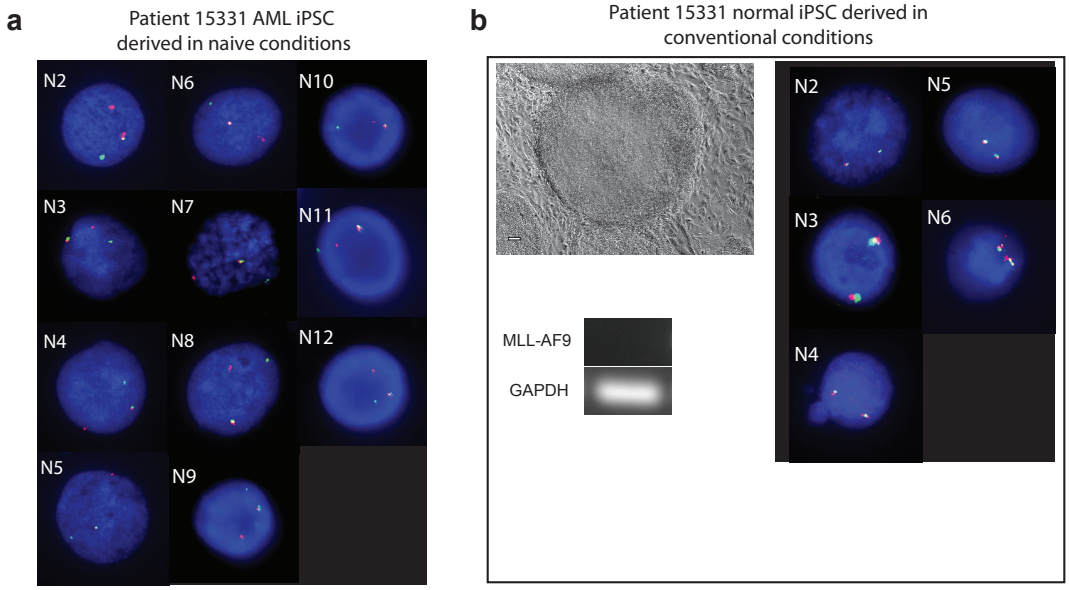
Supplementary Figure 1: Validation of $CD34^+$ enrichment in primary AML PB and BM samples. (**a**) Representative flow cytometric plots depicting CD34 expression in AML samples before and after CD34 enrichment.


a
AML PB CD34+ enrichment efficiency
Pre-enrichment
Post-enrichment
Side Scatter
CD34
89.7
99.5
b
AML BM CD34+ enrichment efficiency
Pre-enrichment
Post-enrichment
Side Scatter
CD34
66.9
99.3

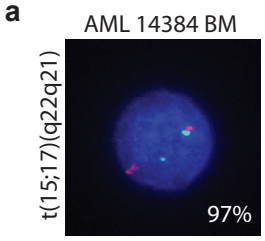
Supplementary Figure 2: OCI-AML3 cell line is refractory to reprogramming despite exposure to conventional reprogramming conditions that are sufficient for iPSC generation from normal MPB. (**a**) Schematic illustrating experimental design in which indicated ratios of OCI-AML3^{GFP+} cells (sorted on GFP^{+} expression) and normal MPB cells were combined, transduced with OSKM, cultured in conventional reprogramming conditions and assessed for the acquisition of pluripotency as demarcated by TRA-1-60 expression. GFP expression was used to distinguish OCI-AML3 cells from normal MPB cells. (**b**) Flow cytometric plot depicting the efficient transduction and expression of OCT4-SOX2 in OCI-AML3. (**c**) Full well images of 50:50, 25:75, and 0:100 depicting GFP and TRA-1-60 expression. GFP^{+}TRA-1-60^{+} (OCI-AML3 derived) colonies were not observed in any well format. Image with yellow border is 10x magnification of indicated region in 50:50 well. (**d**) Total number of TRA-1-60^{+} colonies detected per indicated well. Error bars represent n=2 technical replicates.


a
Normal MPB
OCI-AML3GFP+
50 50
75 25
100 0
+OSKM
ICC for
GFP and TRA-1-60
b
OCI-AML3eGFP+
0.953
26.3
61.2
11.5
OCT4
SOX2
c
Day 20 post-transduction
50:50
75:25
100:0
GFP TRA-1-60
d
GFP-
GFP+
Number of TRA-1-60+ colonies
0:100
50:50
75:25
100:0

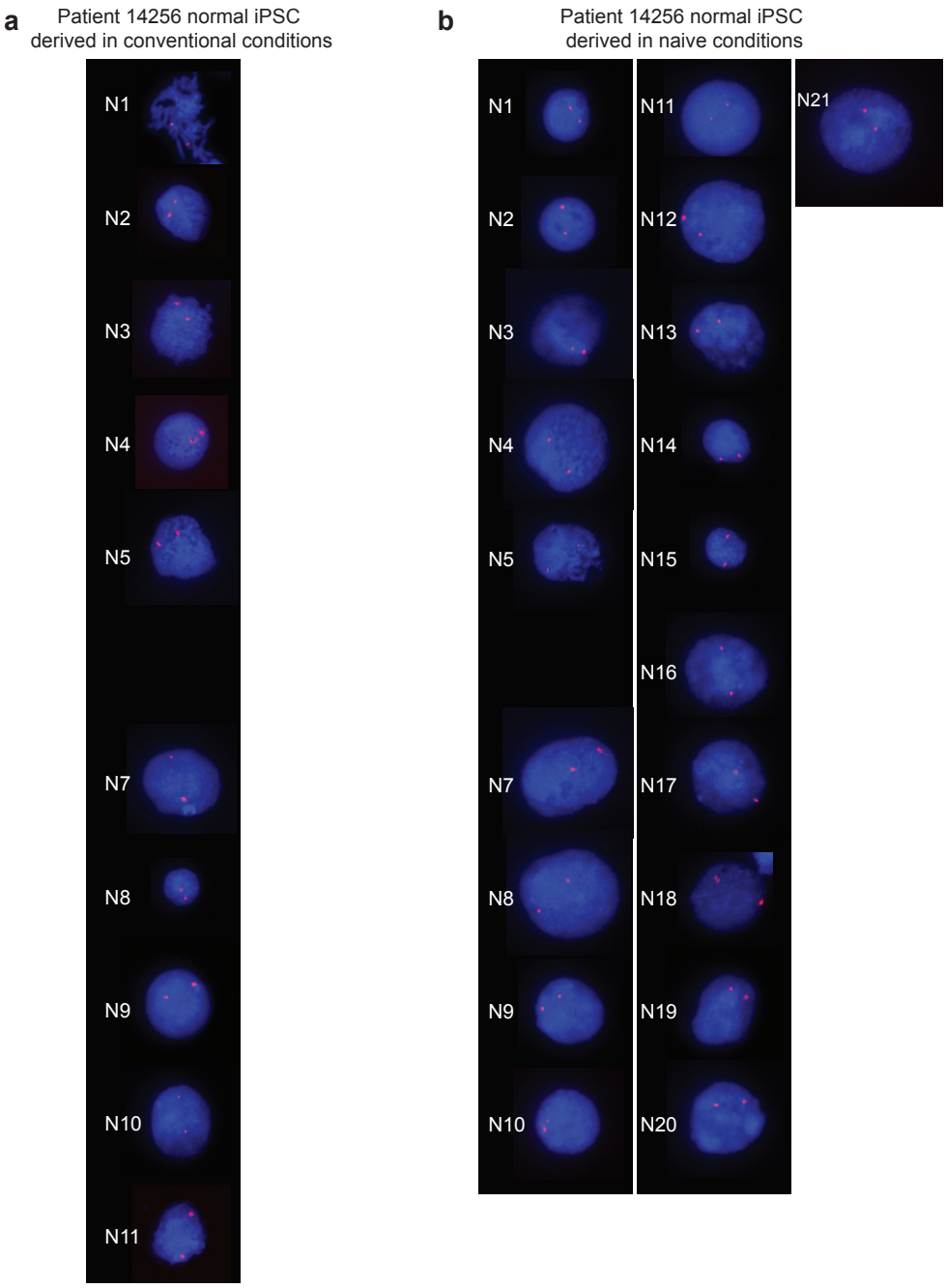
Supplementary Figure 3: Generation of AML and normal iPSCs from AML 15331 BM. (**a**) FISH depicting the presence of leukemic aberration in all AML 15331 iPSC lines derived in naïve reprogramming conditions suggesting their leukemic origin. (**b**) FISH performed in iPSCs derived from AML 15331 BM in conventional reprogramming conditions reveals the absence of leukemic aberration in all lines, suggesting their normal blood cell origins. RT-PCR confirms the absence of the leukemic aberration in these normal iPSCs.


a
Patient 15331 AML iPSC derived in naive conditions
N2
N6
N10
N3
N7
N11
N4
N8
N12
N5
N9
b
Patient 15331 normal iPSC derived in conventional conditions
MLL-AF9
GAPDH
N2
N5
N3
N6
N4

Supplementary Figure 4: AML 14384 BM harbours the PML/RARα mutation. (**a**) FISH performed in AML 14384 BM confirms the presence of the t(15;17)(q22q21) leukemic aberration, indicated by the co-localization of the PML (red) and RARα (green) genes, in 97% of the cells. The iPSC line generated from this sample (**Fig. 1c-e**) was devoid of the leukemic aberration. 200 nuclei were scored.


a
AML 14384 BM
t(15;17)(q22q21)
97%

Supplementary Figure 5: Generation of normal iPSCs from AML 14256 BM using conventional and naïve reprogramming conditions. (**a**) FISH performed in iPSC lines derived from AML 14256 BM sample do not harbour the trisomy 8 mutation found in the leukemic cells (**Fig. 1b**). ≥ 50 nuclei were scored per iPSC line.

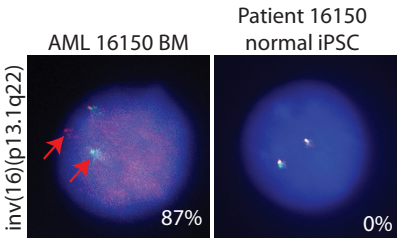

a
Patient 14256 normal iPSC
derived in conventional conditions
N1
N2
N3
N4
N5
N7
N8
N9
N10
N11
b
Patient 14256 normal iPSC
derived in naive conditions
N1
N2
N3
N4
N5
N7
N8
N9
N10
N11
N12
N13
N14
N15
N16
N17
N18
N19
N20
N21

Supplementary Figure 6: Naïve reprogramming conditions do not selectively enable generation of AML iPSCs from other AML patient BM samples. (**a**) Reprogramming using naïve conditions was attempted in two additional AML BM samples to further assess if naïve conditions selectively enabled AML iPSC generation. (**b**) Representative FISH image depicting that only normal iPSCs were generated from AML 16150 BM, as they did not possess the leukemic mutation depicted by loss of co-localization of chromosome 16q22-specific green and red probes. iPSCs were not generated from AML 16158.1 BM.

a

Patient Sample	Disease Status	Source	Blast Frequency	Aberration
16150	Diagnosis	BM	> 90%	inv(16)(p13.1q22)
16158.1	Diagnosis	BM	> 80%	del5q22q33, -7

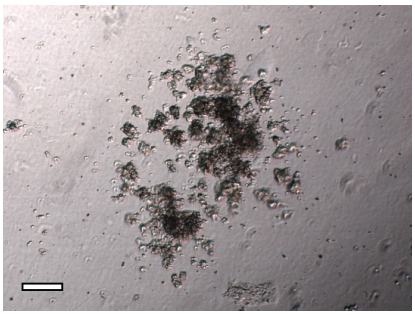
b



Supplementary Figure 7: Normal iPSCs generated from AML 15331 BM differentiate to HPCs that are capable of forming the erythrocytic lineage. (**a**) Representative image of a BFU-E colony derived from plating normal iPSC-derived HPCs in CFU assay. Scale bar represents 100 μm.

a

Patient 15331 normal iPSC-derived BFU-E



CHAPTER 5

DISCUSSION

5.0 Preamble

This thesis set out to investigate whether cellular reprogramming technology had the potential to address unmet needs in the field of human AML research. I hypothesized that ***cellular reprogramming of human AML patient somatic cells to iPSCs is possible and will enable derivation of autologous sources of normal and dysfunctional HPCs.*** I believed that investigating this hypothesis would provide initial proof of concepts and insights in the human AML system required for future development of novel sources of safe HSPCs for transplantation and dysfunctional hematopoietic cells for practical *in vitro* disease modeling and drug development. Ultimately my hypothesis was correct, as we were able to generate normal and dysfunctional hematopoietic progenitors through reprogramming human AML patient fibroblasts and leukemic cells to iPSCs, respectively (**Chapter 2** and **4**). While testing this hypothesis we also encountered and shared initial insights into the current limitations of applying cellular reprogramming to human AML somatic cells, which should be addressed in future experiments. Here I discuss the significance, limitations and future directions of our findings, and share additional insights into other uses cellular reprogramming technologies may have in addressing novel questions in AML research.

5.1 Addressing the clinical shortage of hematopoietic cells with AML patient-specific iPSCs

We first demonstrated that generation of AML patient-specific iPSCs from dermal fibroblasts was possible, and allowed for derivation of $CD34^{+}CD45^{+}$ HPCs devoid of leukemia-associated aberration(s) and capable of normal differentiation *in vitro,* unlike matched patient leukemic cells (**Chapter 2**). These findings established the proof of principle that cellular reprogramming allows for the generation of normal autologous hematopoietic progenitors from AML patients, suggesting that iPSCs may represent a suitable source of healthy autologous cells to address the clinical shortage of blood cells required for transplantation.

The combination of hematopoietic-specifying differentiation conditions and ectopic HoxB4 expression enables generation of HSPCs with long-term reconstitution capacity from mPSCs (Kyba et al., 2002; Wang et al., 2005c). Although this did not apply to the human system (Wang et al., 2005a), it continued to encourage efforts using hPSCs. As such, recent differentiation protocols that use extrinsic and intrinsic hematopoietic-specifying factors provide evidence that generation of transplantable HSPCs from hPSCs is possible (Amabile et al., 2013; Doulatov et al., 2013; McIntyre et al., 2013; Suzuki et al., 2013). As I briefly discussed in **Chapter 2**, our findings should continue to motivate these efforts toward robust and practical generation of transplantable HSPCs. Conversely, these current efforts (Amabile et al., 2013; Doulatov et al., 2013; McIntyre et al., 2013; Suzuki et al., 2013), although not practical for clinical implementation, should allow for next-step investigations required to further understand the potential clinical utility of

AML Fib iPSCs. For instance, *in vivo* differentiation conditions in the mouse that mimic the human BM niche (Amabile et al., 2013; Suzuki et al., 2013) could be used to generate transplantable HSPCs from AML Fib iPSCs. This would enable longitudinal studies in mice to evaluate the ability of the generated AML patient-specific HSPCs to reconstitute and sustain normal hematopoiesis in primary and secondary mouse transplants. Combination with next-generation whole genome sequencing technologies would provide further insights into the long-term genetic stability of AML Fib iPSC-derived HSPCs. Together, these studies would provide further evidence required toward addressing the clinical need for HSPCs using cellular reprogramming.

Independent of the advances required for the generation and evaluation of safe transplantable HSPCs, AML patient-specific iPSCs and derivative HPCs represent cell sources that may have a more immediate clinical impact. Repeated blood transfusions are an essential component of AML management to combat anemia, bleeding and infection (Dawson et al., 2007; Lowenberg et al., 1999; Perl and Carroll, 2007). However, a substantial number of donors are required on a per patient basis to meet these demands (Dawson et al., 2007), and many patients become refractory to transfusion products due to alloimmunization (Schiffer, 2001). We have demonstrated that AML patient-specific HPCs are capable of differentiation to mature myeloid lineages devoid of leukemia-associated aberration (**Chapter 2**). As such, transplantation of these autologous HPCs could serve as a means of providing short-term hematopoietic recovery in AML patients throughout therapy. Alternatively, AML Fib iPSCs could be utilized as a platform from which to derive platelets and RBCs that could be used as transfusion products.

Independent groups have recently demonstrated that megakaryocyte progenitors can be established from human iPSCs (Feng et al., 2014; Nakamura et al., 2014). These megakaryocytes generate platelets *in vitro* that are functionally capable of contributing to formation of blood clots *in vivo* following transfusion into mice (Feng et al., 2014; Nakamura et al., 2014). Similarly, enucleated RBCs with oxygen-carrying capacity have been derived from hESCs (Lu et al., 2008). Although these RBCs predominantly express fetal hemoglobins and resemble RBCs found in the early embryo, our group has recently demonstrated that temporal inhibition of the Hedgehog pathway during hPSC differentiation can enable derivation of RBCs that express adult hemoglobins (McIntyre et al., 2013). Accordingly, generation of AML patient-specific transfusion products using these methods may have immediate clinical utility for AML patients.

Considering the above discussion, I envision that AML patient-specific iPSCs (**Chapter 2**) have the potential to provide a novel source of autologous blood cells that circumvent obstacles associated with current transplantation and transfusion sources. AML Fib iPSCs are autologous, devoid of leukemia-associated aberrations and differentiation features, and are capable of unlimited growth *in vitro*, thereby eliminating the difficult task of finding compatible donors, the risk of immune-responses and leukemic reinfusion, and the shortage of HSPCs that limit current transplantation therapies. Ultimately, our findings (**Chapter 2**) and future perspectives discussed above should enable more AML patients to receive safe HSPC transplantations and blood transfusions during therapy that will increase their rates of disease-free survival and quality of life.

5.2 Developing unique reprogramming conditions that allow for robust generation of iPSCs from diverse primary AML samples

During our attempts to generate disease-specific iPSCs, we discovered that cellular reprogramming of human AML patient leukemic cells is difficult to achieve (**Chapter 4**). Although other groups have also experienced that cancer cell reprogramming is highly inefficient (Carette et al., 2010; Kim et al., 2013; Kim and Zaret, 2015; Kumano et al., 2012), and that only a minority of primary patient samples are capable of undergoing reprogramming to iPSCs (Kim et al., 2013; Kim and Zaret, 2015; Kumano et al., 2012; Stricker et al., 2013), the basis for failure was not experimentally explored within these papers. To our knowledge, we have provided the first experimental insights on the difficulties of reprogramming human cancer cells by demonstrating that reprogramming blockade occurs downstream of reprogramming TF delivery and expression, and can be overcome in one AML sample when conventional reprogramming conditions are modified (**Chapter 4**). However, this limitation needs to be experimentally addressed before reprogramming conditions that facilitate robust reprogramming across diverse AML samples can be developed.

First, why is AML so difficult to reprogram? Despite stable expression of pluripotency TFs required for reprogramming we were unable to generate iPSCs using conventional reprogramming conditions. Perhaps abnormal epigenomes associated with the majority of AML samples (The Cancer Genome Atlas Research Network, 2013) prevent OSK from accessing and activating pluripotency genes during the first 48 hours of reprogramming (Soufi et al., 2012) or are unable to undergo extensive chromatin

remodelling required for pluripotency acquisition (Buganim et al., 2012; Hussein et al., 2014; Koche et al., 2011; Rivera and Ross, 2013; Takahashi et al., 2007). Along these lines, previous works using heterokaryons indicate that the pluripotent state typically reprograms the somatic cell state, causing somatic cells to adopt epigenetic and transcriptional programs associated with pluripotency (Cowan et al., 2005; Tada et al., 2001). However, when Friend erythroleukemia cells were fused with pluripotent ECCs, the pluripotent cells adopted hematopoietic features and began to express hemoglobin genes (McBurney et al., 1978), suggesting that the leukemic epigenome may be more powerful than that of the PSCs and therefore, resistant to change. This is supported by further evidence using SCNT, whereby leukemic cells cannot be reprogrammed by the oocyte to allow for derivation of ESCs (Hochedlinger et al., 2004), and by our findings here that conventional reprogramming conditions are insufficient for iPSC generation. Further investigations that use chromatin-immunoprecipitation sequencing (chIP-seq) of OSK-bound regions, and that examine chromatin marks and DNA methylation throughout reprogramming of AML should help to delineate whether the AML epigenome is refractory to reprogramming toward establishing robust reprogramming methods. These studies will also yield invaluable insights into mechanisms that contribute to aberrant regulation of the AML epigenome.

Second, can barriers to AML reprogramming be overcome? Many AML samples possess unique epigenomic features that correlate with the cytogenetic and/or molecular aberrations they harbour (The Cancer Genome Atlas Research Network, 2013; Figueroa et al., 2010). It has also been demonstrated that experimental manipulation of chromatin

remodelling complexes can help overcome epigenetic barriers that exist for reprogramming normal cells to iPSCs (Rais et al., 2013). As discussed in **Chapter 4**, perhaps the expression of MLL-AF9 AML coupled with LIF/2i treatment provides an extensively demethylated/accessible chromatin state in which reprogramming roadblocks can be overcome. Attempts to reprogram additional AML samples harbouring MLL-AF9 in conventional versus naïve reprogramming conditions are required to establish whether this is the case. These experiments could be supplemented by mechanistic studies in which MLL-AF9 is ectopically expressed in AML samples that were previously refractory to reprogramming, to assess if MLL-AF9 mediates epigenetic changes that "prime" refractory AML samples for successful reprogramming to iPSCs. Along these lines, attempting to reprogram other AML samples such as those harbouring DNMT mutations (defective DNA methylation) (The Cancer Genome Atlas Research Network, 2013), or enforcing DNA and histone demethylation in other AML samples using epigenetic drugs such as valproic acid (VPA) and 3-deazaneplanocin A (DZNep) (Gottlicher et al., 2001; Milutinovic et al., 2007; Miranda et al., 2009; Sarkar et al., 2011) might provide additional insights into whether a "relaxed" AML epigenome is more conducive to reprogramming.

I believe that our initial experimental insights into the inefficiencies of AML cell reprogramming (**Chapter 4**) will lead to further investigation into these barriers, as discussed above, toward the development of more robust reprogramming methods that allow efficient iPSC generation from a range of clinically diverse AML samples (**Chapter 1: Tables 1** and **2**). This will not only provide model platforms in which to

perform mechanistic and drug screening studies in AML, as discussed in Section 5.3 below, but may also extend to other difficult-to-reprogram cancers. Moreover, I envision that better understanding why AML is difficult to reprogram may also reshape our understanding and treatment of AML. For instance, a mechanistic understanding of VPA's dual ability to increase reprogramming efficiency/allow for CML reprogramming and to act as a differentiation treatment of transformed cells *in vitro* (Dr. Keiki Kumano, personal communication; Gottlicher et al., 2001; Milutinovic et al., 2007) may identify unique molecular features of the leukemic state that could be therapeutically targeted. Therefore, I believe that developing robust reprogramming conditions will serve two purposes: to allow for establishment of AML modeling platforms and to provide key insights into unique molecular features of AML that together, improve our understanding and treatment of disease.

5.3 Using AML iPSCs to model disease

Through successful generation of AML iPSCs harbouring leukemia-associated aberration we found that their derivative HPCs were dysfunctional in their differentiation capacity (**Chapter 4**). Surprisingly, we discovered that AML iPSCs also exhibited limited differentiation abilities *in vitro* and *in vivo*, and maintained expression of the leukemia-associated aberration. Moreover, we were also able to generate isogenic normal iPSCs devoid of leukemia-associated aberration from the same AML patient that were capable of normal hematopoietic maturation. Together, these findings establish the proof of concept that cellular reprogramming allows for development of an *in vitro* model of disease that may allow for further investigation of disease mechanisms and development

of drug discovery platforms. Generation and characterization of additional AML iPSC lines as discussed above and in **Chapter 4** will be required to make more concrete statements about the utilities of AML iPSCs for disease modeling. However, here I discuss the potential applications of AML iPSCs as a novel model for the study of human AML based on our current findings.

The ability to achieve physiologically-relevant gene dosing of leukemia-associated aberrations is critical to evaluating their contributions and relevance to disease phenotype, as variations in gene expression resulting from retroviral over-expression or non-endogenous promoter regulation can lead to conflicting conclusions (Brown et al., 1997; Chen et al., 2008; Early et al., 1996; Krivtsov et al., 2006). As such, a more powerful method in which to understand the contributions of leukemic mutations may be to correct endogenous mutations that are under the control of normal regulatory circuitry and assess how this affects disease phenotype in the human system. However, these experiments would not have been possible prior to our findings here, as current culture methods are not sufficient to maintain primitive AML cells for genetic manipulation and subsequent analyses of functionality (Montesinos et al., 2006). Therefore, the ability to generate AML iPSCs that harbour endogenous leukemia-associated aberrations should provide a more suitable and renewable cellular platform in which to perform these studies. Accordingly, pairing targeted CRISPR-mediated correction of mutations in AML iPSCs with subsequent differentiation to $CD34^{+}CD45^{+}$ HSPCs for *in vitro* CFU and *in vivo* transplantation assays (Amabile et al., 2013; Chen et al., 2015; Doulatov et al., 2013; Suzuki et al., 2013), would allow for the first experimental opportunity to assess whether

AML cells could be reverse engineered back to normal HSPCs. These studies would offer novel insights into the contributions of endogenous leukemia-associated aberrations to disease phenotype, and may also allow for identification of other genetic contributors to disease. Moreover, in genetically complex AML samples, correcting mutations in a stepwise manner could help to identify their individual contributions to the disease phenotype. Due to the renewable nature of iPSCs, these studies would also be accompanied by large-scale analyses of chromatin marks, DNA methylation and global gene expression in AML iPSC- and corrected AML iPSC-derived HPCs to tease apart the contributions of the aberrations to the epigenome and transcriptome. Finally, by resetting the AML epigenome by reprogramming to pluripotency, this may allow for studies that elucidate how aberrant epigenetic profiles are established upon redifferentiation to the hematopoietic lineage. This may yield mechanistic insights into why unique epigenetic profiles are correlated with distinct leukemia-associated aberrations (The Cancer Genome Atlas Research Network, 2013; Figueroa et al., 2010). As such, I believe that cellular reprogramming of AML cells to iPSCs could hold great utility as a model system in which to advance our limited knowledge of the functional contributions of the many cytogenetic and molecular aberrations associated with the leukemic state.

With minimal changes to frontline disease treatment over the past 40 years, the poor therapeutic index of AraC, and a dismal rate of disease-free survival, novel therapeutics are required for the treatment of AML patients (Shipley and Butera, 2009; Tallman et al., 2005). Unfortunately, the lack of practical cell-based models of human AML has hindered the development of high-throughput drug screening platforms. The ability to

generate isogenic AML iPSCs and normal iPSCs (either from AML Fibs or normal HSPCs in the BM) from the same AML patient presents an opportunity to address this. We have demonstrated that AML iPSCs derived from a patient with the MLL-AF9 aberration possess limited differentiation ability, drawing a biological parallel to the dysfunctional primary leukemic cells (**Chapter 4**). Accordingly, this may provide a surrogate hPSC-based screening platform in which to identify AML-specific therapeutics similar to our previous demonstration that drug screening on transformed hESCs can effectively identify cancer stem cell-specific drugs (Sachlos et al., 2012). Therefore, performing preliminary screens for compounds that promote normal differentiation of AML iPSCs *in vitro* may identify compounds that also induce differentiation of primary leukemic cells. This concept may also apply to cell viability, whereby differential effects on AML versus normal iPSCs may allow for the discovery of small-molecules that selectively mediate cytotoxic effects in leukemic cells. The effects of candidate compounds could be subsequently validated using *in vivo* humanized mouse xenotransplants. We also determined that AML iPSCs actively expressed the MLL-AF9 fusion transcript. By using CRISPR technology to insert GFP downstream of the MLL-AF9 aberration, fluorescent-based assays could be developed to identify small-molecules that inhibit expression of the leukemic aberration. This would also provide a cellular context in which to explore drug mechanisms of action. Given the poor specificity of inhibitors of leukemic aberrations that are currently in use (Wander et al., 2014), this could also allow for better development of targeted therapies. Although the above assays would be more practical using AML iPSCs, they could also be performed using derivative

HPCs in the event that disease features are not recapitulated until hematopoietic differentiation is enforced. This is suggested based on the observations that CML iPSCs do not recapitulate disease features until differentiation to the hematopoietic lineage (Kumano et al., 2012). Ultimately, the development of robust reprogramming methods for AML samples may allow for the development of iPSC libraries that encompass the cytogenetic and molecular diversity of AML. Combined with the screening platforms described above, this may allow for the development of AML- and/or mutation-specific therapies that greatly advance the management and treatment of human AML similar to previous demonstrations with APL (Ades et al., 2010; Zhou et al., 2007).

Taken together, I envision that AML iPSCs represent suitable *in vitro* systems in which to begin understanding the functional contributions of the remarkable number of cytogenetic and molecular aberrations to disease pathogenesis/maintenance, and to develop much-needed therapies that selectively target AML. Considering the current lack of practical human cell-based model systems, I believe that our findings (**Chapter 4**) provide the first opportunities to perform these studies in the human system. This should ultimately translate to an increased understanding and more targeted treatment of disease that will improve our therapeutic management of AML and increase patient survival.

5.4 Capturing disease heterogeneity and evolution through iPSC generation

Little is known about the first hit mutations that predispose cells to become leukemic and whether these mutations occur in a common ancestral cell in earlier development or later on in the developed adults' blood compartment. The study of monochorionic twins, where only one twin developed leukemia, demonstrated that there exists a common

cellular, pre-natal, first hit mutation that bestows the potential for future tumorigenicity, but is insufficient for leukemia onset (Hong et al., 2008). This provided the first evidence for the existence of pre-leukemic cells harbouring the first hit mutations required for leukemogenesis, and suggested that the pre-leukemic compartment may persist and later reinitiate leukemia even after overt disease has been eradicated beyond detection. This is corroborated by recent work from the lab of John Dick, which identified that the DNMT3A mutation was found in "normal" HSPCs isolated from the BM of AML patients with $NPM1^{+}DNMT3A^{+}$ leukemic blasts (Shlush et al., 2014). These HSPCs appeared normal based on multilineage reconstitution capacity *in vivo*, but possessed an increased proliferative and repopulation advantage over non-mutated HSPCs. Moreover, they were able to survive chemotherapy. Together, these works indicate that pre-leukemic HSPCs exist within the AML BM compartment and are capable of acquiring secondary mutations that result in the development of overt leukemia. However, it remains unclear whether these pre-leukemic cells are predisposed to acquiring secondary mutations, and if additional pre-leukemic mutations exist in AML patient BMs.

During our attempts to generate AML iPSCs, we observed that reprogramming of AML patient BM cells also allows for generation of normal iPSCs, that is, iPSCs that were determined to be devoid of the leukemic aberration that we probed for using FISH. Moreover, these normal iPSCs demonstrated normal differentiation capacity to the hematopoietic lineage. These results suggest that these iPSC lines were derived from normal HSPCs residing within the leukemic BM (**Chapter 4**), and corroborate recent findings by Hoffman et al. who generated normal iPSCs from $t(8;21)^{+}$ AML BM samples

(Hoffmann et al., 2015). However, based on the above works by Hong et al. and Shlush et al., it remains possible that these "normal" iPSCs may have been derived from pre-leukemic HSPCs residing within the leukemic BM. The ability to also generate AML iPSCs that harbour leukemia-associated aberration (**Chapter 4**) and to derive AML Fibs which are devoid of leukemia-associated aberration (**Chapter 2**) from the same patient may provide an opportunity to better understand disease progression within AML patients. Accordingly, by performing whole genome sequencing in AML Fibs, "normal" iPSCs (≥10 independent clones in attempt to capture genetic heterogeneity) and AML iPSCs (≥10 independent clones) this may allow for mapping of clonal/genetic evolution of disease within a given AML patient to reveal insights into how disease progresses (Kern et al., 2002; Raimondi et al., 1993) or to allow for identification of novel pre-leukemic mutations that may be captured in "normal" iPSCs derived from the leukemic BM. If we are able to "capture" these normal, pre-leukemic and leukemic cell states through iPSC generation this could allow for functional and molecular studies of disease progression and/or screening for novel therapeutics to target pre-leukemic stem cells that are able to survive chemotherapy (Shlush et al., 2014). Moreover, we could assess whether pre-leukemic iPSCs are predisposed to acquiring secondary mutations toward modeling disease initiation events. Together, these studies may provide additional insights into pre-leukemia and mechanisms of disease progression.

By using cellular reprogramming to better understand leukemic progression, I envision that this will ultimately allow for the development of new clinical approaches designed to prevent the development of overt leukemia in humans. The identification of

novel molecular pre-leukemic mutations could serve as markers for routine diagnostic screening in at-risk populations, while an understanding of pre-leukemic progression to overt AML could allow for the development of therapies designed to prevent this transition by targeting pre-leukemic cells. Together, this may serve to identify and treat patients susceptible to developing overt AML before the devastating pathophysiological effects are felt, and effectively reduce the number of patients who suffer from AML.

5.5 Viewing leukemogenesis through a cellular reprogramming lens

Cellular reprogramming studies have taught us that cell fate can be altered, and that this can be achieved by stochastic cytoplasmic factors or defined TFs that reset the epigenome and transcriptome (Gurdon, 1962; Takahashi et al., 2007). If we think about leukemogenesis in this light, it is conceivable to think that normal hematopoietic cells are similarly being "reprogrammed" to leukemic cells *in vivo*. This may occur due to epigenetic modifications (DNMT, TET, MLL mutations), dysfunctional/over-expression of master regulators of hematopoiesis (RUNX1, CBFß) and/or proliferation and survival (FLT3, NPM1), with extrinsic support from the BM microenvironment. Here I pose questions that originate from viewing leukemogenesis through a cellular reprogramming lens toward developing novel experimental strategies to better understand AML progression.

In **Chapter 3**, I discovered that OCT4 TF delivery in combination with pluripotent-supportive culture conditions was minimally sufficient to induce pluripotency, but required prolonged culture as compared to conventional reprogramming using OSKM. These observations, combined with my experiences in **Chapters 2** and **4,** and review of

the reprogramming literature have indicated to me that 1) epigenetic barriers exist that prevent reprogramming from occurring in all somatic cells (Rais et al., 2013; Soufi et al., 2012); 2) many reprogramming cocktails can be used to induce pluripotency (Theunissen and Jaenisch, 2014), but OCT4 activation is a unifying theme required for this process; and 3) appropriate culture conditions are required to facilitate reprogramming (Mitchell et al., 2014a).

I now believe that these similar principles can be applied to leukemogenesis: 1) not all normal hematopoietic cells are capable of becoming leukemic following MLL-AF9 expression (Chen et al., 2008), suggesting that a permissive epigenetic context may be required for disease initiation in the leukemic cell of origin; 2) a number of leukemia-associated aberrations have been identified (Vardiman et al., 2009), suggesting that multiple leukemia "reprogramming factor" cocktails exist; and 3) leukemia has been associated with aberrant BM niche components (Raaijmakers et al., 2010; Walkley et al., 2007), suggesting that conducive "culture conditions" are required for leukemic "reprogramming." This latter principle is further illustrated by cases of donor cell leukemia where AML patient relapse occurs from leukemic initiation in donor cells, yet the donor remains healthy (Hertenstein et al., 2005; Wiseman, 2011).

Perhaps by better understanding: 1) why all hematopoietic cells are not amenable to becoming leukemic, we can develop therapies to prevent leukemogenesis; 2) what the roles of each of the leukemia "reprogramming factors" is, we can target their contributions to the initiation and maintenance of the leukemic state; 3) how the BM niche contributes to disease, we can therapeutically alter it so that leukemic

reprogramming cannot occur even if all of the "reprogramming factors" are expressed. Together, these insights may form the bases for future experiments designed to change how we view leukemia and to develop novel approaches for disease treatment that complement current and theoretical therapies discussed previously.

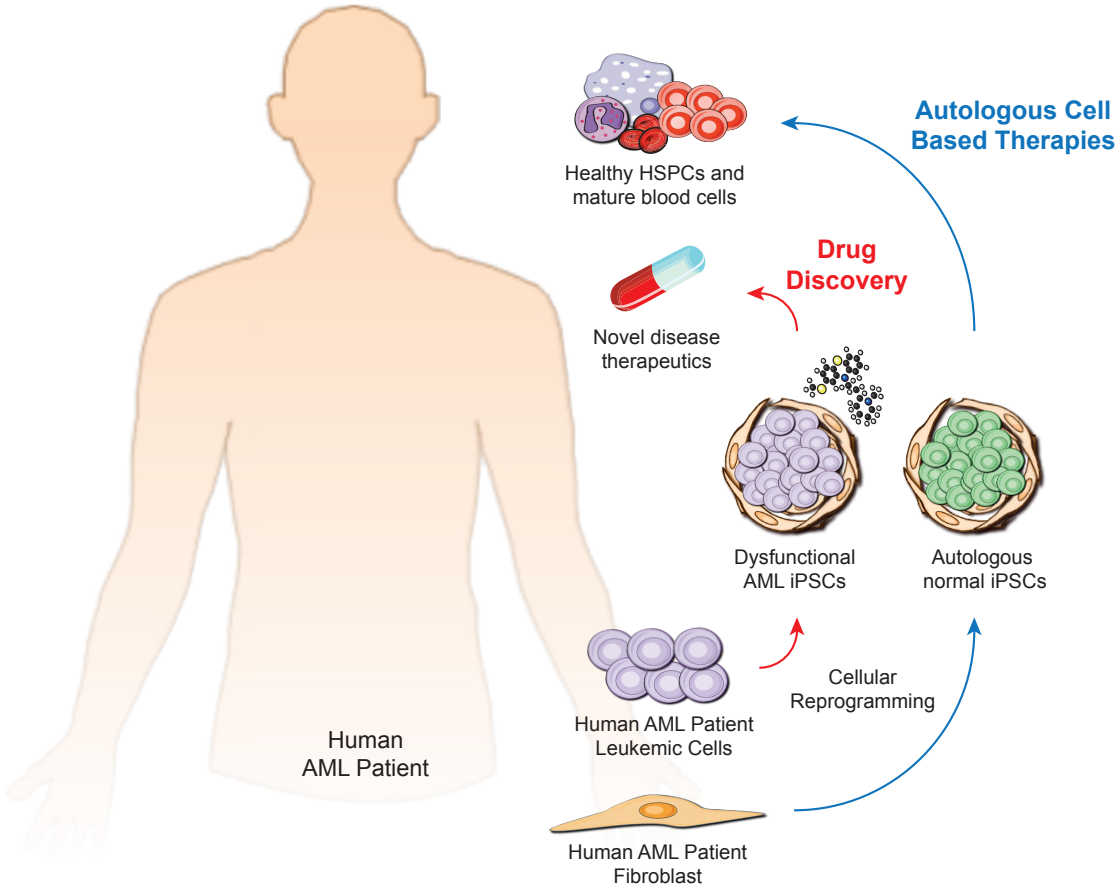
5.6 Concluding remarks

Since the first reported clinical cases of human AML in the 1940's the field has waged war on this cancerous infiltration of the human hematopoietic system (Tefferi, 2008). From advances in disease modeling to next-generation technologies, and guidance from years of clinical observation, we have come to better identify our enemy (Vardiman et al., 2009). However, this has not translated into meaningful clinical tactics, as current frontline chemotherapeutic treatments only serve to ward off AML for a short time before it re-emerges more aggressively (Shipley and Butera, 2009; Tallman et al., 2005). Although effective combat approaches have been exemplified by the success of HSPC transplantations (Burnett et al., 1998) and disease-selective therapies in cases of APL (Zhou et al., 2007), their large-scale adoption has been limited: safe sources of HSPCs for transplantation are rare, while our limited understanding of most AML cases has prevented further development of targeted therapies.

The recent advent of patient-specific cellular reprogramming technology potentially represents a novel source of reinforcements in this ongoing battle (Takahashi et al., 2007). In this thesis, we have performed the initial proof of concepts that cellular reprogramming of human AML patient somatic cells to pluripotency allows for the generation of normal (**Chapter 2**) and dysfunctional (**Chapter 4**) HPCs. These early insights should motivate

additional research efforts toward the development of much-needed transplantation sources and disease models/drug discovery platforms to reinforce and reshape how we currently attack AML (**Figure 1**). It is my belief that continued research into applications of cellular reprogramming technologies to human AML patient somatic cells will considerably bolster our ever-growing arsenal in the war against AML.

Figure 1. Cellular reprogramming of human AML patient somatic cells to pluripotency provides an opportunity for the development of cell-based therapies and disease models/drug discovery platforms. Limited sources of safe hematopoietic stem/progenitor cells (HSPCs) for transplantation and incomplete mechanistic understandings of disease initiation, progression and maintenance have impeded advances in therapy required for improvement of long-term AML patient survival rates. Toward addressing these unmet clinical needs, the ability to generate induced pluripotent stem cells (iPSCs) from human somatic cells may provide platforms from which to develop patient-specific (autologous) cell-based therapies and disease models. Here we provide the proof of principle that cellular reprogramming can be applied on a personalized basis to generate normal and dysfunctional HPCs from AML patient somatic cells. These foundational findings should motivate further studies aimed at developing iPSC-based autologous cell therapies and disease models for drug discovery toward improving AML patient quality of life and long-term survival rates.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Human AML Patient
Fibroblast
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Appendix II: List of Scientific Publications, Abstracts, and Patents

PUBLISHED REFERRED PAPERS

Salci KR, Lee JB, Mitchell RR, Orlando L, Fiebig-Comyn A, Shapovalova Z, Bhatia M. Derivation of human induced pluripotent stem cells through continued exposure of OCT4- induced plastic human fibroblasts to reprogramming media. Stem Cell Res. 2015 Jun 17;15(1):240-242. http://dx.doi.10.1016/j.scr.2015.06.005

Salci KR, Lee JB, Mitchell RR, Orlando L, Fiebig-Comyn A, Shapovalova Z, Bhatia M. Acquisition of pluripotency through continued environmental influence on OCT4-induced plastic human fibroblasts. *Stem Cell Res*. 2015 Jun 17;15(1):221-230. http://dx.doi.10.1016/j.scr.2015.06.006

Salci KR*, Lee JH*, Laronde S, Dingwall S, Kushwah R, Fiebig-Comyn A, Leber B, Foley R, Dal Cin A, Bhatia M. Cellular Reprogramming Allows Generation of Autologous Hematopoietic Progenitors From AML Patients That Are Devoid of Patient-Specific Genomic Aberrations. *Stem Cells*. 2015 Jun;33(6):1839-49. http://dx.doi.org/10.1002/stem.1994. **authors contributed equally*.

McIntyre BAS, Cantas A, Mechael R, **Salci KR**, Lee JB, Fiebig-Comyn A, Guezguez B, Wu Y, Sheng G, Bhatia M. Expansive generation of functional airway epithelium from human embryonic stem cells. *Stem Cells Transl Med*. 2014 Jan;3(1):7-17. http://dx.doi.org/10.5966/sctm.2013-0119

Boyd AL, **Salci KR**, Shapovalova Z, McIntyre BAS, Bhatia M. Non-hematopoietic cells represent a more rational target of *in vivo* hedgehog signaling affecting normal or acute myeloid leukemic progenitors. *Exp Hematol*. 2013 Oct;41(10):858-869.e4 http://dx.doi.org/10.1016/j.exphem.2013.05.287

Salci KR, McIntyre BAS, Bhatia M. Foundational concepts of cell fate conversion to the hematopoietic lineage. *Curr Opin Genet Dev*. 2013 Oct;23(5):585-90. http://dx.doi.org/10.1016/j.gde.2013.04.007

MANUSCRIPTS UNDER PEER REVIEW

Casado FL, **Salci KR**, Shapovalova Z, Guezguez B, Collins TJ, Bhatia M. Expansion of primary human AML by aryl hydrocarbon receptor antagonism minimally affects leukemic transcriptional profiles but alters cellular metabolism. Under review at *Experimental Hematology.*

Boyd AL, Aslostovar L, Fiebig-Comyn A, Shapovalova Z, Almakadi M, Reid J, Casado FL, **Salci KR**, Xenocostas A, Foley R, Leber B, Bhatia M. Cellular and Molecular Targeting of Recurrence in Acute Myeloid Leukemia. Under review at *New England Journal of Medicine.*

MANUSCRIPTS IN PREPARATION FOR SUBMISSION

Lee JH*, **Salci KR***, Tanasijevic B, Shapovalova Z, Fiebig-Comyn A, Bhatia M. Induced pluripotent stem cells and hematopoietic progenitors generated from primary human AML cells harbouring the MLL-AF9 leukemic aberration exhibit dysfunctional differentiation features. Prepared for submission to *Blood*. **authors contributed equally.*

Kushwah R, Guezguez B, Lee JH, **Salci KR**, Lee JB, Mechael R, Shapovalova Z, Fiebig-Comyn A, Makondo K, Bhatia M. Identification of human non-monocytic precursors capable of generating a unique dendritic cell repertoire for immunotherapy. Prepared for submission to *Science Translational Medicine*.