DIRECT CONVERSION OF FIBROBLASTS TO HEMATOPOIETIC PROGENITORS
THE DIRECT CONVERSION OF HUMAN FIBROBLASTS TO HEMATOPOIETIC PROGENITORS WITH LYMPHOPOIETIC POTENTIAL

By

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Abstract

The direct conversion of human fibroblasts to hematopoietic progenitors with lymphopoietic potential

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Immunodeficient-causing diseases such as HIV and leukemia have no cures, often require meticulous treatments and result in high morbidity or mortality. Although bone marrow transplants are an option for a subset of leukemia patients, the shortage of donors and the requirement for donor matching restricts the efficacy of this treatment option. Therefore there is a prominent clinical need for alternative sources of hematopoietic stem/progenitor cells with lymphopoietic potential. Recently we described the direct conversion of human dermal fibroblasts to multilineage hematopoietic progenitors by ectopic expression of OCT4. This direct conversion method was used to assess whether OCT4-transduced fibroblasts had the capacity to derive cells of the lymphoid lineage. This work shows the transient co-expression of CD34 and CD45 of fibroblasts within 7 days of OCT4 transduction followed by stable expression of CD45 on fibroblasts by day 15. The acquisition of hematopoietic markers, however, did not coincide with colony formation as previously described. Furthermore,
CD45+ cells that were enriched and cultured in hematopoietic conducive conditions did not acquire co-expression of CD34 as previously shown. Interestingly, CD34 expression was shown to be inversely correlated with OCT4 expression. Therefore the constitutive expression of OCT4 may have (1) inhibited the acquisition of CD34 expression on CD45+ cells (2) downregulated the expression of CD34 on the day 7 CD34+CD45+ fibroblasts, thereby resulting in the transient expression of these markers. Furthermore, this work shows that expression of CD45 on OCT4-transduced fibroblasts is required for survival on the MS5 stromal cell line used to support hematopoietic progenitors with lymphopoietic potential, while supplementation of CD45+ fibroblasts with hematopoietic progenitor supportive conditions resulting in co-expression of CD34 and CD45 is required for acquisition of CD19, a pan-B cell marker on CD45+ fibroblasts. These findings suggest OCT4-transduced fibroblasts have lymphopoietic potential.
I would like to first and foremost, thank my mentor and supervisor, Dr. Mick Bhatia for giving me the opportunity to work in a state-of-the-art facility with some of the greatest and, coincidently, smartest people in the world. Furthermore I’d like to thank Mick for challenging me to think critically and creatively endowing me with an invaluable innovative edge I will apply in all my future endeavors. Mick challenged me to improve on my weaknesses while fostering my strengths, undoubtedly making me a very competitive candidate in the workforce. Lastly, Mick taught me to strive for excellence, especially when it is difficult and rare, because otherwise it wouldn’t be excellence. For all these reasons, Mick I deeply thank you.

I would like to, next, thank my lab family. Although there are so many wonderful people that made working in this lab an absolute delight there are a few that have really touched my heart. Firstly, I would like to thank Eva Szabo for all her mentorship and guidance over the last 2 years, which, without, I’d still be trying to figure out how to put on my lab coat. Id also like to thank Eva for making my crude jokes sound like childsplay, I will sorely miss your candidness. I would like to thank Kyle Salci for being one of my best friends inside and out of the lab. We have laughed and almost cried together and the last two years just wouldn’t have been as amazing without you Kyle. There are no words for how thankful I am to have you as a friend. Lili Ostovar, you surprised me with your humor and kindness and friendship. You are a wonderful scientist and an even better friend, keep your head up, you will do amazing things with your scientific contributions.
I would like to thank my two best friends in the whole world; Emilie Harvey and Victor Rogozovsky. Emilie, you are truly the most selfless, caring and forgiving person I have ever met and it doesn’t hurt that I have just the most amount of fun with you. I strive daily to follow your example of what a person should be. I admire your free spirit and courage to do the things that would scare me. Your friendship has meant more to me than you will ever know, thank you boo! Victor, you are the most charismatic, entertaining and absolute fun person I have ever met. I trust no ones opinion or judgment more than yours and that will never change. You are just going to take the world by storm and I’ll be watching. I can’t thank you enough for being such a wonderful person in my life.

I would like to thank Melania Saakian and Jorge Rodriguez, my wonderful parents for encouraging and supporting me to follow my passion in science. You guys most definitely have gone through adversity to get where you are now and I could not be more proud of you. Thank you for giving Alex and I the best opportunity to lead happy and successful lives. I love you guys with all my heart.

Lastly, I would like to thank my broski, Alex Rodriguez, for being the best brother a sister could ever ask for. Your big sister hugs and kisses, although backbreaking, always brighten my day. Your intelligence and creativity at the age of 7 scares me but it also makes me so proud to know you are going to achieve unbelievably amazing things.
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### List of Abbreviations

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<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>7AAD</td>
<td>7-amino actinomycin</td>
</tr>
<tr>
<td>AGM</td>
<td>aorta-gonal-mesonephoros</td>
</tr>
<tr>
<td>Ascl1</td>
<td>Achaete-scute homolog 1</td>
</tr>
<tr>
<td>Atoh1</td>
<td>atonal homolog 1</td>
</tr>
<tr>
<td>BCR</td>
<td>b cell receptor</td>
</tr>
<tr>
<td>BMP</td>
<td>bone morphogenic protein</td>
</tr>
<tr>
<td>Brn2</td>
<td>brain-specific homeobox/POU domain protein 2</td>
</tr>
<tr>
<td>CB</td>
<td>cord blood</td>
</tr>
<tr>
<td>CEBPα</td>
<td>CCAAT/enhancer-binding protein alpha</td>
</tr>
<tr>
<td>CLP</td>
<td>common lymphoid progenitor</td>
</tr>
<tr>
<td>Dll1</td>
<td>Delta-like 1</td>
</tr>
<tr>
<td>DN</td>
<td>double negative</td>
</tr>
<tr>
<td>DP</td>
<td>double positive</td>
</tr>
<tr>
<td>EB</td>
<td>embryoid body</td>
</tr>
<tr>
<td>EMP</td>
<td>erythroid-myeloid progenitors</td>
</tr>
<tr>
<td>ESC</td>
<td>embryonic stem cells</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorosence activated cell sorting</td>
</tr>
<tr>
<td>FBS</td>
<td>neonatal bovine serum</td>
</tr>
<tr>
<td>FCS</td>
<td>fetal calf serum</td>
</tr>
<tr>
<td>fib\textsuperscript{OCT4}</td>
<td>OCT4-transduced fibroblast</td>
</tr>
<tr>
<td>Flt3L</td>
<td>fms-like tyrosine kinase receptor-3 ligand</td>
</tr>
<tr>
<td>Foxa2</td>
<td>forkhead box a2</td>
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Foxy3  forkhead box a3
G-CSF   granulocyte - colony stimulating factor
Gata4   GATA binding protein 4
GM-CSF  granulocyte macrophage - colony stimulating factor
GVHD    graft vs host disease
Hb9     motor neuron and pancreas homeobox 1
HEK     human embryonic kidney cells
hes1    hairy enhancer of split-1
hESC    human embryonic stem cells
HOXB4   homeobox B4
HSC     hematopoietic stem cells
HSPC    hematopoietic stem progenitor cell
ICC     immuno cyochemistry
ICN     intracellular domain of Notch
Id1     inhibitor of DNA binding 1
Ig      Immunoglobulin
IL-3    Interleukin 3
IL-6    Interleukin 6
iPSC    induced pluripotent stem cells
Lhx3    LIM homeobox 3
lin-    lineage-depleted
LMO2    LIM domain only 2
Lmx1a   LIM homeobox transcription factor 1
LTR long terminal repeat
M-CSF macrophage colony stimulating factor
MafA musculoaponeurotic fibrosarcoma oncogene family protein A
Mef2c Myocyte-specific enhancer factor 2
mESC mouse embryonic stem cells
MHC major histocompatibility complex
MLL myeloid/lymphoid or mixed lineage leukemia
MPP multipotent progenitor
MS5 Mouse clonal stromal cell line 5
MyoD myogenic determinant
Myt1l myelin transcription factor 1-like
NeuroD1 neurogenic differentiation 1
Ngn 2 neurogenin 2
Ngn 3 neurogenin 3
NOD SCID nonobese diabetic severe combined immunodeficiency
NSG NOD/SCID/IL2Rgamma(null)
OKSM OCT4 Klf4 SOX2 c-Myc
olig2 oligodendrocyte marker 2
OSNL OCT4 SOX2 Nanog Lin28
P-Sp para-aorta splanchnopleura
Pax6 Paired box 6
PBS phosphate buffered saline
Pdx1 pancreatic and duodenal homeobox 1
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>Runx1</td>
<td>Runt-related transcription factor 1</td>
</tr>
<tr>
<td>SCF</td>
<td>stem cell factor</td>
</tr>
<tr>
<td>SCL/tal1-1</td>
<td>T-cell acute lymphocytic leukemia-1 protein</td>
</tr>
<tr>
<td>SP</td>
<td>single positive</td>
</tr>
<tr>
<td>SRC</td>
<td>SCID repopulating cell</td>
</tr>
<tr>
<td>TAT</td>
<td>trans-activator of transcription</td>
</tr>
<tr>
<td>Tbx5</td>
<td>T-box 5</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TGFbeta</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>VPA</td>
<td>valproic acid</td>
</tr>
<tr>
<td>VSV</td>
<td>Vesicular stomatitis virus</td>
</tr>
<tr>
<td>zic1</td>
<td>zinc-finger protein 1</td>
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Declaration of Academic Achievement

This thesis was completed mainly by the work of Linda Rodriguez with the following contributions from individuals in Dr. Mick Bhatia’s lab. Monica Graham and Borhane Guezguez processed and lineage-depleted cord blood samples used in co-culture experiments. Marilyne Levedoux-Martin assisted with interpretation of flow cytometry data.
Chapter 1

Introduction

In a multi-cellular organism, cells are differentially specialized in order to optimize fitness. The spatial and temporal organization of cells in an evolutionarily determined hierarchal manner is critical for the proper formation of a multi-cellular organism. The process by which this occurs is termed development. In the 17th century, Jan Swammerdam provided one of the first published examples of the study of development with his description of the varying stages of insect maturation (i.e. larva, pupa)[1]. In the article entitled Theoria Generationis (Theory of Generation), Caspar Friedrich Wolfe was first to suggest that organs did not simply come into existence during gestation, but that instead undifferentiated material acquired cues and formed organs in an ordered and sequential manner[2]. Thus, it was with this hypothesis, prior even to cell theory, that a rudimentary concept of stem cells was inadvertently introduced. However, it wasn’t until the pioneering work of Till and McCulloch that reproducible evidence emerged for a modern conceptualization of stem cells[3]. The simplest and most readily agreed upon definition of a stem cell is a cell with both self renewal capacity, as well as the ability to give rise to differentiated progeny[4](Figure 1.1). The potential progeny a stem cell is
capable of giving rise to is defined by its potency. There are two main classifications of stem cells, embryonic stem cells (ESCs) and adult stem cells. Although many differences exist between these two classifications, the definitive distinction is that embryonic stem cells have the capacity to give rise to all three germ layers and are therefore termed pluripotent (Figure 1.2), while adult stem cells give rise to all cells of a particular system or organ and are hence termed multipotent (Figure 1.3). To date, several adult stem cells have been described including neural stem cells[5, 6], hematopoietic stem cells (HSCs)[3] and skin stem cells[7]. By understanding and studying the unique properties of these cells, their therapeutic potential can be harnessed for clinical applications.

1.1 Reprogramming

Reprogramming refers to the erasure and remodeling of the epigenetic landscape of a particular cell type such that a different cell type is established. It was the pioneering work of King, Briggs[8] and later Gurdon[9] that first demonstrated the phenomena of reprogramming through cloning. King and Briggs developed the nuclear transfer technique, in which the nuclei of somatic cells at different developmental stages were introduced into enucleated oocytes, to address whether a differentiated cell retained the information required to give rise to a fertile organism. Although their findings suggested that successively more developed cell types lost their ability to confer a fertile organism it was Gurdon, with serial transplantation that showed fully fertile frogs could be derived from terminally differentiated epithelial cells (Figure 1.4). The phenomena of cloning in itself
Figure 1.1: Stem cell hierarchy. Stem cells reside at the top of a hierarchy where they have the capacity to self-renew, generating another stem cell, and differentiate into a committed progenitor. The committed progenitor has the ability to proliferate but is committed to differentiate into terminally differentiated specialized cell types.
Figure 1.2: Embryonic stem cells have the capacity to give rise to all the cell types of the embryo proper, which is made up of the three germ layers. Embryonic stem cells are termed pluripotent.
Figure 1.3: Adult or somatic stem cells reside in a developed organism and give rise to a particular tissue type. In this case, the hematopoietic stem cell resides in the bone marrow and gives rise to all the cells of the hematopoietic system. Adult stem cells are termed multipotent.
was a revolutionary accomplishment however what would prove to be more influential to our current understanding and study of stem cells and regenerative medicine was the conclusion that the nuclei of terminally differentiated cells retained the genetic material required to confer an undifferentiated state. Furthermore, this work highlighted that developmental commitment was a reversible epigenetic phenomena. This observation was strengthened by the work of Miller and Ruddle[10] in which mice injected with embryonic carcinoma cells (which are related to ESCs but are derived from tumors) that had been fused with thymus cells developed teratocarcinomas. Furthermore the thymocyte marker Thy-1 was silenced while the cells adopted characteristics of pluripotent stem cells (PSCs) such as X-chromosome inactivation. These data suggested the embryonal program was once again dominant and, like oocytes, embryonic-like cells had dominant determinants that conferred a pluripotent state.

Figure 1.4: Serial nuclear transfer of epithelial cell nuclei into enucleated xenopus eggs gives rise to adult fertile frogs.
It was in 1981 that G. R. Martin[11] and Evans et al.[12] independently described the isolation and subsequent stable in vitro propagation of undifferentiated mouse embryonic stem cells (mESCs), isolated from the inner cell mass of blastocysts (Figure 1.5). These cells were deemed pluripotent based on their ability to give rise to teratocarcinomas in vivo as well as differentiate into the 3 germ layers in vitro. Implications of this discovery include elegant mouse genetic manipulation experiments that have facilitated the study of cellular biology, development, pathophysiology, and perhaps most importantly, it paved the way for the successful isolation of human embryonic stem cells (hESCs) approximately two decades later. Thomson et al.[13] were first to successfully isolate and culture in vitro hESCs, which were derived from the inner cell mass of donor blastocysts. This achievement was not only monumental for the future study of human development and disease biology, but also provided a foundation for stem cell-based cell replacement therapies. Today, cell replacement therapy, or regenerative medicine, is an exciting and growing field of study that aims at developing a therapeutic approach for treating a plethora of human disease and trauma, by regenerating and replacing damaged body tissue.

However, arguably the most profound technique for cellular reprogramming to a pluripotent state was first described in 2007, when Takahashi and Yamanaka[14] were able to reprogram mouse embryonic fibroblasts by the transduction of 4 defined factors, Oct4, Klf4, Sox2 and c-Myc (OKSM), later termed the Yamanaka factors (Figure 1.6). This was achieved by the elegant experimental designs in which 24 candidate factors associated with pluripotency were, through successive rounds of elimination, narrowed
Figure 1.5: In vitro propagated embryonic stem cells are derived from the inner cell mass of blastocysts. The blastocyst stage of development occurs following the formation of the blastocoel (fluid filled space) within the morula relatively shortly after fertilization.
down to a core set of genes, capable of inducing pluripotency of murine fibroblast cells. Within 2 years of optimization, generated induced pluripotent stem cells (iPSCs) were capable of forming adult chimeric mice and functional germ cells[15, 16]. This pivotal discovery demonstrated that the global gene expression, epigenetic and phenotypic status of a specific cell, especially one as developmentally distinct and complex as a pluripotent cell, can be appreciably mimicked by the expression of a relatively minute number of definable factors. In 2007 Yu et al.[17] and Takahashi et al.[18] independently reprogrammed human fibroblasts to iPSCs using Oct4, Sox2, Nanog and Lin28 (OSNL) and OSKM respectively, providing another method of deriving autologous sources of tissues, which can potentially be utilized for cell replacement therapy. However, in order to derive tissues suitable for clinical use, efforts were aimed at eliminating the use of integrating factors as well as for increasing the efficiency of the process. To date, iPSCs have been derived using non-integrative viruses such as adenovirus[19], ectopic supplementation of reprogramming proteins[20], and even modified mRNA[21]. Furthermore, the oncogene c-Myc was found to be dispensable to reprogramming [22, 23]. With the use of the histone deacetylase inhibitor valproic acid (VPA), only OCT4 and SOX2 are required to derive iPSCs[24]. Other small molecules that have been identified to promote reprogramming include CHIR99021, a glycogen synthase kinease-3 inhibitor, tranylcypromine, an H3K4 inhibitor[25], BIX091294, a histone methyl transferase inhibitor and Bayk8644, an L-channel agonist[26].
1.2 Induced direct lineage conversion

Yamanaka’s use of cell lineage-associated transcription factors to drive cell fate decisions is an idea that was inspired by the work of Davies et al., in which mouse fibroblasts were directly converted to myofibres through the ectopic expression of the muscle specific master regulator MyoD (myogenic determinant)[27]. Furthermore, MyoD was also shown to convert other cell types such as fibroblasts, smooth muscles, adipocytes and kidney cells to myofibres[28]. This work demonstrated the ability of committed cell types to transverse germ layer boundaries without first becoming pluripotent. Similarly, ectopic expression of CEBPα (CCAAT/enhancer-binding protein alpha) was shown to convert B cells into functional macrophages with intact immunoglobulin rearrangements[29]. The same phenomena was even demonstrated in sensory ‘hearing cells’ where the ectopic expression of Atoh1 (atohal homolog 1) was shown to convert non-sensory cells of the auditory epithelium into functional inner hair cells[30]. Furthermore, the phenomenon of

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Figure 1.6: Ectopic expression of OCT4, SOX2, NANOG and c-MYC reprograms fibroblasts to induced pluripotent stem cells.
direct conversion has also been demonstrated \textit{in vivo} by Douglas Melton’s group in which a screen of 1100 transcription factors identified 3 factors, Pdx1 (pancreatic and duodenal homeobox 1), Ngn3 (neurogenin 3) and MafA (musculoaponeurotic fibrosarcoma oncogene family, protein A) capable of converting over 20\% of endocrine cells into $\beta$-like cells in vivo. Furthermore, these $\beta$-cells had the capacity to alleviate hyperglycemia caused by insulin deficiency in the type-1 diabetes mouse model[31]. More recently, derivation of hepatocyte-like cells with \textit{in vivo} hepatic function from mouse fibroblasts was shown using Hnf4$\alpha$ (hepatocyte nuclear factor 4alpha), Foxa1 (Forkhead box a1), Foxa2 (Forkhead box a2) and Foxa3 (Forkhead box a3)[32]. Additionally, mouse cardiac fibroblasts were converted into beating cardiomyocytes using Gata4(GATA binding protein 4), Mef2c (Myocyte-specific enhancer factor 2), and Tbx5(T-box 5)[32].

Work demonstrating the generation of neural subtypes through conversion has been extensively studied. The combination of Ascl1(Achaete-scute homolog 1), Brn2(Brain-specific homeobox/POU domain protein 2), and Myt1l (myelin transcription factor 1-like) with Lmx1a (LIM homeobox transcription factor 1) and Foxa2 allows for the generation of dopaminergic neurons, the neural cell type lost during Parkinson’s disease, from fibroblasts[33]. The addition of NeuroD1 (Neurogenic differentiation 1) with Ascl1, Brn2 and Myt1l greatly enhances neuron generation from fibroblasts[34]. This cocktail in combination with Lhx3 (LIM homeobox 3), Ngn2 (Neurogenin 2), Isl1 (ISL LIM homeobox 1) and Hb9 (Motor neuron and pancreas homeobox 1) allows for the generation of cholinergic motor neurons[35] while, with zic1 (zinc-finger protein 1) and Olig2 (oligodendrocyte marker 2) allows for glutamatergic neurons to be derived[36]. Lineage conversion can
even be induced by microRNAs. For instance human fetal fibroblasts were converted to MAP2 neuron-like cells using miR-9/9 and miR-124[37].

To note, all of these methods derived committed cell types without progenitor capacity thereby limiting their efficacy in therapeutic settings (Figure 1.7). Efe et al.[38] took a different approach in an attempt to standardize conversion by inducing an unstable intermediate state in which the epigenetic state is such that it allows for direction by culture conditions. As such, the reprogramming factors OCT4, SOX2 and KLF4 were used in concert with cytokines required for the signaling of the following pathways: Transforming growth factor beta (TGFβ), bone morphogenetic protein (BMP), hedgehog, Wnt and Notch to efficiently derive cardiomocytes. The same group used a similar method to derive neural progenitors [39]. Interestingly Vierbuchen et al.[40] used Ascl1 and Myt1l to reprogram mouse embryonic fibroblasts to neurons, however the addition of Brn2, a POU family transcription factor like OCT4, was required to derive a more progenitor type of neural cell. In a similar manner, multipotent neural stem cells were derived from sertoli cells using Ascl1, Ngn2, Hes1(hairy enhancer of split-1), Id1 (inhibitor of DNA binding 1), Pax6 (Paired box 6), Brn2, Sox2, c-Myc and Klf4[41]. We recently described the direct conversion of human dermal fibroblasts to multi-lineage hematopoietic progenitors by ectopic expression of OCT4[42]. These OCT-4 expressing fibroblasts, hereafter referred to as fibsOCT4, were able to give rise to myeloid, erythroid and megakaryocytic lineages and demonstrated in vivo hematopoietic reconstitution capacity in a xenograft model (Figure 1.8). Additionally, these cells had activated definitive blood programs, consistently lacking from hESC-derived HSCs. In all examples in which progenitor types
were derived either the reprogramming factors were used or related transcription family members, thereby highlighting the potential role of these factors to possibly partially dedifferentiate terminally committed cell types to a progenitor/stem cell identity while culture conditions or master regulators of the target cell type direct the fate of the cell (Figure 1.7).

Figure 1.7: Current methods of direct conversion. The use of master regulator genes to specify cell fate result in terminally differentiated cell types predominantly. However the use of pluripotency factors or pluripotency-related factors (ie. Brn2) with lineage specific cytokines or master regulator genes results in more progenitor-like phenotypes that are expandable.
Figure 1.8: Ectopic expression of OCT4 directly converts human fibroblasts to multi-lineage hematopoietic progenitors.

Table 1.1: Examples of direct conversion using mainly lineage specific master regulatory genes or pluripotency factors. Examples of direct conversion have also been demonstrated using microRNAs and chemical compounds.

<table>
<thead>
<tr>
<th>Initial cell type</th>
<th>Factors</th>
<th>Target cell type</th>
<th>Organism</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>fibroblasts, smooth muscles adipocytes and kidney cells to myofibres</td>
<td>MyoD</td>
<td>myofibres</td>
<td>mouse</td>
<td>[27, 28]</td>
</tr>
<tr>
<td>B cells</td>
<td>CEBPα</td>
<td>macrophages</td>
<td>mouse</td>
<td>[29]</td>
</tr>
<tr>
<td>auditory epithelium</td>
<td>Atoh1</td>
<td>inner hair cells</td>
<td>mouse</td>
<td>[30]</td>
</tr>
<tr>
<td>endocrine cells</td>
<td>Pdx1, Ngn3 and MafA</td>
<td>β-like cells (<em>in vivo</em>)</td>
<td>mouse</td>
<td>[31]</td>
</tr>
<tr>
<td>fibroblasts</td>
<td>Hnf4α, MafA and Foxa3</td>
<td>hepatocyte-like cells</td>
<td>mouse</td>
<td>[32]</td>
</tr>
<tr>
<td>cardiac and dermal fibroblasts</td>
<td>Gata4, Mef2c and Tbx5</td>
<td>beating cardiomyocytes</td>
<td>mouse</td>
<td>[32]</td>
</tr>
<tr>
<td>fibroblasts</td>
<td>Ascl1, Brn2, and Myt1l with Lmx1a and Foxa2</td>
<td>dopmingic neurons</td>
<td>mouse and human</td>
<td>[33]</td>
</tr>
<tr>
<td>fibroblasts</td>
<td>NeuroD1, Ascl1, Brn2, and Myt1l</td>
<td>Enhanced neuron generation</td>
<td>human</td>
<td>[34]</td>
</tr>
<tr>
<td>Cells Type</td>
<td>Transcription Factors</td>
<td>Differentiation Outcome</td>
<td>Species</td>
<td>Reference</td>
</tr>
<tr>
<td>--------------------------------</td>
<td>----------------------------------------</td>
<td>---------------------------------------------</td>
<td>----------</td>
<td>-----------</td>
</tr>
<tr>
<td>fibroblasts</td>
<td>Ascl1, Brn2, and Myt1l with Lhx3, Ngn2, Isl1 and Hb9</td>
<td>cholinergic motor neurons</td>
<td>mouse</td>
<td>[35]</td>
</tr>
<tr>
<td>Porcine embryonic fibroblasts</td>
<td>SB431542 (TGF-beta inhibitor), Thiazovivin (ROCK inhibitor)</td>
<td>Adipocytes</td>
<td>porcine</td>
<td>[43]</td>
</tr>
<tr>
<td>fibroblasts</td>
<td>zic1 and Olig2</td>
<td>glutamatergic neurons</td>
<td>human</td>
<td>[36]</td>
</tr>
<tr>
<td>fibroblasts</td>
<td>miR-9/9 and miR-124</td>
<td>MAP2 neurons like cells</td>
<td>mouse</td>
<td>[37]</td>
</tr>
<tr>
<td>fibroblasts</td>
<td>SOX2</td>
<td>Neural stem cells</td>
<td>mouse</td>
<td>[44]</td>
</tr>
<tr>
<td>fibroblasts</td>
<td>OCT4, Sox2 and KLF4 (TGFβ, BMP, hedgehog, Wnt and Notch signaling)</td>
<td>cardiomyocytes</td>
<td>mouse</td>
<td>[38]</td>
</tr>
<tr>
<td>fibroblasts</td>
<td>OCT4, Sox2 and KLF4</td>
<td>neural progenitors</td>
<td></td>
<td>[39]</td>
</tr>
<tr>
<td>fibroblasts</td>
<td>Ascl1, Brn2, and Myt11</td>
<td>Neuronal progenitor generation</td>
<td>mouse</td>
<td>[40]</td>
</tr>
<tr>
<td>sertoli cells</td>
<td>Ascl1, Ngn2, Hes1, Id1, Pax6, Brn2, Sox2, c-Myc and Klf4</td>
<td>multipotent neural stem cells</td>
<td></td>
<td>[41]</td>
</tr>
<tr>
<td>fibroblasts</td>
<td>OCT4</td>
<td>Multipotent hematopoietic progenitors</td>
<td>human</td>
<td>[42]</td>
</tr>
<tr>
<td>astrocytes</td>
<td>OCT4, Nanog, SOX2</td>
<td>Neural stem cells</td>
<td>human</td>
<td>[45]</td>
</tr>
<tr>
<td>fibroblasts</td>
<td>miRNA 1, 133, 208, 499</td>
<td>Cardiomyocyte-like</td>
<td>mouse</td>
<td>[46]</td>
</tr>
</tbody>
</table>
1.3 OCT4 and cell fate reprogramming

OCT4 is one of the core factors common between the two cocktails established to reprogram human fibroblasts to iPSCs[17, 18]. In fact, OCT4 is the only core factor originally identified by Yamanaka[18] that is indispensable to reprogramming. The other factors can either be replaced by small molecules, such as VPA and BIX[24, 47], or can be omitted in certain starting cell contexts (ie. Neural cells can be reprogrammed with the use of OCT4 solely due to the endogenous expression of SOX2 and c-MYC[48]). This truly underscores the unique function and activity of Oct-4 during the cell fate decision process. OCT4 (POU5F1) is part of the POU domain family of transcription factors, which were identified by the homologous region shared between the mammalian genes, Pit-1, Oct-1 and Oct-2 and the c. elegans gene unc-86, hence owing to its name[49, 50, 51, 52, 53, 54]. POU domain containing transcription factors have been isolated from different stages of development, from a variety of tissues and have been shown to bind native and predicted octamer DNA sequences, such as “5’nTGCAnnn3’” (“n” can be replaced with any nucleotide) and “5’TGTGCG3’” [55]. The POU domain consists of 2 highly conserved regions and a variable linker which allows conformational flexibility resulting in their ability to recognize different octamer sequences[56, 57]. The 75-82 amino acid N-terminal region is designated the POU specific subdomain for its ability to recognize a 4 nucleotide specific sequence found within octomer binding sites while the 60 amino acid C-terminal region is designated the POU homeodomain for its ability to recognize the AT rich region of the octomer sequence much like conventional homeodomains[58, 59, 60, 61]. POU domain proteins are grouped into 6 (sometimes 7) classes based on the amino
terminal sequence of their POU domains and the conservation of the variable linker region [62]. Amongst the POU proteins, POU5F1 (Oct-4) has been the most widely studied, given its pivotal role in pluripotency, nonetheless its functional role and transcriptional targets are yet to be fully delineated. For example, Oct-4 is expressed in pluripotent cells of the embryo, from which embryonic stem cells are derived, however recent studies have demonstrated that Oct-4 is expressed in a subset of adult somatic cell compartments such as skin[63] and in bone marrow derived mesenchymal stem cells[64]. In fact OCT4 is expressed in all three germ layers of gastrulation-stage embryos[65] and studies have implied that Oct-4 levels play a critical role during differentiation of pluripotent stem cells towards different lineages[66]. Furthermore a recent study has also shown that Oct-4 can alter the epigenetic landscape of somatic cells allowing their conversion into mature cell types under the appropriate culture conditions[67]. This has been functionally well characterized in which, when tailoring the culture conditions, either iPSCs [14], induced epiblast stem cells [68], induced neural cells [39] or induced cardiac cells [38] can be derived using the same factor combination including OCT4. OCT4 is therefore being currently coined as the gatekeeper into the reprogramming expressway.

1.4 Hematopoiesis

Hematopoiesis in a developing embryo occurs in distinct anatomical areas and in sequential waves. The initial wave consists of the generation of predominantly erythrocytes from the extra-embryonic mesoderm of the yolk sac, allowing for the first intrinsic supply
of oxygen to the embryo. This initial wave of hematopoiesis is termed primitive due to the transient production of erythroid cells expressing embryonic globins[69]. A second wave of hematopoiesis occurs in which first erythroid-myeloid progenitors (EMPs) arise from the yolk sac and fetal liver[70], however HSCs with lymphopoiesis potential arise from the para-aortic splanchnopleura (P-Sp), which is the presumptive aorta-gonadal-mesonephros (AGM) region in mammals[71, 72]. The HSCs travel and colonize the fetal liver, spleen, thymus and finally bone marrow[73]. It is speculated that the development of the different blood cells from different anatomical regions of the developing organism provide cues toward their identity. It is the prevailing idea that HSCs emerging from the hemogenic endothelial cells in the AGM region give rise to most (if not all) bone marrow hematopoietic cells.

In an adult organism, HSCs reside in the bone marrow. HSCs are capable of giving rise to all the cell types that comprise the hematopoietic system, as well as have the capacity to self-renew for the life-span of the organism. HSCs are often classified into long-term and short-term HSCs [74]. Long-term HSCs are predominantly quiescent while the short-term HSCs are proliferative however have limited self-renewal potential. HSCs give rise to multipotent progenitors (MPPs) that expand by proliferating but lose the self renewal potential endowed to HSCs. MPP further differentiate into two main lineages, the erythro-myeloid lineage and the lymphoid lineage. Classically, the erythro-myeloid lineages progressively differentiate while losing potency, giving rise to terminally differentiated granulocytes, monocytes, erythrocytes and platelets. Similarly, lymphoid progenitors differentiate to naive T, B and Natural Killer cells. Recent data showed
that human hematopoiesis does not follow the classical model of myeloid-lymphoid segregation. For instance, a distinct population of multi-lymphoid progenitors, with the Thy-1\(^{\text{neg-lo}}\)CD45RA\(^+\)CD34\(^+\)CD38\(^-\) profile, give rise to all lymphoid cells as well as monocytes, macrophages and dendritic cells [74].

1.5 Master regulators of early hematopoiesis

The cell fate decisions governing primitive and definitive hematopoiesis have been extensively investigated in order to provide insights into how the hematopoietic system develops and maintains homeostasis thereby allowing for the exploitation of these programs for therapeutic and regenerative medicine purposes. SCL/tal-1 (T cell acute lymphocytic leukemia-1 protein) and its associated partner LMO2 (LIM domain only 2) are individually required for both primitive and definitive hematopoiesis[75]. Developmentally, hematopoiesis does not occur without these transcription factors. Runx1 (Runt-related transcription factor 1) and MLL (myeloid/lymphoid or mixed lineage leukemia) on the other hand are required for the generation of HSC from the AGM[76]. In the absence of Runx1 the classic hematopoietic clusters do not form in the AGM of mice. MLL on the other hand is required for the activation of HOX genes, particularly HOXB4 (homeobox B4) for HSC specification. During further lineage commitment GATA-1 is highly expressed in megakaryocyte and erythroid progenitors while C/EBP\(\alpha\) is predominantly expressed in granulocyte/macrophage progenitors[77]. Furthermore high expression of PU.1 (spi-1) results in macrophage differentiation whereas low level expression favors
1.6 Human lymphopoiesis: T- and B-cell lineages

Lymphocytes are comprised of two lineages: T- (thymus) cells and B- (bursal or bone) cells can be defined as a subset of hematopoietic cells derived from a common lymphoid progenitor (CLP) [79, 80]. CLPs produce cells that express clonally diverse cell surface immunoglobulin (Ig) receptors for the purposes of recognizing specific antigen epitopes and mounting an immune response, Fig ???. The Ig B cell receptor is made up of 2 identical heavy and 2 identical light chain peptides[81]. Both chains have variable and constant domains, however the constant domain on the heavy chain is much longer, hence its name[82]. The variable domain is so named because at the genomic level genes are rearranged on the VDJ (heavy chain) or VJ (light chain) genes to create completely unique, non-germline peptide sequences allowing for antigen-specific antibody production towards mounting an adaptive immune response[81].

1.7 Human B-cell maturation and development

In the bone marrow, CLPs [79, 80, 82] give rise to B-progenitors as depicted in Fig ???. B-lineage progenitors have committed to the B-cell lineage, but lack the Ig receptor on the cell surface. Thus acquisition of the Ig receptor expression represents a critical maturation step. The commitment to the B-cell lineage is delineated by the activation
and assembly of the recombinase complex (RAG1/2 and TdT) [83] which is required for the recombination of the VDJ genes on the heavy chain of the immunoglobulin receptor [84] a process that coincides with the rearrangement is the co-expression of pan-B-cell markers CD19 and CD10 on the cells surface [85]. Once the heavy chain immunoglobulin gene has been rearranged, its expressed on the surface of the cell, however the light chain, which has not yet been assembled, is required for proper folding of the receptor [82]. To circumvent this problem, maturing B-cells also express a surrogate light chain gene (VpreB). The surrogate complex on the surface of the cell is termed the pre-B-cell receptor (BCR), hence, pre-B-cell [82, 86] (Fig 1.1). Once the VJ genes of the light chain are rearranged and expressed, the chains come together to form an Ig cell surface receptor [87].

1.8 Human T-cell maturation and development

CLPs also give rise to thymocyte precursor cells which leave the bone marrow and migrate to the thymus [88], their earliest marker being the expression of CD7 [89]. Subsequent expression of CD1a on CD7+ thymocytes indicates commitment to the T-cell lineage. During commitment, thymocytes are termed double negative (DN) because they do not express the surface receptors CD4 or CD8, which are critical for determining specific T-cell function [90]. CD4 recognizes the antigen-presenting receptor that mediates helper T-cell function, while CD8 recognizes the antigen-presenting receptor that mediates cytotoxic T-cell function [81, 89]. The pre-T cell receptor (TCR) is assembled
in a similar manner to B-cells via specific and unique genomic rearrangements signifying progressive commitment steps [91, 92] (See Fig ??). Once the T-cell receptor is assembled on the surface of the cell, it becomes double positive (DP) for both CD4 and CD8. At the DP stage, thymocytes undergo positive and negative selection for recognizing major histocompatibility complex (MHC) molecules critical for mounting a proper immune response and for binding self-antigens respectively[81, 93, 94, 95]. Finally, thymocytes downregulate either CD4 or CD8 on their cell surfaces becoming single positive (SP) helper T-cells or cytotoxic T-cells respectively [96, 97]. Taken together, and similar to B-cell development, these combinations of cell surface markers and molecular genomic rearrangements provide a definitive and discrete set of measureable steps during human T-cell development.

Figure 1.9: Lymphopoietic Development from common lymphoid progenitors.
1.9 Hematopoiesis and co-culture methods

Following the advent of Till and McCullogh’s [3] seminal work in experimentally proving the existence of stem cells, the study of the hematopoietic system became a prevalent model of study for the newly emerging field of stem cells. By the late 1980s, *in vitro* models for the study of hematopoiesis were developed in which a long-term culture system of unfractionated marrow samples were cultured for several weeks [98, 99]. Distinct blood colonies would appear with different compositions of erythro-myeloid lineage cells. It was inferred that the resulting colonies originated from a primitive progenitor cell present from the initiation of the culture. This *in vitro* system was further optimized to utilize pre-established irradiated human bone marrow stroma, which could be co-cultured with different fractions of sorted hematopoietic cells [100]. In a seminal study conducted to investigate the role of colony-stimulating cytokines in augmenting hematopoiesis, investigators sought to create more controlled culture conditions. Therefore they transduced a mouse marrow stromal cell line with human granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF) or interleukin 3 (IL-3) expressing retroviruses [101]. The intrinsic cytokines released by the mouse marrow stroma are not species cross-reactive and therefore their effect was negligible. Through the use of genetically engineered growth factor producing feeders vs. ectopic addition of human growth factors, it was hoped to more closely mimic how hematopoietic cells are stimulated *in vivo*. Since this work, the hematopoietic-inductive capacity of different murine stromal cell lines have been well established. Of particular interest, the murine clonal stromal cell line 5 (MS5), originally derived for long-term cultures of murine
hematopoietic cells, was shown to expand a subpopulation of very early human multipotent progenitors [102]. Furthermore, due to a lack of in vitro culture systems for human B cell development, MS5 stroma was shown to support early B lymphopoiesis [103].

Following the isolation of mESCs, efforts to differentiate them to hematopoietic stem cells were underway. However both feeder and non-feeder methods resulted in the generation of myeloid cells only. Nakano et al. [104] hypothesized that macrophage-colony stimulating factor (M-CSF) could be responsible for the generation of predominately myeloid lineage cells. As such, the authors derived the OP9 cell line from the calvaria of the op/op mouse that lack a functional M-CSF gene. The investigators were able to generate both myeloid and early B-lymphoid cells however T lymphopoiesis was not observed.

More recently, several lines of evidence suggested that Notch signaling is required for T cell development. Specifically bone marrow transplants of mice with Notch expressing cells induced ectopic development of T cells in the bone marrow [105]. In a complementary experiment, Notch-1 deficient mice had a severe block in their ability to generate T cells with a concomitant development of B cells in the thymus [106]. This data together suggest that Notch signaling is required for T cell development at the expense of B cell development. Notch is a heterodimeric transmembrane receptor, which is activated by interaction with adjacent cells. In mammals, Notch has 2 ligand families; Jagged ligands (serrate like), and Delta-like ligands based on earlier nomenclature when originally identified in Drosophila [106]. The cell-surface bound ligand causes a self-cleavage event to occur in the adjacent cell expressing the Notch receptor allowing the intracellular domain
of Notch (ICN) to act on target genes. With this in mind, the Zuniga-Pflucker [89] group generated functional T cells \textit{in vitro} through the co-culture of mouse hematopoietic progenitors on OP9 stromal cells ectopically expressing the Delta-like-1 (Dll-1) ligand. This group later showed T cell development from mESCs and human cord blood (CB) using the same method [107].

1.10 Differentiation of PSCs to the hematopoietic lineage

Through identification and exploitation of molecular pathways critical for cell fate decisions, PSCs have been induced to differentiate into several cell types of interest. The differentiation methods employed on human PSCs have largely stemmed from differentiation studies performed on mouse PSCs. In the context of blood derivation, PSCs can be induced to differentiate using two main methods (Figure 1.10). The first, known as the embryoid body (EB) method, utilizes low adherence plastic in order to form 3D floating structures composed of differentiating PSCs cells [108]. The second method utilizes feeder cells and inductive cytokines to differentiate the PSCs towards a particular lineage. Several feeder cell lines have been developed for co-culture with PSCs including murine stroma such as the OP9 cell line as well as human fetal liver and AGM cell lines [109, 104].

Differentiation of hESCs to the hematopoietic lineage follows temporally defined ac-
Figure 1.10: Pluripotent stem cells can be differentiated to the blood lineage through embryoid body formation or through co-culture on supportive stroma such as OP9, AGM- or fetal liver-derived stroma.

quisition of more restricted markers indicative of hematopoiesis. During early hESC differentiation, a population of cells expressing CD31 and CD34 cell surface markers are capable of generating both endothelial and hematopoietic lineages suggesting this population contains cells with hemangioblastic properties[110]. Recently, Vodyanik et al. [111] described an early CD45− population of hematopoietic cells. This CD43+CD34+CD45− population had the capacity to derive myeloid-monocytic, erythrocytic and NK precursors as well as demonstrated B cell lymphopoiesis potential. Currently the CD45+CD34+ population of hematopoietic cells enriches the stem cell/progenitor pool although CD43+CD34+ cells appear to be an earlier precursor of hematopoiesis during development.

To assess the functionality of a long-term HSC, the gold standard in the field is to assess its serial repopulating capacity in the nonobese diabetic/severe combined immun-
odeficiency (NOD/SCID) xenograft model (SCID repopulating cell or SRC) [112]. There has been limited success in the field for generating a bona fide HSC. Through ectopic expression of Hoxb4 in mouse embryonic stem cells, Daley and colleagues demonstrated that these cells had in vivo self renewal capacity via the xenograft assay and multi-lineage differentiation capacity in vitro [113]. Unfortunately, this could not be recapitulated in the human [114], highlighting the often subtle disparity in the developmental processes between species. Ledran et al.[115], however, were able to show 1% engraftment of hESCs-derived blood progenitors in NOD-scid IL2Rγnull (NSG) mice and 1-2% engraftment of secondary mouse transplants. These results are promising, however, they have not been reproduced yet. The inability to recapitulate the functional characteristics of true HSCs when subjected to in vivo mouse repopulation assays has been attributed to the failure of differentiation protocols to activate essential HSC genetic programs [116], and to inactivate certain embryonic gene expression patterns [117]. The embryonic-like state of PSC-derived blood progenitors is believed to restrict long-term engraftment due to the lack of long-term progenitor capacity of embryonic blood. Particularly, primitive blood development during embryogenesis generates erythro-myeloid precursors without HSC capacity [118, 119]. During definitive hematopoiesis later in embryogenesis, the first detectable HSC is generated with the capacity to differentiate into all blood lineages including lymphoid cells[120]. A hallmark of definitive blood development is the expression of adult globins vs. embryonic and fetal globins [116]. Acquisition of definitive blood [117] from PSC-derived blood progenitors defined by serial engraftment with the capacity to form all blood lineage cells, particularly B and T cells has yet to be demonstrated.
1.11 Lymphopoiesis from human PSC sources

There has also been limited success in deriving fully functional naïve lymphocytes from PSC sources. Zambidis et al. [117] showed low expression of CD19, a pan marker of B cells, on a small number of cells, through EB differentiation and subsequent co-culture on OP9 stroma. Vodyanik et al. [111] used the OP9 stroma and subsequent culture on MS5 stroma to generate robust expression of CD19 (Figure 1.11). Interestingly, the CD19+ cells were only derived from the CD34+CD43+CD45− population of cells suggesting the commonly denoted primitive progenitor CD34+CD45+ is not the earliest hematopoietic progenitor. Using this same co-culture method, Carpenter et al. [121] derived CD19+ B cells from iPSCs. However, unlike CB-derived CD19+ B cells, PSC-derived CD19+ cells have never been demonstrated to express surface IgM, a hallmark of a naïve functional B cell.

T cells have been generated from hESC both in vitro and in vitro. Galic et al. [122, 123] used the EB method and OP9 co-culture method to generate blood progenitors and then using the SCID-humanized mouse model in which SCID mice are transplanted with human fetal thymic material and human fetal liver to allow for human T cell development, to generate T cells in vivo. Timmermans et al. [124] were able to generate functional T cells in vitro from hESCs using a combination of OP9 stroma and OP9-Dll1. Interestingly only the CD34+CD43+ population of blood progenitors generated T cells, irrespective of CD45 expression. These data taken together suggests the HSC compartment is enriched in CD34+CD43+CD45− cells unlike previously thought, since CD34+CD45+ cells lose
Figure 1.11: B cell lymphopoiesis can be derived from both somatic and pluripotent stem cell sources however only somatic stem cell derived B cells express IgM, a hallmark of functional B cells.
B lymphopoiesis capacity. Having said that, mature B cell development (expression of IgM) has never been demonstrated from PSC sources and T cell development has never been demonstrated from iPSC sources.

1.12 Rationale

Human immuno-deficient diseases caused by a lack of functional T- and B-cells such as HIV [125] and leukemia require meticulous monitoring and lack robust curative treatments [126]. Treatments do exist, but are complex to administer, ie lymphoid leukemias with high incidence of relapse following post-induction chemotherapy [127] can be induced into remission if chemo/radiotherapy is intensified and coupled with hematopoietic stem-progenitor cell (HSPC) transplantation [128, 129]. Even with treatment plans lymphoid leukemias cause severe immunodeficiency and are the most common cause of death due to opportunistic infection [130]. Bone marrow transplants are required after chemotherapy/radiation due to the destructive actions of the treatment on healthy hematopoietic cells [131],[132]. Unfortunately, sources of patient-specific autologous HSPCs are scarce. Accordingly, such patients are reliant on allogenic (matched donor) and autologous (same patient) HSPC transplantations to rescue the patients hematopoietic/immune system. While allogenic transplants have added curative potential when combined with chemotherapy, they also pose the potential risk of graft vs. host disease (GVHD) [133]. The use of autologous HSPCs circumvents the issues of GVHD, but risks reinfusion of contaminating tumor cells in peripheral tissue and circulation[133]. In addition, our cur-
rent understanding of the basic cellular and molecular biology underlying autoimmune diseases is lacking [134]. This is largely due to the absence of model systems that recapitulate the disease in vitro for robust and detailed analysis that can be easily manipulated and studied using modern genetic approaches. For example, development of effective treatments for autoimmune diseases such as type-1 diabetes [135] and arthritis [136] requires a more detailed understanding of the underlying biology that initiates autoimmune reactions in lymphoid cells that mediate and sustain the disease process and subsequent tissue damage to joints and pancreatic β-cells respectively. Therefore, novel approaches to obtain patient-specific HSPCs capable of reconstituting healthy T- and B-cells in vivo or for in vitro disease modelling/testing are required.

Given our recent results for conversion of hFibs to myeloid and erythroid progenitors displaying a definitive/adult phenotype, we are uniquely positioned to determine whether our approach can be combined with existing strategies to generate functional B- and T-cells from somatic/adult sources. Although other sources of cells have provided limited success for B- and T-cell derivation, the intrinsic nature of hFibs to avoid embryonic states/programs during direct conversion to hematopoietic fate provides a unique and critical experimental advantage.

1.13 Hypothesis

I hypothesize that, through optimized culturing conditions conducive to hematopoiesis and lymphopoiesis, functional hematopoietic progenitors with the lymphopoietic potential
can be efficiently generated from hFibs\textsuperscript{OCT4}.

### 1.14 Objectives

Therefore, this thesis aims to:

1. Reproduce the direct conversion of OCT4-transduced fibroblasts to hematopoietic progenitors.

2. Evaluate the capacity of directly converted hematopoietic progenitors to commit to the lymphoid lineage using well-established co-culture protocols.
Chapter 2

Methods and Materials

2.1 Cell culture

Primary human dermal adult fibroblasts derived from breast dermal tissue were maintained in fibroblast medium (DMEM (Gibco) supplemented with 10% v/v FBS (Neonatal Bovine Serum, HyClone), 1 mM L-glutamine (Gibco), and 1% v/v nonessential amino acids (NEAA; Gibco)) or with conversion media (complete F12 medium (F12 DMEM; Gibco) supplemented with 10% knockout serum replacement (Gibco), 1% NEAA (Gibco), 1 mM L-glutamine (Gibco), and 0.1 mM b-mercaptoethanol containing 16 ng/ml, bFGF (BD Biosciences) and 30 ng/ml, IGFII (Millipore)) 10 days prior to transduction. Human dermal fibroblasts transduced with OCT4 were seeded on matrigel-coated 12-well dishes 16 hours prior to transduction at cell density of 1 000 or 10 000 cells/4 cm² surface area. 30 minutes before transduction, 50% conversion media was added to 50%
fibroblast media with 8mg/ml of polybrene. 2 days post-transduction the media was changed for complete conversion media or conversion media supplemented the following hematopoietic cytokines: 300ng/ml of SCF (stem cell factor), 300ng/ml of Flt3L (fms-related tyrosine kinase 3 ligand), 10ng/ml of IL-3, 10ng/ml of IL-6 (interleukin 6), 50ng/ml of G-CSF, 25 ng/ml of BMP4. Cells were passaged to freshly matrigeled 12-well plates after confluence or colony emergence through either manual colony plucking, 0.25% trypsin (GIBCO) passage or passage of non-adherent cells (without trypsin) for at least 21 days, for which the media was changed every 1-2 days. All cell cultures were maintained at 37°C and at 5% CO₂.

2.2 Hematoptoic cytokine embryoid body assay

The arising CD45⁺ OCT4-transduced cells were manually transferred by well scraping onto low attachment 6-well plates in hematopoietic medium (80% knockout DMEM (KO-DMEM) (Gibco), 20% v/v non-heat inactivated fetal calf serum (FCS) (HyClone), 1% v/v NEAA, 1mM L-glutamine and 0.1mM b-mercaptoethanol (Sigma)), for 16 days supplemented with hematopoietic differentiation cytokines (SCF, G-CSF, FLT3L, IL-3, IL-6 and BMP-4; RDSystems) after 1 day and changed every 3-4 days.
2.3 Lentivirus production

The OCT4 Lentiviral vector (pSIN) was obtained from Addgene. These vectors were transfected with the packaging plasmids pMD2.g and psPAX2 (addgene) in a 293-FT human embryonic kidney cells (HEKs) packaging cells line. Specifically, a ratio of 1:1.4:2.4 of pMD2.g, psPAX2, and pSIN-E2a-OCT4-puro or pSIN-E2a-GFP-puro plasmids respectively were incubated together in OPTI-mem media (Gibco) for 5 minutes. 45 µl of lipofectamine, an agent that optimizes transfection was added to 1.5 ml of OPTI-mem and incubated for 5 minutes. The mixture of plasmids and lipofectamine were incubated together at room temperature for 15 minutes. 5 ml of OPTI-mem was added to 90% confluent 293-FT HEK cells. The mixture of the plasmids and lipofectamine was added dropwise to the 293-FT HEK cells. 16 hours later, fresh media was added to the HEK cells. Viral supernatants were harvested 48 h after transfection and ultracentrifuged for 2 hours to concentrate the virus. An equal amount of each virus, which varied between experiments, was used for fibroblast transduction in the presence of 8 mg/ml of polybrene.

Lentivirus transduction: Human adult dermal fibroblasts (Fibs) (derived from breast skin; age 30-40 yr) were infected with lentivirus expressing OCT4 16 hours after seeding of fibroblasts. 7 Days post transduction, fibroblasts were intracellular stained with OCT4-alexa647 antibody and analyzed for the presence of OCT4 through flow cytometry.
2.4 Live immuno cytochemistry (ICC) staining

For live staining, hFibs\textsuperscript{OCT4} colonies were washed once with F12 medium and incubated with sterile CD45-PE antibody (Miltenyi) for 30 min at room temperature. Cultures were then washed twice to remove unbound antibody with F12 medium. Cells were visualized by an Olympus IX81 fluorescence microscope and Metamorph software at 4x and 10x magnification under the “Multi-Dimensional Acquisition” settings of bright field and and Cy3 for the visualization of CD45-PE stained live cells.

2.5 Flow cytometry

Live cells were identified by 7-amino actinomycin (7AAD) exclusion and then analyzed for cell surface marker expression using an LSRII flow cytometer (Becton Dickinson). Collected events were analyzed using FlowJo 8.8.6 Software (Tree Star). hFib\textsuperscript{OCT4} were disassociated with either 0.25% trypsin or TrypLE (Gibco) and analysed for expression of hematopoietic markers. Hematopoietic cells were identified by staining single cell suspensions with fluorochrome-conjugated monoclonal antibodies: CD34-APC (1:100 dilution) and CD45-PE (1:400 dilution), CD19-APC (1:80 dilution) or -PE (1:200 dilution) and CD10-PE (1:200 dilution)(miltenyi) and Tra-1-85 (1:200 dilution). Optimal working dilutions were determined for individual antibodies and are delineated in the brackets. Cells were first resuspended in PEF (3% v/v FBS, 1 mM EDTA and 97% Phosphate Buffered Saline (PBS)) and incubated with the aforementioned antibodies for 30 minutes at room temperature in the dark before being washed 3 times. 7AAD dye (1:50) was
then added to resuspended antibody-bound cells and incubated for 10 minutes before being analyzed using the LSRII flow cytometer.

2.6 Intracellular staining for OCT4

hFibs\textsuperscript{OCT4} were first stained for surface markers CD45PE and CD34APC as described above. Cells were then stained with live/dead violet in PBS, an amine-reactive fluorescent dye that stains dead cells 50 times more intensely than live ones, for 30 minutes at 4°C at an optimized titration (1:7500). The cells were then washed with PBS, fixed and permeabilized using the CytoFix/CytoPerm kit (BD Sciences). Specifically CytoFix was added at a dilution of 1:2 in PBS for 20 minutes at 4°C. The cells were then washed 3 times and resuspended in a 1:10 dilution of CytoPerm Wash Buffer. Once permeabilized cells were incubated with OCT4-alexa647 (1:100 dilution) overnight at 4°C. Cells were than washed and resuspended in PEF and analyzed using the LSRII flow cytometer and FlowJo 8.8.6 Software.

2.7 IFN bioassay

The vero cell line, a mammalian kidney epithelial cell line that cannot produce interferon but responds to the cytokine, was graciously provided to us by Karen Mossman as was vesicular stomatitis virus- GFP (VSV-GFP). Vero cells were maintained in 90% v/v of DMEM, 10% v/v FBS, 1mM L-glutamine (Gibco), and 1% pen/strep. Vero cells were
seeded at a confluency of 50-60%. 24 hours later, fibroblasts were transduced with OCT4 using the method described above. 1 hour and 24 hours later, transduced fibroblasts were washed 3 times with DMEM and supplemented with fibroblast media. 5 hours later the supernatants of the transduced fibroblasts were serially diluted 1:2 in triplicate and transferred onto the vero cells in volumes of 100 µl and incubated for 24 hours. Standard recombinant IFN-alpha (Sigma-Aldrich) was also serially diluted 1:2 in triplicate and transferred onto the vero cells in order to quantify the results. The supernatants were then removed, and the vero cells were infected with 30 µl of VSV-GFP with predetermined viral titers for 1 hour (1:10000 dilution). A 1% methylcellulose overlay was added and incubated for 24 hours at 30°C. The expression of GFP was used to qualitatively assess IFNα production.

2.8 Ectopic addition of Interferon alpha (IFNα) to fibsOCT4

1 hour after transduction of fibroblasts with OCT4 lentivirus, viral supernatant was removed and a 2x serial dilution of IFNα (Sigma-Aldrich) was added from 4000 biological U to 0 U (biological units were provided by manufacturer and were not tested in-house). Additionally, 1000 U of IFNα were added to OCT4-transduced fibroblasts without removing lentiviral supernatant.
2.9 CD34 or CD45 enrichment using magnetic sorting

A single-cell suspension was labelled with either CD45 microbeads or CD34 microbeads (Miltenyi) and as recommended by the manufacture. Specifically 45 ul of the beads was added to up to 1x10^7 cells (cell number varied between experiments). CD45 microbead bound cells were applied to LS+ seperation columns attached to a QuatroMACs seperator (Miltenyi). Columns were washed 3 times to remove non-microbead bound cells (negative fraction) before the magnet-retained fraction of either purified CD34+ or CD45+ hfib^{OCT4} was recovered. The negative fraction was also collected by retaining the flow through.

2.10 MS5 co-culture

MS5 stromal cells were maintained in 90% v/s of alpha-mem (Cellgro) 10% FBS (Neonatal Bovine Serum, HyClone) 1 mM L-glutamine (Gibco). MS5 cells were plated at 50% confluency. 24 hours later MS5 cells were 90% confluent and ready for co-culture. In the case of hfib^{OCT4} cells were scraped and directly added to MS5 stromal cells. Lineage-depleted (lin-) cord blood cells were added as single cell suspensions to MS5 stroma. MS5 media was supplemented with 100ng/ml of SCF and 10ng/µl of G-CSF upon initiation of co-culture. Half media changes were performed every 3-4 days for up to 5 weeks.
2.11 Lineage-depletion of cord blood cells

Cord blood cells, prior to co-culture, were handled by Dr. Borhane Guezguez. Dr. Borhane Guezguez used the Miltenyi Lineage Cell Depletion Kit with the MS column as per the manufacturers specifications to lineage-deplete Cord blood cells before providing them for co-culture.
Chapter 3

Results

We recently described the direct conversion of human skin fibroblasts to multi-lineage hematopoietic progenitors by ectopic expression of the transcription factor OCT4. Much like the reprogramming of fibroblasts to induced pluripotent stem cells, colonies would emerge after 14-21 days\cite{14}. In keeping with the propagation techniques developed and perfected from the reprogramming field, these fib^{OCT4} colonies were manually plucked and isolated from the original initiation culture dish. This method of propagation enriches for the cells undergoing the direct conversion process allowing for a purer expansion of hematopoietic cells with progenitor potential. However, a major drawback of this method that has plagued the reprogramming field for many years is the partially-reprogrammed phenomena\cite{137, 138}. There is often a greater proportion of colonies that arise which are not bona fide iPSCs. These partial-reprogrammed colonies arising from a single cell appear morphologically identical to iPSCs however upon further phenotypic and functional characterization lack the properties of a bona fide pluripotent stem cell. We
have similarly found that plucking the correct colony is an art form that we do not completely understand (Figure 3.4B). In fact, direct conversion of fibroblasts to colonies occurs at a far lower and unpredictable frequency than reprogramming. As the method is in its infancy, it is clear that the exact requirements for consistent conversion still need to be delineated. Unfortunately, as the event of conversion, at least measured by colony formation, is so infrequent, determining which combination of factors is responsible for conversion is proving to be challenging. Particularly, although it is clear that OCT4 is responsible for the conversion, what remains to be fully investigated is whether there is a pre-disposed population of fibroblasts that are amicable to conversion, whether a specific level of OCT4 must be expressed, whether OCT4 must be expressed for a specific and/or finite amount of time or whether is it a combination of all these factors. To add to the complexity, it is unclear if conversion is a cell autonomous process or whether cell density and/or niche are critical factors.

3.1 Cellular growth dynamics during conversion

To speak to the effect of cell density, the acquisition of colonies with the potential to express CD45 is always proceeded with a period of quiescence, where, by empirical observation, the culture of fibroblasts phenocopies the process of senescence observed following continual passage of fibroblasts. After 10 days of what is termed the quiescent phase, a subset of fibroblasts regain proliferative capacity in the form of colonies comprised of rounded cells with hematopoietic progenitor potential. (Figure 3.1A). However, if the
quiescent phase is not observed, for which the governing factors are unknown, not only do colonies not arise but the non-colony forming fibroblasts morphologically alter and become highly proliferative (Figure 3.1B,C). Furthermore, the fibroblasts acquire the expression of CD34, an endothelial marker (Figure 3.2). In order to evaluate which factors were contributing to the acquisition of CD34 expression in non-converting fibroblasts, fibroblasts were maintained in fibroblast media as well as cultured in conversion media in parallel with fibroblasts that were transduced with OCT4 and GFP-pSIN. Acquisition of CD34 was not correlated with viral transduction but solely required incubation of the fibroblasts with conversion conditions (media and growth factors) (Figure 3.2A). In fact, the frequency of CD34 expression on non-transduced fibroblasts or pSIN-GFP transduced fibroblasts (70-90%) was 1.4-6 times greater than on OCT4-transduced fibroblasts. Upon further investigation, aberrant acquisition of CD34 was inversely correlated with expression of OCT4 (Figure 3.2B,C). This pattern was observed regardless of the amount of OCT4-expressing virus used or the media used to administer the virus (PBS vs. conversion media). These data suggest that co-expression of CD34 and OCT4 is unfavorable, and, if OCT4 expression is required for conversion, the ectopic acquisitions of CD34 may be the factor hindering the conversion process. However, what remains unclear is whether acquisition of CD34 expression inhibits expression of OCT4, or whether transduction with OCT4 downregulates CD34 expression. Furthermore, it is unclear how this inhibitory relationship functionally manifests in inhibiting conversion, for instance (1) do the highly proliferative fibroblasts inhibit acquisition of colonies through sub-optimal cell density conditions, (2) is the potential pre-disposed conversion population of fibroblasts lost due to their change in the phenotype or (3) is the required niche lost, so, although
the fibroblasts retain the capacity to convert they are unable to survive due to the lack of supportive niche. To begin addressing these questions, the effect of cell density was first investigated. Cells were seeded at cell densities of 1000 and 10 000 cells / 4 cm$^2$ to determine if the quiescent phase of conversion could be re-established (suggesting it is an artifact of proper cell density) or whether an optimal cell density could be determined that allowed for colony emergence. However, the range of cell densities used did not confer slower growth kinetics nor had an effect on colony emergence (Figure 3.2D). It is therefore evident that the quiescent phase of conversion does not depend on cell density or establish an optimal cell density that can be recapitulated.
Figure 3.1: **Cell growth dynamics of direct conversion culture:** A. The cell morphology and proliferation of hfibs$^{OCT4}$ observed prior to robust acquisition of CD45 expression. The arrows depict senescent-like fibs while colonies are circled B. The cell morphology and proliferation of hFib$^{OCT4}$ observed during my direct conversion attempts over 10 days. C. The 3D structures and cell morphologies observed during my direct conversion attempts.
Figure 3.2: Correlation between CD34 vs. OCT4 expression: A. Expression of CD34 of untransduced fibroblasts in fibroblast growth media and conversion media and pSIN-GFP transduced fibroblasts in conversion media. B. Expression of CD34 and OCT4 of pSIN-OCT4-transduced fibroblasts. Two different media and amounts of virus were used. C. Correlation of OCT4 expression with blood marker expression. D. Empirical observation of the effect of seeding density on hFib^{OCT4} growth kinetics and expression of hematopoietic markers through flow cytometry.
3.2 Effect of induced quiescence on conversion

These results, however, did not address whether the quiescent phase is required for conversion. For instance, induced quiescence may establish optimal growth kinetics that allow for the correct integration of genetic and epigenetic signals initiated by OCT4 for colony emergence. To conclusively determine whether the growth kinetics conferred by the quiescent phase allow for conversion or whether the quiescent phase is an artifact of the genetic and epigenetic changes of conversion, IFNα, an innate immunity inducer, was used to promote quiescence of OCT4-transduced fibroblasts. Although quiescence was achieved with a serial range of concentrations of IFNα, no colonies emerged after 14 days (Figure 3.3A). Furthermore hfibs\textsuperscript{OCT4} were assayed for IFN α production using an IFN bioassay. There was no significant difference in IFN production between mock and hfibs\textsuperscript{OCT4} (Figure 3.3B). These data suggests that the quiescent phase of conversion utilizes a different molecular path than that controlled by IFNα and the molecular pathway used is cell density independent. Furthermore, it suggests that establishing similar growth kinetics observed during the quiescent phase of conversion is insufficient for conversion and other factors are at play for which quiescence is indirectly induced.
Figure 3.3: The effect of induced quiescence by IFNα on acquisition of CD45+ colonies. A. Ectopic addition of IFN to hFib^{OCT4} 1 hour following transduction. B. A representation of the IFN bioassay designed to detect the presence of biologically active IFN from hFib^{OCT4} supernatant. *The quantitative biological units of IFN could not be calculated due to the sub-optimal expression of the VSV-GFP virus in IFN negative controls, which are required for the calculation.
3.3 Alternative approaches to inducing conversion of hfibs$^{OCT4}$

It became evident that attempting to recapitulate the quiescent phase would not induce conversion, as it would appear other factors are at play. As an alternative to trying to recapitulate the quiescent phase new methods were employed to induce the formation of colonies instead, as it is possible that the genetic and epigenetic conversion changes are occurring, but at a different rate than previously observed. Different methods of passing cells were employed to assess whether, given the chance, colonies would emerge later on, as a stochastic model of conversion would suggest. Particularly morphologically distinct colonies were manually plucked, hFibs$^{OCT4}$ were enzymatically passed, non-adherent hFibs$^{OCT4}$ were manually passed or a sheet of fibroblasts that forms was mechanically removed revealing morphologically distinct structures of fibroblasts underneath (Figure 3.4B,C). Interestingly, one method of ‘passage’, which contradicted the stochastic model of conversion, yielded unexpected results (Figure 3.4C). Upon mechanically removing a sheet of cells that forms following transduction of fibroblasts with OCT4, an assortment of structures and colonies composed of morphologically distinct cells remained in the culture. Of the remaining cells a small but significant percentage of the cells expressed CD45 (2%), a pan-hematopoietic marker. In order to determine if a morphologically specific colony or ‘structure’ marked the CD45$^+$ cells, thereby allowing us to correlate CD45 emergence with a specific cellular morphology as a surrogate marker, ICC staining was performed on hfibs$^{OCT4}$ that were passaged in the same manner. Unfortunately no dis-
tinct cellular morphology could be confidently detected (Figure 3.4D). However, this led us to ask whether the acquisition of colonies is required for the acquisition of hematopoietic cells. In fact, upon comparing the emergence of CD45$^+$ cells between the original method of propagation (manually plucked and propagated colonies) to those from the aforementioned method, it became evident that although the frequency of CD45 expression was 15x greater in the original method (27% vs 2%) the number of CD45$^+$ cells was only 2x greater (∼4000 cells vs. ∼2000 cells) (Figure 3.4E). These data suggested the hFib$^{OCT4}$ cells were still converting to CD45$^+$ cells with a similar frequency as originally described, however CD45$^-$ fibroblasts were also proliferative unlike previously, perhaps augmenting only colony formation but not CD45 emergence. Furthermore, CD45$^+$ cells were detected within 14 days of OCT4 transduction without the need to continually passage the cells. In fact, other methods of serial passage did not yield CD45$^+$ cells. This suggested that the cells are primed for conversion from the originally transduced culture and, at least using our current understanding and the methods of conversion, the stochastic model of reprogramming does not correspond.

3.4 Effect of lineage specific culture conditions on conversion

Data in our lab and work from others [39, 38, 139] suggest that OCT4 may be inducing plasticity of cells while the culture conditions direct differentiation to a particular lineage. To determine whether the acquisition of CD45 could be influenced by blood con-
Figure 3.4: Direct conversion experimental attempts: A. Schematic of direct conversion method. B. Morphology and blood marker expression of colony-like hfib^OCT4 manually isolated and allowed to propagate for 6 days. C. Cell morphology and blood marker expression of hfib^OCT4 passed using alternative methods. D. ICC staining of Day 14 hfib^OCT4 for CD45PE. E. Comparison CD45 frequency of expression and cell count between robust direct conversion vs. the peeled skin-like layer method.
ducive conditions, hematopoietic inductive cytokines were added to the conversion media following transduction with OCT4 lentivirus. Indeed, an increase in CD45 acquisition was observed as illustrated in Figure 3.5A. What was most striking was the capacity of this method to generate comparable numbers of CD45\(^+\) cells to those seen in original experiments in which manually plucked colonies yielded robust frequencies of CD45.

In order to induce progenitor capacity, as described in the original experiments, these cells were cultured using the EB method with hematopoietic cytokines. Unlike using hematopoietic cytokines to induce conversion in which 5\% of hfibs\(^{OCT4}\) were CD45\(^+\), the original method of manually plucking colonies allowed for the crude enrichment of CD45\(^+\) so that 50\% of the hfibs\(^{OCT4}\) establishing the embryoid body assay were CD45\(^+\). In order to mimic the original composition of CD45\(^+\) cells in the embryoid body assay, a subset of cells were enriched for CD45. Furthermore, to compare the effects of enriching CD45\(^+\) hfibs\(^{OCT4}\) to more closely resemble original experimental parameters to using whole samples of the hematopoietic cytokine induced CD45\(^+\) hfibs\(^{OCT4}\), CD45\(^-\) and non-enriched cells were cultured in EB conditions in parallel. Strikingly, the enriched CD45\(^+\) cells appeared apoptotic within 4 days of culture (Figure 3.5B), while the negative CD45 fraction and non-enriched CD45 cells could form embryoid-like bodies, although and they did not acquire co-expression of CD34 (Figure 3.5C). Unfortunately, the enrichment method used (antiCD45 beads and column) cannot discriminate between cellular debris and target antigen (CD45) and according to the manufacturers specifications the combination of a dim and low frequency of CD45 cannot be properly enriched. It stands to reason the CD45\(^+\) were not sorted out, and instead mostly cellular debris was enriched in its place. This would account for the increased cell death observed in the enriched CD45
fraction and the lack of phenotypic difference between the negative fraction and the bulk fraction of fibs$^{OCT4}$. Therefore it still remains to be seen if the CD45$^{-}$ negative population plays an inhibitory role or whether the CD45$^{+}$ cells acquired through non-colony forming methods are not functionally equivalent to those derived in original experiments, suggesting the method of emergence (i.e. colony formation) of the cells that have the propensity to become progenitors is critical for derivation of hematopoietic progenitors. In order to answer these questions the CD45$^{+}$ cells must be sorted out using fluorescence activated cell sorting.

3.5 Transient acquisition of CD45$^{+}$CD34$^{+}$ hfib$^{OCT4}$

It has recently become apparent that within the first 7-12 days of ‘conversion’ there is a low and transient, however consistent, co-expression of CD34 and CD45 by hfib$^{OCT4}$ (Figure 3.6A,B). A new paradigm is coming to light in which the reprogramming factors confer a meta-stable plastic state throughout reprogramming that can be exploited through the provision of conducive/inductive conditions of the cell type of interest[39, 38, 139]. For instance, through the transient expression of the reprogramming factors, cells that have been cultured in neural-supportive conditions phenotypically and functionally become neuronal progenitors. It is possible that during the initial stages of conversion in which a subset of cells silence the expression OCT4 lentivirus, these cells become epigenetically primed to alter their cell identity, however due to the lack of inductive cues do not survive or lose the propensity. This would explain the transient expression of CD34
Figure 3.5: **Effect of blood cytokines on the direct conversion process:**

A. Comparison of CD45 expression following blood cytokine treatment from initiation of culture vs. regular conversion media.  

B. Cell viability of CD45 enriched hfibs$^{OCT4}$ after 4 days of EB cytokine treatment.  

C. Blood marker expression of negative CD45 fraction of enriched hfibs$^{OCT4}$ and non-enriched CD45$^+$ hFib$^{OCT4}$ after 16 days in EB cytokine culture.
and CD45 observed. Therefore it would be interesting to sort out the CD45\(^+\)CD34\(^+\) cells to determine if they are truly functional hematopoietic progenitors through the CFU assay and determine if indeed OCT4 is silenced through assaying for (1) integration of the lentivirus and (2) protein expression.

### 3.6 Lymphopoietic potential of hfibs\(^{OCT4}\)

Although the incidence of conversion is infrequent and inconsistent, it is a repeatable phenomenon. As such, the functional potential of these fibroblast-derived progenitors was further characterized. Particularly, although the myeloid progenitor potential was extensively delineated, the potential of these cells to derive the lymphocytic lineage required elucidation. In conjunction with defined cytokines, the MS5 stromal cell line has been shown to induce robust B cell lymphopoiesis demonstrated by the co-expression of CD45 and CD19 (early proB cell markers) from several sources of CD34 enriched blood including iPSCs and hESCs [121, 111]. In order to assess whether OCT4-transduced fibroblasts had the same potential to derive cells of the lymphocytic lineage the MS5 stromal cell line was similarly used. Although, during the conversion process, acquisition of CD45 and CD34 co-expression conferred myeloid progenitor potential, it was unclear whether the same stage of conversion could confer lymphoid progenitor potential. For instance, although the acquisition of CD45 is the earliest marker of conversion, lymphoid cells derived from iPSC and hESCs sources were derived from the CD34\(^+\)CD43\(^+\)CD45\(^-\) fraction of differentiating cells (not the CD34\(^+\)CD45\(^+\) cell fraction) suggesting the commonly denoted
Figure 3.6: **Transient Expression of CD34\(^+\)CD45\(^+\) hfib\(^{OCT4}\)**: A. Schematic of hematopoietic marker expression over time. B. Comparison of hematopoietic progenitor marker expression between day 7 and 14 hFib\(^{OCT4}\) cells using flow cytometry.
primitive progenitor CD34<sup>+</sup>CD45<sup>+</sup> may not have lymphopoietic potential. As such, 3 distinct stages of conversion were used to assess the potential of fibs<sup>OCT4</sup> to derived B lymphocytes, here denoted as stage (1) CD45<sup>-</sup>CD34<sup>-</sup> hFib<sup>OCT4</sup> (2) CD45<sup>+</sup>CD34<sup>-</sup> hFib<sup>OCT4</sup> and (3) CD45<sup>+</sup>CD34<sup>+</sup> hFib<sup>OCT4</sup>. Ideally, fibroblasts from the same OCT4 transduction experiment would have been used, however due to infrequency and resultant low cellular yield of the conversion process, fibroblasts from different OCT4 transduction experiments were used. Newly OCT4-transduced fibroblasts were used to represent stage one of conversion (CD45<sup>-</sup>CD34<sup>-</sup>hFibs<sup>OCT4</sup>) while an in-house established cell line of CD45 expressing fetal fibs<sup>OCT4</sup> was used to represent stage 2 of conversion (CD45<sup>+</sup>CD34<sup>-</sup>fibs<sup>OCT4</sup>) (Figure 3.7A,B).

**Figure 3.7:** **CD34 induction of CD45<sup>+</sup> fetal fibs<sup>OCT4</sup>:** Flow cytometry analysis of CD45<sup>+</sup> fetal fibs<sup>OCT4</sup> acquisition of CD34 expression following culture in inductive hematopoietic cytokines or co-culture with contact inhibited OP9 stromal cells.

Furthermore upon using 2 different established protocols for generating hematopoietic
progenitors (by acquisition of CD34 expression on CD45^+ hFibs) the in-house established fetal fibs^{OCT4} cell line was also used to represent stage 3 of conversion (CD45^+CD34^+ fibs^{OCT4}). As a positive control, lineage-depleted cord blood was also co-cultured on the MS5 stromal cell line in parallel to the co-culture of hfibs^{OCT4} (Stage 1-3) (Figure 3.8A,B).

Figure 3.8: Acquisition of pro-B cell markers from lineage-depleted cord blood co-cultured on MS5 stroma: A. Flow cytometry analysis of lineage-depleted cord blood (CB^{lin-}) negative for the expression of CD19 (pan-B cell marker). B. Flow cytometry analysis of CB^{lin-} following co-cultured on MS5 stroma with SCF and G-CSF for co-expression of CD45 and pan-B cell marker CD19 as well as co-expression of CD19 with CD10.

Commitment to the B-lymphoid lineage was measured by co-expression of CD45 with CD19 (pan-B cell marker). First, survival of hfibs^{OCT4} on the stroma was measured. Whole co-cultures were collected (consisting of the murine MS5 stroma and hfibs^{OCT4})
and were incubated with a Tra-1-85 antibody which stains exclusively human cells (Figure 3.9B). Interestingly, stage 1 fibs$^{OCT4}$ could not survive on the MS5 stroma identified by the lack of expression of Tra-1-85 (a pan-human marker) after 2 weeks of co-culture (Figure 3.9C). Furthermore, although stage 2 fibs$^{OCT4}$ could survive on the MS5 stroma, (Figure 3.9D) only the stage 3 fibs$^{OCT4}$ derived CD45$^+$CD19$^+$ cells (Figure 3.10C-E).

Figure 3.9: **Survival of fibs$^{OCT4}$ requires CD45 expression:** **A.** Schematic representing the method used to derive B cell marker expression from fibs$^{OCT4}$. **B.** Flow cytometry analysis of Tra-1-85 staining of fibs$^{OCT4}$ without cross staining of MS5 stroma. **C.** Flow cytometry detection of Tra-1-85 from CD45$^-$ fibs$^{OCT4}$ co-cultured on MS5 stromal cells with SCF and G-CSF after 2.5 weeks of co-culture. **D.** CD45$^+$ fibs$^{OCT4}$ co-cultured on MS5 stromal cells with SCF and G-CSF after 2.5 weeks of co-culture.
Figure 3.10: Acquisition of pan-B cell marker CD19 requires expression of CD34 on fibs$^{OCT4}$: A. Schematic representing the method used to derive B cell marker expression from fibs$^{OCT4}$ B. Flow cytometry analysis of hematopoietic markers (CD34 and CD45) and pan-B cell marker (CD19) demonstrating lack of cross-staining of antibodies against MS5 stroma. C. Flow cytometry analysis of Day 18 and Day 37 fibs$^{OCT4}$ for the expression CD45, CD34 and CD19. D. Flow cytometry analysis of CD45 expression of CD45$^+$ fibs$^{OCT4}$ following co-culture on MS5 from 2.5 weeks and 7 weeks. E. Flow cytometry analysis of CD45 and pan-B cell marker CD19 expression of CD45$^+$CD34$^+$ fibs$^{OCT4}$ following MS5 co-culture for 2.5 weeks
Particularly almost 50% of CD45$^+$ cells in co-culture expressed CD19. These data suggest that pre-stimulation with hematopoietic conducive cytokines that induce hematopoietic progenitor generation (demonstrated by acquisition of CD34) is required for the derivation of CD45$^+$CD19$^+$ fibs$^{OCT4}$. 
Chapter 4

Discussion

Recently, we described the direct conversion of fibroblasts to multipotent hematopoietic progenitors via the ectopic expression of OCT4. This work shows that acquisition of CD45\(^+\) cells, the first marker of direct conversion, can be acquired without colony formation by day 14. Furthermore the frequency of CD45\(^+\) cells can be increased 5 times by the incubation of hFibs\(^{OCT4}\) with hematopoietic cytokines shortly following transduction. Additionally, the transient co-expression of CD34 and CD45 on fibroblasts can be detected at day 7. However when CD45\(^+\) cells are cultured using the embryoid body assay with hematopoietic cytokines, they do not acquire co-expression of CD34. This is likely due to the inverse relationship seen between OCT4 and CD34 expression, although it is unclear as to why a negative correlation exists. Interestingly, if OCT4 cannot be downregulated in the hFibs\(^{OCT4}\) than it would explain why CD45\(^+\) cells do not acquire co-expression of CD34 or why the expression of CD34\(^+\)CD45\(^+\) on cells is transient. Furthermore, this work shows that stimulation of CD45\(^+\) hFibs\(^{OCT4}\) with hematopoietic
cytokines or co-culture on OP9 stoma cells results in acquisition of CD19 on CD45 expressing fibroblasts when cultured on the lymphopoietic supportive stroma, suggesting lymphopoietic potential of hFibs$^{OCT4}$.

4.1 Affect of niche on direct conversion

During attempts to develop alternative methods of deriving hematopoietic progenitors using the direct conversion process, it became evident that CD45$^+$ cells could be generated without the generation of the conventional colony of rounded cells (Figure 3.4C-E). The difference in growth kinetics displayed between original experiments and current ones by the fibroblasts likely explains this. Firstly, in original experiments, the non-converting fibroblasts remained mainly quiescent while converting fibroblast were proliferative. Due to the relatively small number of converting fibroblasts (1-3 colonies/10 000 cells seeded), the culture had several distinct areas of proliferation. Therefore, it was easy to visualize the proliferative colonies. However, if the whole culture were proliferative, as I have experienced, then it would be difficult to identify colonies. As detailed in the results, the removal of the sheet of fibroblasts that forms following OCT4 transduction of fibroblasts yielded consistent CD45 acquisition while passaging did not. Indeed, during normal maintenance of fibroblasts, if left to become over confluent, a similar sheet of cells forms. In contrast to hFibs$^{OCT4}$ however, untransduced fibroblasts form single monolayer forms such that its removal leaves no cells behind. In the OCT4-transduced fibroblast cultures, underneath the layer of cells, remains distinct gross morphological structures of which
a subset of cells express CD45 (Figure 3.4C). It would appear that perhaps the niche created by these cells is required for the acquisition and retention of CD45 expression because passage of hFibs$^{OCT4}$ results in no expression of CD45 and elevated expression of CD34 by hFibs$^{OCT4}$ (Figure 3.4C). This isn’t surprising since the establishment of niche-like cells prior to hematopoietic cell generation from PSC sources has been extensively detailed in the literature [121, 123, 124, 111, 109, 117]. Indeed during our direct conversion experiments in which non-converting fibroblasts were quiescent, the proliferative converting fibroblasts first formed distinct squamous-like cells upon which rounded non-adherent cells arose resting atop these supportive niche-like cells (Figure 3.1). Therefore when these colonies were mechanically plucked and passaged it was easy to pluck both the supportive-like cells and the rounded cells such that the niche was passaged as well as the converting cells. In current conversion experiments, where CD45$^+$ cells and niche cells cannot be visualized (Figure 3.4), it is difficult to mechanically passage these cells such that the CD45$^+$ cells are still provided with the proper niche. Furthermore, enzymatic passage appears to disrupt the relationship between the niche and the CD45$^+$ such that CD45 expression of fibroblasts cannot be maintained (either through downregulation of CD45 or cell death). Alternatively CD34 expressing cells may survive more readily or CD45 expressing cells cannot survive as well upon being passed, irrespective of a niche, and therefore CD45$^+$ cells are obscured from detection. These data further highlight the importance of developing the correct maintenance for the derivation of target tissues.

These data also challenges the current ideology that reprogramming is a stochastic process and that prolonged culture of reprogramming fibroblasts will lead to increased
reprogramming yield. Indeed, in our hands, for the direct conversion of fibroblasts to hematopoietic progenitors, passing hFibs\textsuperscript{OCT4} does not yield an increase in conversion, and in fact if CD45\textsuperscript{+} cells are not observed with the first 21 days of conversion then they do not emerge.

### 4.2 Transient expression of OCT4 and direct conversion

Apart from the need for the proper development of the required niche, it is likely that a particular temporal and level of expression of OCT4 is also critical. From Their et al.\textsuperscript{s} [139] work, in which expandable neural progenitors were derived, a specific amount of transient expression of OCT4 is absolutely critical for cell fate specification. Furthermore, reprogramming of fibroblasts to iPSCs is concomitant with silencing of the exogenously expressed pluripotency genes. From our own original work, derived hematopoietic progenitors had silenced the expression of OCT4, while CD45\textsuperscript{+} hfib\textsuperscript{OCT4} that did not acquire hematopoietic progenitor potential continued to express OCT4. Therefore it is evident that expression of OCT4 must be tightly regulated to allow for conversion and reprogramming.

Recently, it has come to light that the OCT4 lentivirus that had been used in the original experiments had been unconventionally prepared, but perhaps had conferred the ability to direct conversion more readily.
The original experiments conducted to directly convert fibroblasts to blood utilized a generation 2 OCT4 plasmid, however were packaged with generation 3 helper plasmids, which lacked the required TAT protein. As such, it stands to reason that many of the lentiviral particles produced contained truncated and therefore nonsense mRNA, while a fraction of lentiviruses contained, in the least, a portion of the OCT4 POU domain if not the whole transcript. Therefore, when the fibroblasts were transduced, the majority of fibroblasts received the nonsense mRNA containing lentiviruses. This type of insult naturally activates a cellular innate immune response, which in turn induces quiescence. While the fibroblasts transduced with either a portion of the POU domain of OCT4 or whole OCT4 transcript were able to express a protein product. It is also likely that since both long terminal repeats (LTRs) are required (found at the terminal ends) for genetic integration, the partial or whole OCT4 transcripts did not integrate. Therefore OCT4 was probably transiently expressed. Additionally, if fibroblasts had indeed received functional POU domain transcripts with truncated C-termini it is possible that it was these fibroblasts that had converted, given the fact that the POU domain may have the propensity to mimic the activity of OCT4s POU family members OCT1 or OCT2, factors that have been implicated in hematopoiesis. The combination of quiescence from non-converting fibroblasts to the transient expression of OCT4 (or a portion of the POU domain) most likely allowed for more efficient conversion.

Furthermore, as mentioned, the bypassing of the quiescent phase occurred concomitantly with the acquisition of CD34 expression by non-converting fibroblasts, which was evidently due to the conversion media(Figure 3.2A). Interestingly there was an inverse
relationship between OCT4 expression and CD34 expression (Figure 3.2B,C). This observation would explain why OCT4 must be downregulated in order for CD34+CD45+ hematopoietic progenitors to be derived and why CD45+ cells that do not acquire CD34 still express OCT4. The molecular mechanism is yet to be investigated but may shed light on how to manipulate this relationship to induce more efficient conversion.

Interestingly the transient expression of the CD34+CD45+ cells supports the hypothesis that OCT4 establishes a plastic state from which cells can be directed to or selected by culture conditions to a particular lineage. It is possible the hematopoietic progenitors derived from original experiments were only derived following the degradation of the non-integrated OCT4 in the fibroblasts. Indeed it has been previously demonstrated that lentiviruses with nonfunctional integrase activity at first express the lentivirus in 90% of cells however expression diminishes to 0.5% of cells within 30 days [140]. The culmination of these results would explain why it has been difficult to derive CD34+CD45+ since our use of the 2nd generation helper plasmids with the 2nd generation OCT4 lentivirus integrate into the fibroblasts and cannot be downregulated, thereby inhibiting acquisition of CD34 expression even in hematopoietic inductive cytokines.

Whether the CD34+CD45+ cells had functional hematopoietic progenitor potential, or whether the CD45+ cells could be induced to express CD34 is yet to be fully investigated. However the yield of these cells, especially when hematopoietic conducive conditions are used in combination with the conversion media, were comparable to those observed in original conversion experiments and furthermore take a fraction of the time to establish (7 days vs. 21)(Figure 3.5).
4.3 Lymphopoietic potential of hFibs$^{OCT4}$

There has also been limited success in deriving fully functional naïve lymphocytes from PSCs sources. This limited success [121, 123, 124, 111, 109, 117] may be due to the embryonic-like state of PSCs. Interestingly, Vodyanik et al. [111] were able to derive robust expression of CD19, an pan-B cell marker from iPSCs solely from the CD34$^+$CD43$^+$CD45$^-$ population of cells. However, naïve B cells, expressing IgM have been derived from the CD34$^+$CD45$^+$ fraction of CB. Similarly, only the CD34$^+$CD43$^+$ population of blood progenitors generated T cells. This disparity between hematopoietic cell fractions with lymphopoietic capacities could be due to an intrinsic developmental difference that occurs normally, suggesting differentiation of PSCs recapitulates early development not adult hematopoiesis or this disparity maybe an aberrant artifact of our current culture methods perhaps explaining our inability to derive bone fide HSCs from PSCs. Furthermore the expression of IgM, a hallmark of a naïve functional B cell has never been derived from PSCs sources either, further highlighting our inability to derive bone fide HSCs. As previously discussed, in contrast to differentiating PSCs to the hematopoietic lineage, our direct conversion approach generates adult vs. embryonic hematopoietic fates.

Interestingly, it appears that the CD45$^+$CD34$^+$ population of hfib$^{OCT4}$ gives rise to CD19$^+$ cells upon co-culture on MS5 stroma(Figure 3.10). However, because CD34$^+$ cells were enriched vs. sorted for CD34$^+$CD45$^+$ expression, it is possible that the small fraction of CD34$^+$CD45$^-$ hfib$^{OCT4}$ contributes to the acquisition of CD19 mimicking results.
demonstrated from PSC derived B cells. In order to assess which population of cells truly contributes to commitment to the B cell lineage, hFib$^{OCT4}$ hematopoietic progenitors should be sorted for CD45$^{-}$CD34$^{-}$, CD45$^{+}$CD34$^{-}$, CD45$^{+}$CD34$^{+}$ and CD45$^{-}$CD34$^{+}$. Furthermore, although the CD45$^{-}$ hfib$^{OCT4}$ were shown to not survive on the MS5 stroma (Figure 3.9), it could be possible that, since the conversion process is so infrequent these hFibs$^{OCT4}$ never acquired the propensity or epigenetic status to become hematopoietic progenitors. By FACS sorting for the CD45$^{-}$CD34$^{-}$ population from a sample that has demonstrated hematopoietic potential, the propensity of this population to derive lymphocytes can more accurately be assessed.

Furthermore, if truly the CD45$^{+}$CD34$^{+}$ population of hFib$^{OCT4}$ gives rise to committed progenitor B cells, it would be interesting to determine if these cells are capable of acquiring IgM potential much like CD45$^{+}$CD34$^{+}$ CB cells do. This result would definitively show that adult molecular programs are required for derivation of functional lymphocytes and that direct conversion of adult somatic cells allows for the retention of the adult molecular programs required. Furthermore if the hFib$^{OCT4}$ truly are able to derive functional lymphoid cells, and since functional myeloid potential has been demonstrated, it is entirely possible that in fact these hFib$^{OCT4}$ cells are early multipotent progenitor or even HSCs. Indeed, partial secondary engraftment was observed as described originally.

In order to assess whether myeloid progenitors and lymphoid progenitors are either independently derived or whether a HSC or MPP is generated from which myeloid and lymphoid cells differentiate from, the MS5 stroma co-culture is an excellent assay. The Ms5 stromal cell line is uniquely capable of supporting both myeloid and lymphoid cells.
simultaneously. The hFib\textsuperscript{OCT\textsuperscript{4}} cells can be cultured at a single cell level and single cells can be tracked clonally based on morphologically distinct colonies that arise. Therefore individual colonies can be plucked and assayed using giemsa-wright staining as well cell surface marker expression to determine if both myeloid and lymphoid cells are present within a colony. Because each colony inherently arises from one cell, it can be reasoned that that cell was in the least a MPP with both myeloid and lymphoid lineage potential. If however colonies that are assayed for myeloid and lymphoid morphology and marker expression are comprised of one or the other hematopoietic lineage than we can deduce that lymphoid and myeloid progenitors are independently derived.

4.4 Conclusion

The process of direct conversion from one cell type to another has become a topic of intense research. Two main methods have been developed from the efforts of generating target tissues directly from somatic cells. The first exploits lineage specifying master regulator genes for their ability to guide cell fate decision to a particular cell type. By the ectopic expression of these master regulator genes, the cell identity of one tissue can be redirected to that of particular interest. The second method employs the transient expression of pluripotency genes such as OCT\textsuperscript{4}, to induce the epigenetic priming of cells such that the cells transverse an unstable or meta-stable plastic state that can be directed based on culture conditions rather than master regulator genes. For instance, by the transient expression of OCT\textsuperscript{4}, SOX2 and NANOG in neural or cardiac growth
media conditions, Efe and Kim et al. [38, 39] were able to derive cardiac and neuronal progenitors respectively while bypassing the pluripotent state. We recently described the direct conversion of human fibroblasts to multipotent progenitors by the ectopic expression of OCT4. Which method of conversion our work employs, however, is currently under debate. For instance, although OCT4 is a pluripotency factor and has been shown to globally alter the epigenetic landscape [67] of cells undergoing reprogramming, OCT4 alone is inefficient to induce pluripotency from fibroblasts. Furthermore, OCT4 related transcription factors OCT1 and OCT2 have been implicated to play a role in hematopoietic development [141, 142, 143, 144]. Moreover, studies have predicted that OCT4 has the capacity to bind the conserved octomer binding sites within the promoters of hematopoietic genes, such as Runx1 and CD45 [145], thus potentially augmenting their expression. The global gene expression data from our study demonstrated that OCT4 binds to the promoters and activates the hematopoietic genes SCL, Runx1, CD45 and GATA1 while not altering the expression of pluripotency genes. This data taken together suggests that perhaps the overexpression of OCT4 may promote an acquired ability to bind octomer domains of hematopoietic genes and thus cause critical cell fate changes allowing for the direct conversion of fibroblasts to blood.

From this work it would appear that acquisition of CD45+ hFibs\textsuperscript{OCT4} and transient acquisition of CD45+CD34+ hFibs\textsuperscript{OCT4} is possible without an initial quiescent phase or the formation of colonies. Furthermore, acquisition of CD45+CD34+ and CD45+ hFibs\textsuperscript{OCT4} occurs within 7-14 days of OCT4 transduction without the requirement of passage. Additionally, the acquisition of these markers by hFibs\textsuperscript{OCT4} is enhanced by
the use of hematopoietic cytokines. It is speculated here that the expression of CD45 and CD34 is transient due to the continued expression of OCT4, also causing the inability of CD45\(^+\) hFibs\(^{OCT4}\) to acquire CD34 expression in the embryoid body assay with hematopoietic cytokines. Indeed, a negative correlation between the expression of OCT4 and CD34 was observed during this study. To circumvent this affect and promote more efficient direct conversion the use of a dosable and transiently expressed vector for OCT4 is suggested to determine which levels and for which period of time OCT4 expression is required for optimal conversion efficency. This data taken together suggests the transient expression of OCT4 may induce plasticity of transduced fibroblasts for which the culture conditions can direct the lineage decisions required to aquire a target cell type. A model is proposed here where OCT4 expression, possibly depending on level, promotes the expression or epigenetic signature of a particular cell type ie blood more than another cell type (Figure 4.1). Once the expression of OCT4 is removed, the cells with the blood genes and epigenetic profiles sufficient to propagate the blood signature go on to acquire a blood cell type (Figure 4.1A). Other cells in which the blood signature was not established as dominant may take on another cell type. Furthermore, if transient expression of OCT4 is coupled with hematopoietic signaling molecules or hematopoietic conducive conditions, than the signaling pathways may inhibit the expression of non-target cell type genes thereby allowing the blood cell type genetics and epigenetics, once again to dominate and survive in conducive conditions increasing the yield of hematopoietic cells (Figure 4.1B). However, with prolonged OCT4 expression no one cell type signature can dominate unless inherently OCT4 is required for its cell fate specification. This model is applied to explain current observations and how to increase efficiency of reprogramming (Figure
4.2). With prolonged expression of OCT4, the propensity to maintain CD45^+CD34^+ expressing cells is lost within 21 days (Figure 4.2A). While in original experiments, the level and timing of OCT4 expression was not optimized possibly leading to suboptimal derivation of hematopoietic progenitors (Figure 4.2B). However if the levels and duration of OCT4 expression can be optimized, than highly efficient conversion can be achieved with a fraction of the time. (Figure 4.2C).

Moreover, if the derivation of hematopoietic progenitors can be optimized to be more robust, then more controlled experiments can be designed to prospectively isolate different populations of hfibs^{OCT4} (ie. CD34^-CD45^-, CD34^+CD45^-, CD34^+CD45^+, CD34^-CD45^+) to assess which population contains lymphopoiesis potential. Furthermore, in vitro and in vivo lymphopoietic potential needs to be further characterized to determine whether this method of deriving hematopoietic progenitors is superior to those derived from pluripotent sources. If it is possible to identify a particular point during the conversion process that confers functional lymphoid lineage and myeloid lineage generation, it may suggest that these cells are epigenetically and phenotypically primed multi-potent progenitors or HSCs that can engraft the xenograft model. This would be the first example of deriving bone find HSC from a somatic cell source other than blood. A finding of this magnitude could revolutionize our understanding of lineage specification and our approach to regenerative medicine.
Figure 4.1: **Proposed model of cell fate determination by transient expression of OCT4** A. A fraction of fibroblasts will activate hematopoietic epigenetic and genetic signatures at a higher level than other cell type signatures while the opposite is true for other fibroblasts. Which cell type signatures are activated may depend on OCT4 levels and duration of expression. Once OCT4 expression is turned off, the epigenetic and genetic programs that are most dominant in that cell can direct the fate of the cell. B. The addition of hematopoietic signaling molecules or conducive conditions may partially inhibit the OCT4 activation of non-blood cell signatures allowing for blood signatures to dominate and direct the fate of the cell.
Figure 4.2: Proposed model of conversion efficiency as a product of OCT4 expression and duration. A. Prolonged expression of OCT4 results in transient expression of CD45⁺CD34⁺ B. Un-optimized transient expression of OCT4 results in suboptimal conversion after 37 days C. Optimized level and duration of expression of OCT4 (4-7 days) with hematopoietic cytokines may lead to efficient conversion within 7 days.
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