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The mechanisms and regulation of lineage commitment in hematopoietic stem cell

Mechanismy a regulace liniové determinace v hematopoetické kmenové buňce

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Bachelor's thesis

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Poděkování:

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Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracoval/a samostatně a že jsem uvedl/a všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

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Podpis

Abstract

Hematopoietic stem cells (HSCs) are crucial for maintaining balanced homeostasis in the human body. HSCs are pluripotent cells, which are able to give rise to many very different cells. HSCs can be found in fetal liver initially during organismal development where they expand and move to their more definitive location, the bone marrow, shortly before birth in humans and mice. HSCs possess to not only recapitulate themselves (self-renew) or proliferate and expand, but are also the first branching point from which subsequent multipotent progenitors and eventually all blood cell lineages are formed thus establishing specific and restricted terminal differentiation pathways. The irreversible decision to initiate and follow a specific differentiation pathway is designated as lineage commitment. The drivers of lineage commitment, which are a base of this thesis, are intrinsic as well as extrinsic factors acting within the stem cell niche, such as transcription factors, chromatin remodeling factors, and cytokines, which are essential for proliferation, survival, self-renewal and lineage commitment decisions. These regulatory factors, working either independently or in mutual coordination, maintain balanced homeostasis of HSC renewal and their differentiation. The goal of this thesis will be to ascribe the mechanisms of lineage commitment of HSCs with regard to the role that key regulation molecules play in this cell fate decision.

Hematopoetické kmenové buňky (hematopoietic stem cells, HSCs) mají rozhodující význam pro udržení vyvážené homeostázy v lidském těle. Jedná se o pluripotentní buňky, které můžeme nalézt ve fetálních játrech vyvíjejícího se plodu, odkud se krátce před narozením jedince šíří a přesouvají do svého cílového orgánu, kostní dřeně. Hematopoetické kmenové buňky mají schopnost se nejen sami obnovovat a rozšiřovat, ale jsou také prvním bodem větvení z kterého jsou vytvářeny multipotentní progenitorové buňky a následně všechny typy krevních buněk v lidském těle, čímž se stanoví specifické a terminálně diferenciované linie krevních buněk. Tento proces je vysoce specifický, nezvratný a nazývá se liniová determinace. Řídící molekuly liniové determinace jsou vnitřní i vnější faktory, působící na kmenové buňky, jako jsou transkripční faktory, chromatin remodelační faktory, a cytokiny. Tyto faktory jsou nezbytné pro proliferaci, přežití, sebeobnovu a liniovou determinaci buněk, a pracují buď samostatně nebo v koordinaci. Udržují vyváženou homeostázu obnovy a diferenciace buněk. Cílem této práce je identifikovat mechanismy zodpovědné za liniovou determinaci hematopoetických kmenových buněk s ohledem na roli, kterou hrají klíčové regulační molekuly při rozhodování o osudu buněk.

Abbreviations

CD - Cluster of Differentiation
CHARM - Comprehensive High-throughput Array-based Relative Methylation
ChIP - Chromatin Immune Precipitation Protocol
CLP - Common Myeloid Progenitor
CMP - Common Myeloid Progenitor
CMRP - Common Myeloid Restricted Progenitor
DMR - Differentially Methylated Region
Dnmt1 - DNA (Cytosine-5-)-Methyltransferase 1
Dnmt3b - DNA (Cytosine-5-)-Methyltransferase 3 beta
Epo - Erythropoietin
Ery - Erythroid
FACS - Fluorescence Activated cell Sorting
Gadd45α – Growth Arrest and DNA Damage-Inducible Protein
Gata-1 - GATA Binding Protein 1
GATA-4 – GATA Binding Protein 4
G-CSF - Granulocyte Colony Stimulating Factor
GM - Granulocyte-Monocyte
GMP - Granulocyte-Monocyte Progenitor
H3K4me1 - Monomethylation of Histone Lysine 4
Hdac7a - Histone Deacetylase 7
HNF3 – Forkhead Box E3
Hoxa9 – Homeobox A9
HSC - Hematopoietic Stem Cell
IT-HSC - Intermediate Hematopoietic Stem Cell
LMPP - Lymphoid Primed Multipotent Progenitor
LT-HSC - Long Term Hematopoietic Stem Cell
M-CSF - Macrophage Colony Stimulating Factor
Meg - Megakaryocyte
Meis1 - Meis Homeobox 1
MEP - Megakaryocyte-Erythroid Progenitor
MERP - Megakaryocyte-Erythroid Repopulating Progenitor
MkRP - Megakaryocyte Repopulating Progenitor
MPP - Multipotent Progenitor
MyB - Myeloid Progenitor with B cell Potential
MyRP - Myeloid Repopulating Progenitor
MyT - Myeloid Progenitor with T cell Potential
NK - Natural Killer Cell
PU.1 - Spi-1 Proto-Oncogene
rCMP - Restricted Common Myeloid Progenitor
SLAM - Signaling Lymphocyte Activation Molecule
ST-HSC - Short Term Hematopoietic Stem Cell
TF - Transcription Factor
vWF - von Willebrand Factor

Table of Contents

1. Introduction 1

1.1 Hematopoietic Stem Cells 1

1.2 The Hematopoietic Map 1

1.3 Lineage Commitment 2

2. Redefining HSCs 3

2.1 HSCs Phenotype and Surface Markers 4

2.2 HSC Subtypes 5

3. Models of Lineage Commitment 7

3.1 Classical Model 8

3.2 Revised Model 9

3.3 Myeloid Bypass Model 10

3.4 Other Models 11

4. Transcription Factors 12

4.1 Transcription Factors GATA-1 and PU.1 14

5. Epigenetic Modification 17

5.1 Enhancers 17

5.2 DNA Methylation 20

6. Extrinsic Cytokine Signaling 22

7. Conclusion 23

8. Bibliography 25

1. Introduction

1.1 Hematopoietic Stem Cells

Hematopoietic stem cells (HSCs) are the prime component in hematopoiesis as they reside at the top of the hematopoietic lineage hierarchy. HSCs are defined as a population of pluripotent cells with varying developmental potentials regulated by a complex intrinsic network of transcription factors located in the bone marrow of adult mammals (Notta *et al,* 2015; Orkin and Zon, 2008) The stem cell niche is a highly specific microenvironment of numerous cell types attached together in an interlinked microtissue, that is necessary for both the self-renewal of HSCs as well as for their differentiation via reception of critical signal inputs (J. Zhang *et al,* 2003). HSCs are responsible for the replenishment and reconstitution of all hematopoietic cells by yielding multipotent progenitors that are gradually more restricted to one or several lineages (Serafini *et al,* 2007). These multipotent progenitors give way to blood precursors that become committed to a specific lineage differentiation and produce all mature cells, including red blood cells, megakaryocytes, myeloid cells and lymphocytes.

1.2 The Hematopoietic Map

Figure 1 (below) illustrates the simplified cartoon that depicts the development of blood lineages originating from the primitive HSC. The dominant position is held by the uncommitted HSCs that are capable of self-renewal through symmetrical cell division producing two daughter cells with the same cell fate. Down the line, HSCs then differentiate into the primitive progenitor cells of the myeloid and lymphoid lineages (Miyamoto *et al,* 2002). These cells lose the potential to self-renew, and are able to differentiate to multiple mature cell types by asymmetrical cell division that gives rise to two daughter cells with differential cell fates (Takano *et al,* 2004). The myeloid progenitor cell is able to specialize to become a megakaryocyte, responsible for the production of thrombocytes (platelets), as well as an erythrocyte (red blood cell), mast cell or a myeloblast that differentiates either into a basophil, neutrophil, eosinophil or a monocyte. Monocytes are then able to further specify into mature macrophages or

dendritic cells (Akashi *et al*, 2000). The lymphoid progenitor can either become a natural killer cell or a lymphocyte that further specifies into a T cell, or a B cell that gives rise to plasma cells (Kondo, Weissman, and Akashi, 1997). These mature cells are fully committed, fulfilling a specific role in the hematopoietic system and are unable to become, or produce any other cell type.

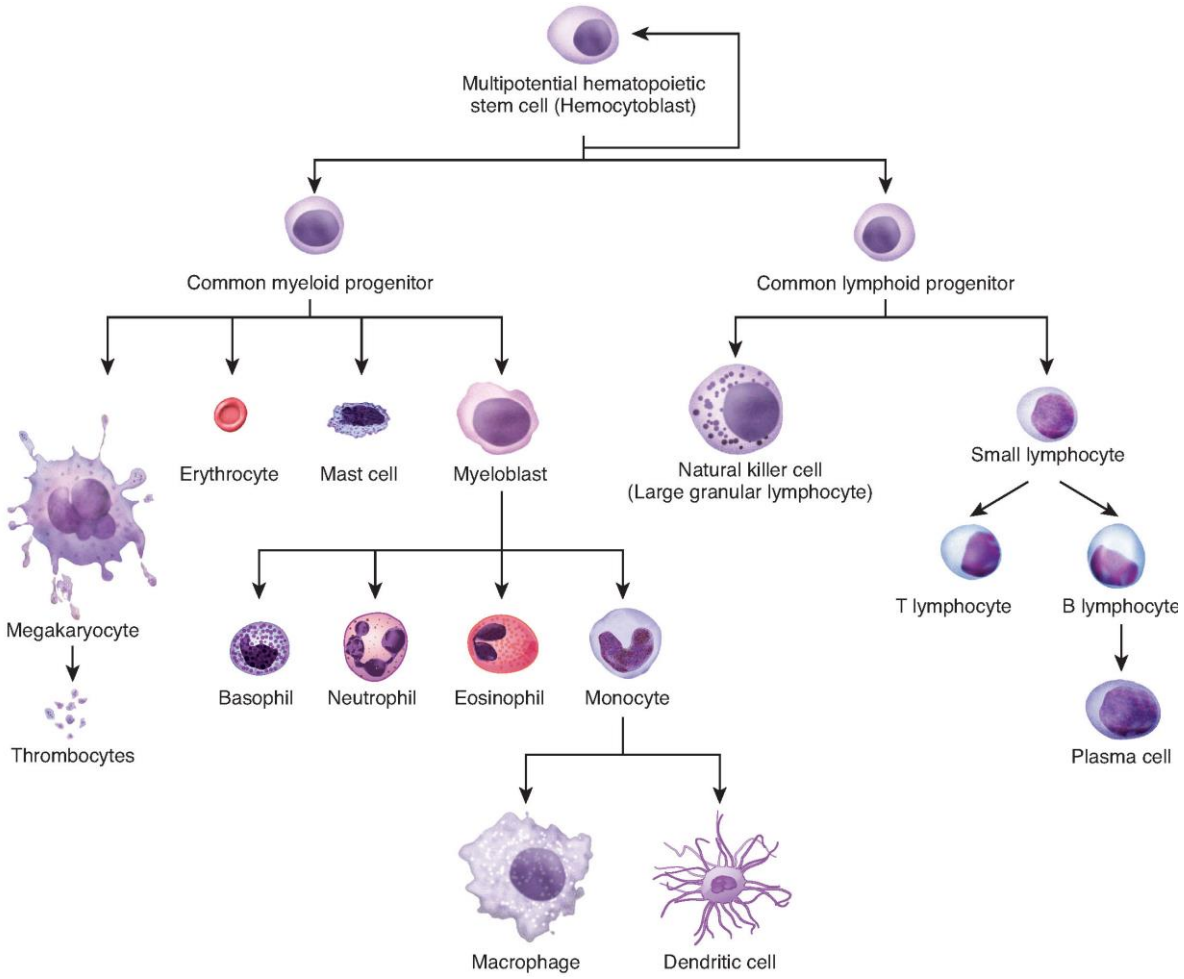

Figure 1 – Developmental Hierarchy of the Hematopoietic Stem Cell
(OpenStax College, 2013, available online: http://cnx.org/contents/FPtK1zmh@6.27:lmwyzD1v@4/Cellular-Differentiation)

1.3 Lineage Commitment

The process by which HSCs become restricted to a specific differentiation pathway is called lineage commitment. Lineage commitment may be defined as the point at which a cell becomes irreversibly restricted to one particular fate, and thus loses any

subsequent potential to differentiate into any other kind of cell (Pina *et al*, 2012). The molecular mechanisms by which individual cells decide to what particular lineage they become committed to are highly complex and poorly characterized. Models depicting lineage commitment range from a stochastic differentiation event to a specifically regulated process controlled by intrinsic transcription factors or chromatin remodeling factors, and extrinsic signal molecules such as cytokines (Rieger *et al*, 2009; Teles *et al*, 2013; Jeong *et al*, 2013; Nimmo, May, and Enver, 2015). Although a number of player molecules have been identified, they may represent only a small subset of a larger network controlling lineage commitment. Furthermore, understanding the mechanisms of lineage commitment may be a crucial stride in our knowledge of blood malignancies, as the disturbance of the HSCs transcriptional network is at the core of oncogenesis (Mueller *et al*, 2002; Orkin and Zon, 2008).

2. Redefining HSCs

Hematopoietic stem cells reside at the top of the lineage commitment tree that becomes over several cell divisions more restricted in its potential to form cells of multiple characteristics. However, classical models depicting this lineage hierarchy are overly simplified, ignoring that HSCs do not conform to a stoic classification, but that they are a rather dynamic class of cells (Notta *et al*, 2015). Experiments have shown that within a single population of HSCs, the levels of expressed genes are highly heterogeneous and promiscuous, being more or less inclined to more specified restriction pathways (Notta *et al*, 2015; Ema, Morita, and Suda, 2014). This heterogeneity suggests that HSCs form functional subtypes, and that the order depicted in the classic bifurcation model may be much more random. Transplantation experiments have utilized reconstitution kinetics as a basis of creating new classifications within HSCs, and suggest that HSCs classification and differentiation is linked to their life span (Ema, Morita, and Suda, 2014; Müller-Sieburg *et al*, 2002). In this chapter we will look at the classification of HSCs, the phenotype surface markers used to identify them to show that HSCs lineage hierarchy doesn't necessarily begin with a single homogenous cell, but rather a group of heterogeneous cells that incline toward a particular programmed pathway.

2.1 HSCs Phenotype and Surface Markers

Population-based studies in the past provided important data to understand HSCs. However, commitment and self-renewal decisions are made by each individual HSC, therefore examination of single cells at the clonal level is also highly required (Muller-Sieburg *et al*, 2002). The expression of cell surface markers such as proteins and cytokine receptors is a crucial aid in the identification and classification of HSCs and their multipotent progeny (Guo *et al*, 2013). Distinct combinations of surface markers expressed in individual cells reflect the inclination and adherence to specific lineages as functionally diverse populations of cells only express certain combinations, enabling the compartmentalization of HSCs and progenitor cells (Macaulay *et al*, 2016). The benefit of compartmentalizing cells into subpopulations based on their expressed surface markers is countered by the limitations of profiling, because choosing to use a small number of surface markers can conceal a higher level of heterogeneity and could compromise HSCs hierarchy (Guo *et al*, 2013). The many surface marker molecules known as CDs (Cluster of Differentiation) can be identified using an antibody clone that binds to the receptor site of the marker (Notta *et al*, 2015). Breakthrough analytic techniques such as single cell mRNA sequencing is able to detect 75 % more genes than the previously used microarray techniques and thus enables to analyze whole transcriptomes (the entire ensemble of RNA molecules within a single cell) (Tang *et al*, 2009). Furthermore fluorescence-activated cell sorting (FACS) flow cytometry is also an essential procedure for the sorting of HSCs populations and subsequent progenitors (Bendall *et al*, 2011). As cells become more lineage restricted, their surface marker expression changes, some CDs are lost while others are gained, which allows the separation of cell populations and lineages based on the markers expressed.

Among the most well-known to define HSCs and progenitor cells is the CD34 surface marker (Debili *et al*, 2001). The presence of $CD34^+$ marks an undifferentiated/progenitor state although it has been observed that $CD34^-$ cells also exist within the most primitive populations of HSCs exemplifying the high degree of heterogeneity within the same class of cells (Goodell *et al*, 1997). The $CD150^+$ marker part of the SLAM (signaling lymphocyte activation molecule) family of markers is yet

another classic example of HSCs characterization with a particular function in myeloid reconstitution in the $CD150^{+}CD34^{-}KSL$ ($c\text{-}Kit^{+}Sca\text{-}1^{+}Lin^{-}$) fraction (Yamamoto *et al*, 2013; Morita, Ema, and Nakauchi, 2010). Another example of an important surface marker is CD135 also known as FLT3 or FLK-2. This surface marker is also an important factor for lineage differentiation as it is crucial for lymphocyte development and characterizes the lymphoid/myeloid progenitor cell (LMPP) although $CD135^{+}$ HSCs have been observed to momentarily become repopulating cells with a short-term reconstitution capability (Boyer *et al*, 2011; Christensen and Weissman, 2001). The examples above demonstrate the usefulness of surface markers to determine HSCs and progenitor phenotypes and their classification. On the contrary, they also demonstrate high heterogeneity among already defined sets of cells indicating the need for further single cell gene expression data (Guo *et al*, 2013).

2.2 HSC Subtypes

Serial single-cell transplants in mice have revealed that it may be practical to classify HSCs into specific subtypes based on their differentiation potential, as it was found that even individual HSCs are capable of producing quite diverse subtypes. (Wilson *et al*, 2015; Babovic and Eaves, 2014). However, varying nomenclature used to describe these subclasses has promoted confusion (Ema, Morita, and Suda, 2014) so a revised classification system should be implemented. For the sake of clarity in this assay, the terms long-term HSCs (LT-HSCs) having a reconstitution time (the duration it takes for a cell to repopulate and proliferate) longer than 12 months correspond to the α subtype. Intermediate HSCs (IT-HSCs) with more than a 6 month reconstitution time for β cells, and short-term HSCs (ST-HSCs) for the γ and δ subtypes having a reconstitution of less than 6 months (Benz *et al*, 2012). The LT, IT and ST-HSC subtypes can be further categorized by their contributive ratio of myeloid versus lymphoid cells present 4-6 months after single cell transplantation in recipient mice (Dykstra *et al*, 2007). On top of that, the LT-HSC and IT-HSC types were found to be linked to self-renewal activity and succeeding daughter cells maintained the same differentiation programs arguing that these patterns have been intrinsically predetermined in cells even before the initial

transplant (Dykstra *et al,* 2007). **Figure 2** below represents the hierarchy of the 4 subtypes of HSCs based on long-term repopulation following single cell transplantation. Furthermore, IT-HSCs were found to have a shorter self-renewal period than LT-HSCs and ST-HSCs completely lacking self-renewal ability (Dykstra *et al,* 2007). Differentiation toward a myeloid pathway becomes increasingly restricted in IT-HSC (β) and ST-HSC (γ, δ) cells which correspond to traditional HSC differentiation hierarchy. (Dykstra *et al,* 2007). LT-HSC (α) cells have long-term repopulating activity with high self-renewal capacity that can be exclusively sustained over multiple cycles. These cells also have a tendency to generate myeloid specified progeny although they are also capable of presenting direct lymphoid repopulation (Dykstra *et al*,2007).

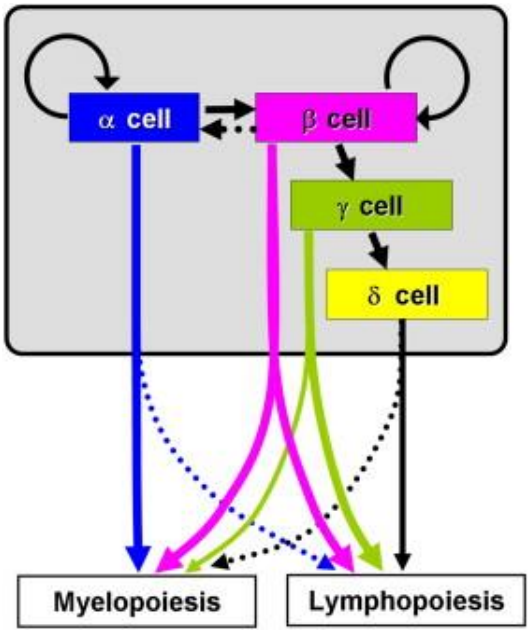

Figure 2 - Hierarchy of long-term repopulating HSCs subtypes after single cell transplantation in mice (Dykstra *et al*, 2007)

The perception that progeny of transplanted cells maintain similar differentiation programming suggests that they are intrinsically regulated, and they challenge the idea that all HSCs have identical multi-lineage differentiation potential. On contrary, it may suggest that the cell environment provides such expected heterogeneity. The attempt to find order in the classification of HSCs has been largely unsuccessful as the findings

indicate that HSCs have a unique intrinsically regulated epigenetic marker that maintains similar lineage differentiation from successive generations of daughter HSCs (Mercer *et al*, 2011; Babovic and Eaves, 2014). Furthermore, and more importantly, the proposition that HSCs have individual intrinsic/extrinsic programming that is maintained in subsequent generations lends to the idea that lineage commitment is a regulated process rather than a random mechanism. The suggestion that each HSC has its own individual programming could also explain the high heterogeneity found within single populations of HSCs and could test the hypothesis that differentiation is a stochastic event. Repopulation experiments using individual stem cells might prove to be a useful tool in further studying not only of HSCs classification but also lineage commitment in potential (Müller-Sieburg *et al*, 2002).

3. Models of Lineage Commitment

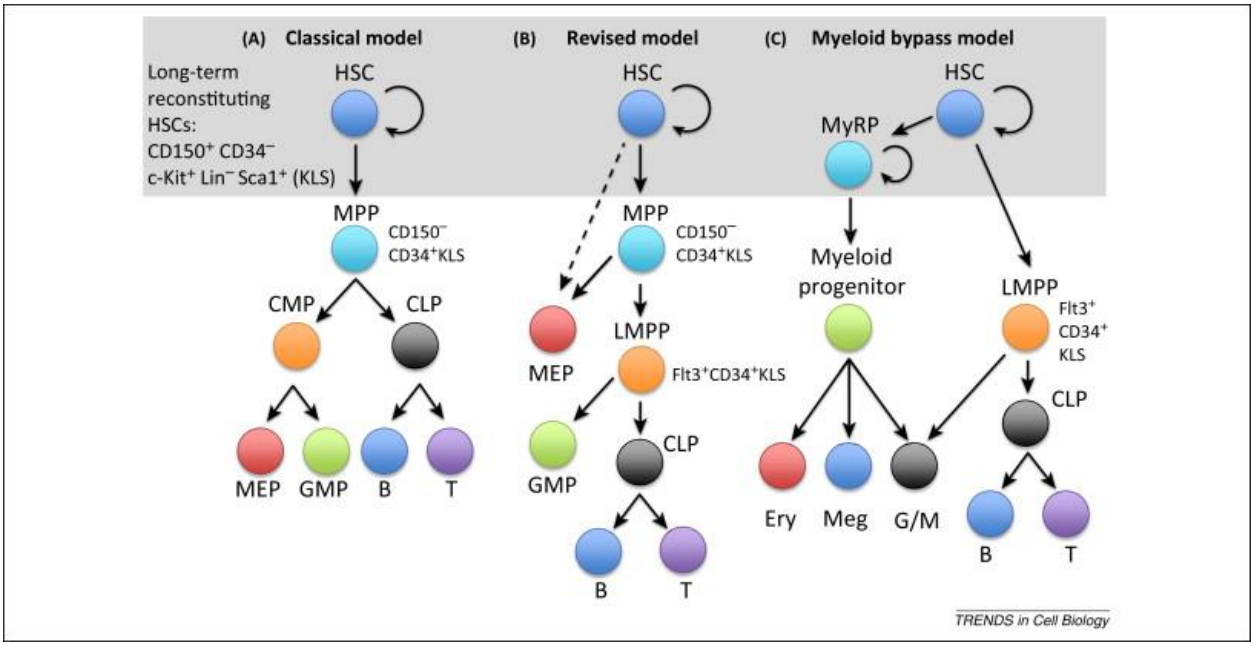

Figure 3 – Models of Lineage Commitment; (A) Classical Bifurcation Model, (B) Revised Model, (C) Myeloid Bypass Model (Nimmo, May, and Enver, 2015)

As I have pointed out in the previous chapter, lineage hierarchy is a dynamic system, such fact was revised over the last decade mirroring HSCs and its subsequent

progenitor capability to differentiate and more importantly reprogram multiple commitment lineages. This chapter will discuss suitable models how to study lineage hierarchy from the outdated classical bifurcation model to modern revised models illustrating the heterogeneous nature of HSCs. In the bone marrow, LT-HSCs have the innate ability of self-renewal, extended life-span as well as the potential to reconstitute the complete immune system for the life of an organism (Notta *et al*, 2015; Spangrude, Heimfeld, and Weissman, 1988). They can, through asymmetrical cell division differentiate into ST-HSCs which also have multipotent properties, but their self-renewal capabilities are quite limited than that of their predecessor as they are active only for a limited time (Mercer, Lin, and Murre, 2011). The differentiation from LT-HSC to ST-HSC is marked by downregulation of previously discussed CD150 marker which was shown to sub classify HSC and MPP (multipotent progenitor) populations. (Yamamoto *et al*, 2013; Oguro, Ding, and Morrison, 2013). Further down, ST-HSCs then differentiate into MPPs that are not capable of self-renewing but do carry potential to differentiate to both myeloid and lymphoid lineages (Akashi *et al*, 2000).

3.1 Classical Model

In the established, but outdated classical bifurcation model seen in **Figure 3A**, primitive LT-HSCs bearing surface markers $CD150^{+}CD34^{-}KLS$ give rise to MPPs $CD150^{-}CD34^{+}$ which then commit to either common myeloid or lymphoid progenitors known as CMPs and CLPs, marking the first major segregation of cell fate (Notta *et al*, 2015). CLPs then give rise to B cells, T cells, as well as natural killer cells (NK), and CMPs give rise to unipotent progenitors GMPs (granulocyte-monocyte progenitors) and MEPs (megakaryocyte-erythroid progenitors) that differentiate to only one cell lineage (Kondo, Weissman, and Akashi, 1997; Akashi *et al*, 2000; Notta *et al*, 2015). In this model CLPs and CMPs can be viewed as reciprocally exclusive populations (Nimmo, May, and Enver, 2015). This model has been proposed over a decade ago (Akashi *et al*, 2000) and suggests that cellular differentiation is a gradual and symmetrical process. However, recent studies are overruling this view as CMP-like progenitors have been found to be more heterogeneous than expected, comprised of unipotent myeloid and erythroid progenitors with little or no megakaryocytic activity (Notta *et al*, 2015). Furthermore, these studies at the clonal level have not been able to identify oligopotent

progenitors (more restricted progenitors that produce only a few cell lineages) suggesting that lineage restricted cells producing entire ranges of myeloid cells may rather be a subset of HSCs poised for myeloid lineage bias (Yamamoto *et al*, 2013; Benz *et al*, 2012). MPPs have also not been experimentally found, and thus the association of MPPs to CLPs or CMPs is not yet clear (Ema, Morita, and Suda, 2014). Single cell gene expression experiments utilizing clonal functional assays discovered an early division of megakaryocyte-erythroid and lymphoid fates as early as in HSCs, and implicated that a subset of HSCs exist with an exclusive platelet reconstitution potential (A Sanjuan-Pla *et al*, 2013).

3.2 Revised Model

Above introduced findings show the necessity to review and redraw the lineage commitment hierarchy, and many models reflecting the heterogeneity of HSCs and progenitor cells have been proposed. One of these is the revised model (**Figure 3B**) that suggests lineage commitment to occur at the MPP and possibly at the HSC level. Studies have shown that the MPP population contains cells that express the lymphoid lineage-specific recombination activation gene (rag)-1, causing them to differentiate into lymphoid-biased cells, but also preserving minor myeloid potential allowing for the differentiation to the MEP lineage independently of MEPs originating from HSC fractions (Arinobu *et al*, 2007). Further studies have shown that a fraction of MPPs expressing $CD135^+$ (Flk-2/$Flt3^+$) lack MEP potential to become specified toward lymphoid differentiation. The $CD135^+CD34^+$KLS MPPs were therefore designated as LMPP (lymphoid-primed multipotent progenitor) (Adolfsson *et al*, 2005). In this revised model, the early separation of MEP lineages in HSC/MPP fractions is key and proposes that most MEPs arise directly from HSCs as there were no single cells found to co-express any megakaryocyte-erythroid and lymphoid markers (Notta *et al*, 2015). The summarization of this model takes into account the early separation of the MEP lineage near to the HSC fraction (Notta *et al*, 2015), but also takes into account heterogeneity found in the MPP fraction as a portion retained slight MEP differentiation potential while the LMPP- biased fraction lacked any MEP potential (Adolfsson *et al*, 2005).

3.3 Myeloid Bypass Model

The myeloid bypass model (**Figures 3C and 4**) is different from previous models because it proposes self-renewal of cells to exist apart from the primitive HSC compartment, specifically in the MyRP (myeloid repopulating progenitor) cells that are lineage restricted to either MkRPs (megakaryocyte repopulating progenitor), MERPs (megakaryocyte-erythrocyte repopulating progenitor), and CMRPs (common myeloid repopulating progenitor) (Yamamoto *et al*, 2013; Nimmo, May, and Enver, 2015).

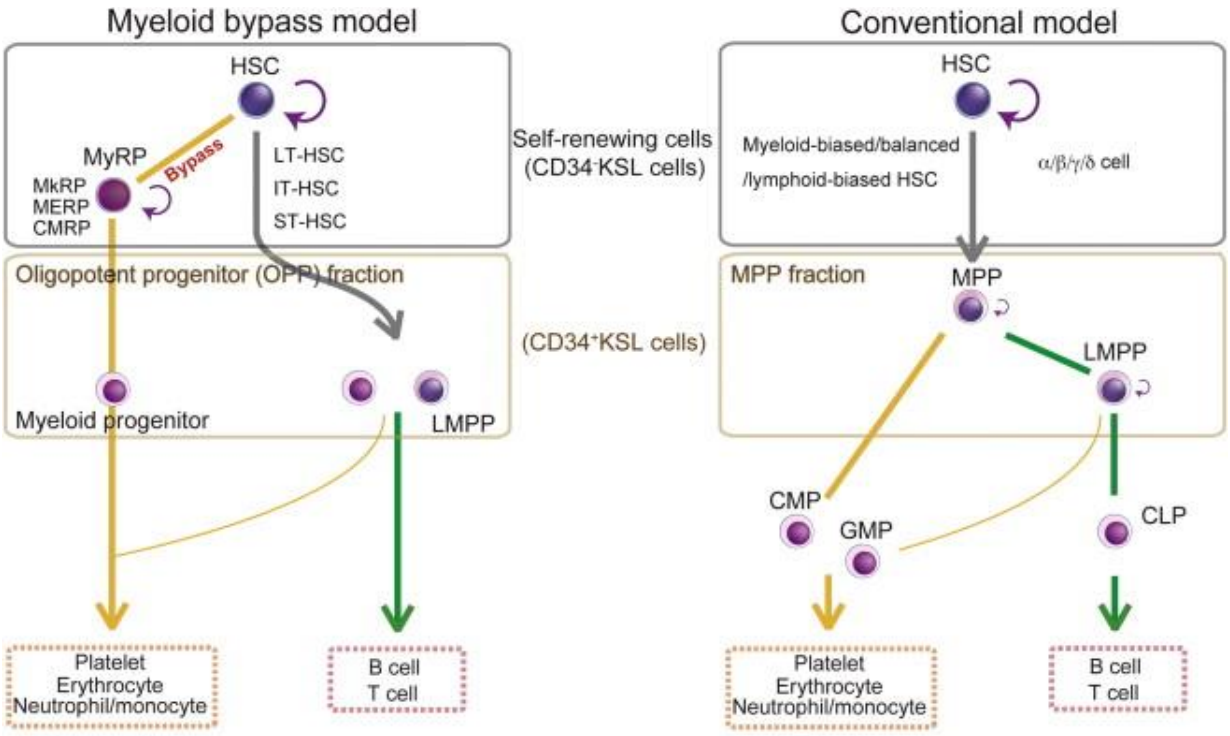

Figure 4 – Myeloid Bypass Model and Conventional (Revised) Model with respect to HSC subtypes (Yamamoto *et al*, 2013)

The self-renewing MyRP populations then give rise to a myeloid progenitor that differentiates to all myeloid cells as in other models, with a fraction of the LMPP compartment contributing to the G/M lineage (Yamamoto *et al*, 2013). Unlike the revised model, the myeloid bypass model categorizes LMPPs with lack of repopulating ability implying that they may originate from ST-HSCs rather than MPPs (Morita, Ema, and Nakauchi, 2010). In the study by Yamamoto *et al* (2013) single MPPs and LMPPs did

not show any repopulation in mice receiving these single cells which indicates that MyRPs rather than MPPs and LMPPs are responsible for the production of myeloid cells. **Figure 4** usefully illustrates the position of LT-, IT- and ST-HSCs on the lineage hierarchy being separate from the MyRP fraction, as well HSC subtypes in the conventional model based on expressed surface markers.

3.4 Other Models

To illustrate the wide degree of variability, I have included the "New differentiation model" created by Ema, Morita, and Suda (2014) seen in **Figure 5** which is centered around the similar concept as the myeloid bypass model that the megakaryocyte/myeloid lineage (rCMPs) is one of the first lineages constituted, directly from the HSC compartment through asymmetrical cell division (Yamamoto *et al,* 2013). This model therefore expects that the myeloid lineage is established prior to and is larger than the lymphoid lineage. In this model, LT-HSCs give rise to myeloid progenitors and IT-HSCs form an intermediate state between LT-HSC and ST-HSC cells. From there, ST-HSCs give rise to MyB (myeloid progenitor with B cell potential) and MyT (myeloid progenitor with T cell potential) (Ema, Morita, and Suda, 2014).

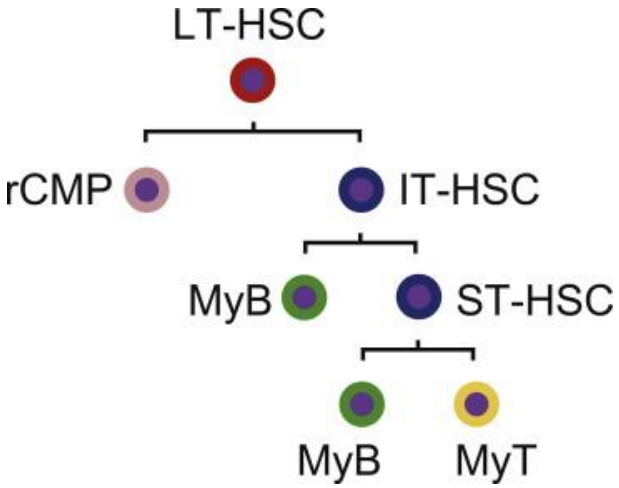

Figure 5 – "New Differentiation Model" (Ema, Morita, and Suda, 2014)

As was demonstrated by the differing models of lineage hierarchy, wide heterogeneity has been found to exist even within the most classically defined and pure HSC compartments. Lack of oligopotent intermediate progenitors found in studies by

Notta *et al* (2015) support a simpler two tier hierarchy seen in **Figure 6,** with an upper tier consisting of HSCs and MPPs and a lower tier made up of committed unipotent progenitors. It is easy to become lost and confused among the constantly changing models, however, there is one clear take away message and that is the importance of single cell studies of HSCs. Our need to find order within HSC and progenitor populations often leads to more questions than answers, as population-based experiments are unable to distinguish between assortments of cells with fluctuating degrees of lineage bias. Therefore, simpler models such as in **Figure 6** should be implemented as emerging evidence suggests that HSCs and their progeny can be efficiently studied and thought of as individual, unique cells rather than populations assumed to be marker and functionally synonymous (Notta *et al*, 2015).

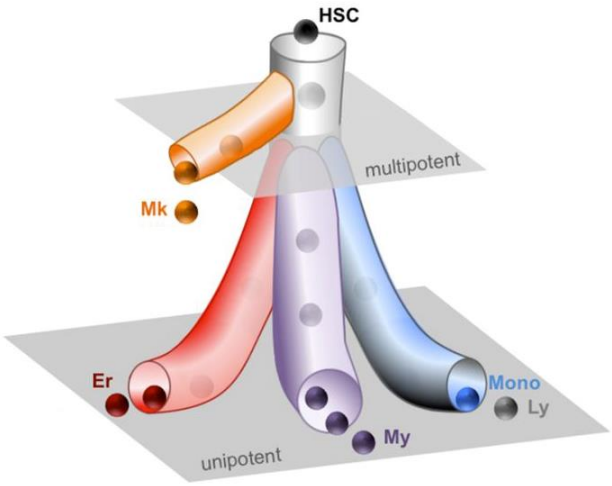

Figure 6 – Simplified model of Mk (Megakaryocyte), Er (Erythroid), My (Myeloid), and Ly (Lymphoid) Differentiation. Acquired and revised from (Notta *et al,* 2015)

4. Transcription Factors

Decisions of HSCs cell fate have been suggested to result from a network of active transcription factors based on a cross-antagonism relationship (Takano *et al*, 2004; Notta *et al*, 2015; Nimmo, May, and Enver, 2015). Experiments attempting to show this connection have revealed that HSCs express multiple lineage-restricting genes at low levels suggesting that multiple differentiation programs, restricting a cell to one particular fate are expressed at the same. The co-expression of multiple antagonistic genes within

one cell in preparation for commitment has been described as multilineage priming (Hu *et al,* 1997). Multilineage priming can predict how the concentration of a particular transcription factor over another one influences lineage choice (see **Figure 7)**. The schematic illustrates how two genes (B, A) or (B, C) are simultaneously expressed, at low levels in multipotent cells. In committed cells, gene expression is locked to one specific differentiation program, while the others are silenced (as represented by lineages A, B and C in **Figure 7**).

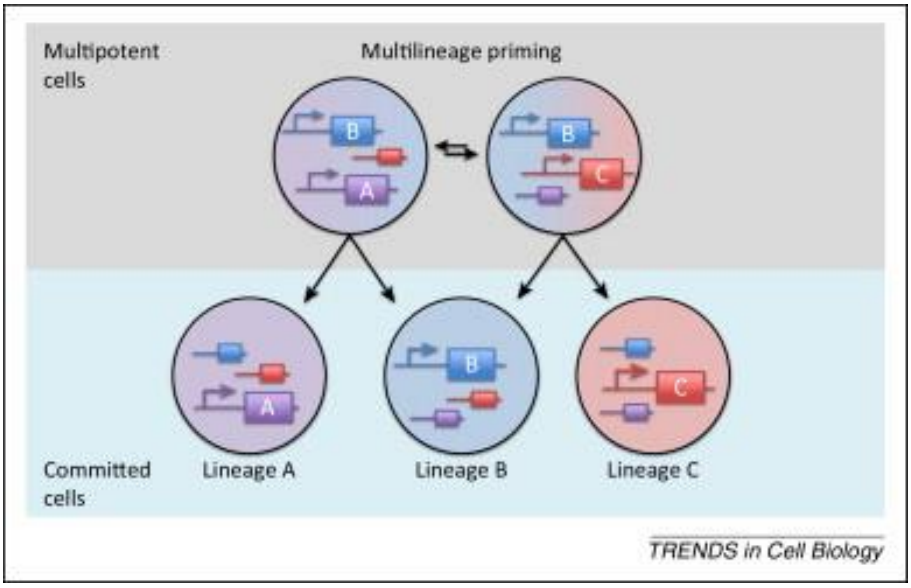

Figure 7 – Schematic Diagram of Multilineage Priming (Nimmo, May, and Enver, 2015)

The upregulation of one gene leads to the downregulation or inhibition of its antagonistic partner, and thus the alternative differentiation pathway becomes silenced locking the cell into a more committed state (Nerlov *et al,* 2000; Orkin and Zon, 2008). Although HSCs express the ability to switch from an undifferentiated state to a committed one, the underlying regulation of this mechanism, the relationship between transcription factors is still unknown and needs to be assessed using more sensitive assays. Physical interactions between transcription factors may be only transient, and it shouldn't be assumed that lineage priming is a regulated process, but that the up and downregulation of transcription factors could be merely a stochastic fluctuation within an inactive transcriptional network (Notta *et al,* 2015; Roeder and Glauche, 2006).

4.1 Transcription Factors GATA-1 and PU.1

The network of interacting transcription factors whose role may be critical for fate choice is vague as many TFs have yet to be identified, and the mechanisms by which TFs regulate lineage commitment is even more of a mystery. For the sake of clarity, I will draw upon the best-studied examples of transcription factors that are critical in the early separation of HSC lineages. These transcription factors are PU.1 and GATA-1, both of which have been extensively studied and found to have an essential role that leads to the separation and maturation of the myeloid/lymphoid cells that are regulated by PU.1 and erythrocyte/megakaryocyte cells controlled by GATA-1 (Nerlov *et al*, 2000; Arinobu *et al*, 2007) .

Furthermore, PU.1 and GATA-1 also have a physical cross-antagonistic relationship that can cause mutual inhibition and cause HSCs to lean towards an erythroid/megakaryocyte fate over a myeloid specification (Nerlov *et al*, 2000). In the earliest steps of lineage commitment, both *PU.1* and *GATA-1* genes are co-expressed in multipotential progenitor cells at low levels, reflecting the theory of lineage priming allowing HSCs to differentiate down multiple pathways (P. Zhang *et al*, 2000; Roeder and Glauche, 2006).

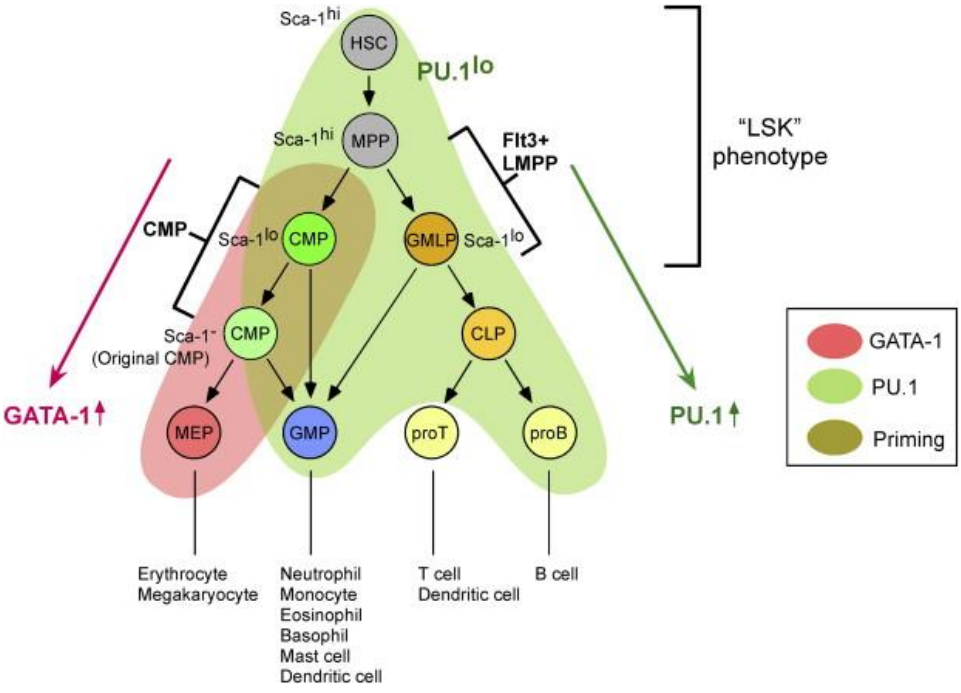

Figure 8 – HSC Development mapped by *GATA-1* and *PU.1* (Arinobu *et al*, 2007)

As either *PU.1* or *GATA-1* gene is upregulated, it inhibits the other causing the cell to become specified to a particular fate as cross antagonism of transcription factors suggests (Notta *et al,* 2015; Orkin and Zon, 2008). **Figure 8** (Arinobu *et al,* 2007) shows the primed states of *PU.1* and *GATA-1* genes and how lineage commitment is affected if either one becomes more dominant. Low expression levels of *PU.1* are present in earliest HSC stages, however, the upregulation of both *PU.1* and *GATA-1* at the MPP stage initiate commitment to myeloid/lymphoid differentiation (Arinobu *et al,* 2007). The absence of *GATA-1* expression marks the cells to specify toward a lymphoid fate eventually producing B and T cells, while the primed state co-expression of both leads HSCs to become myeloid inclined. As *GATA-1* expression upregulates in later stages of the myeloid progenitor, *PU.1* expression is downregulated causing the cell to become affiliated to an erythrocyte/megakaryocyte fate (Arinobu *et al,* 2007) Although it is not clearly known what drives one transcription factor to become dominant over the other, the physical interaction between GATA-1 and PU.1 proteins that causes their cross antagonism has been described (Rekhtman *et al,* 1999; Rekhtman *et al,* 2003)

Experimental studies on both factors in **Figure 9** by Zhang et al (1999) have revealed that GATA-1 protein can actively bind to a specific region of the PU.1 protein known as β3/β4 that interacts with its co activator C-JUN that is responsible for activating target myeloid gene promoters (P. Zhang *et al,* 1999). As GATA-1 occupies the binding site of C-JUN, it inhibits activity of the PU.1 protein and essentially suppresses the cell's ability to differentiate toward a myeloid specification. The interaction of GATA-1 with PU.1 can therefore affect myeloid lineage development without actually suppressing the expression of the *PU.1* gene (Nerlov *et al,* 2000; Burda, Laslo, and Stopka, 2010). As the relationship between the two factors is cross antagonistic, PU.1 also possesses the ability to inhibit GATA-1 and represses erythroid/megakaryocyte development although through a different mechanism (Rekhtman *et al,* 2003) which includes repression of the target genes of GATA-1 at chromatin levels (Stopka *et al,* 2005). GATA-1 transcriptional activity is inhibited by binding PU.1 β3/β4 region to GATA-1 C-finger region disabling the ability of GATA-1 to bind to its promoter (P. Zhang *et al,* 2000).

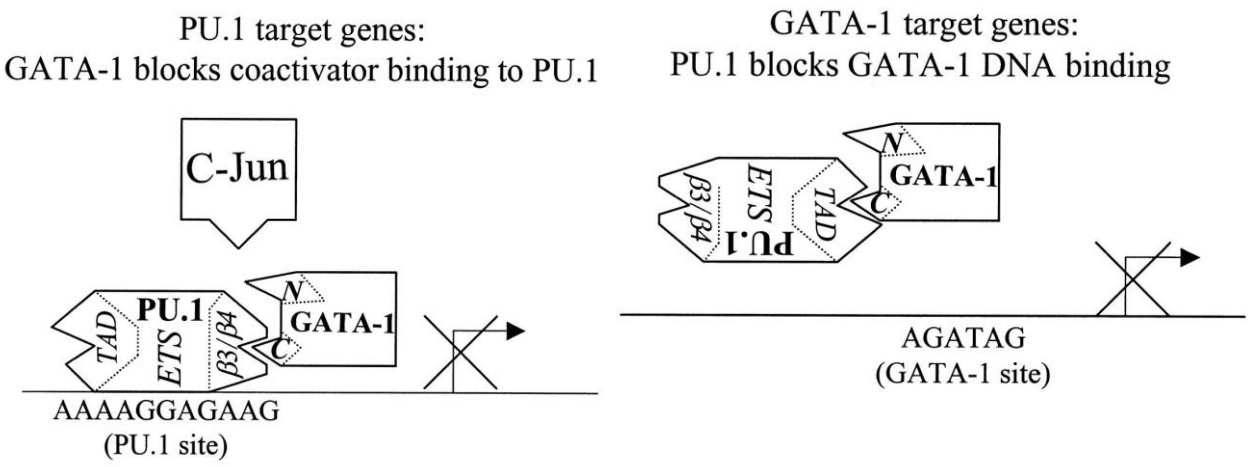

Figure 9 – GATA-1 and PU.1 Reciprocal Physical Interaction (P. Zhang *et al,* 2000)

It has also been reported that PU.1 known to bind to *GATA-1* on DNA, can also repress erythroid/megakaryocyte differentiation through the cooperation of its co-repressor pRB, a tumor suppressing protein without interfering with the ability of GATA-1 to bind to DNA (Rekhtman *et al,* 2003; Burda *et al,* 2016).

Although the relationship between TFs GATA-1 and PU.1 reflects the idea of multilineage priming, recent studies conducted by A Sanjuan-Pla et al (2013) and Notta et al (2015) show that molecular factors such as vWF (von Willebrand factor), a protein known to be associated with platelet aggregation, are near mutually exclusively expressed. These findings do not conform to the idea of lineage priming and rather suggest that lineage commitment occurs without the presence of an instructive differentiation program (Notta *et al,* 2015). The resolution of the debate will largely depend on the individual examinations of TF interactions known to instruct lineage commitment. Until then, theories that lineage commitment occurs primarily through mutual TF cross antagonism while being primed at low levels in multipotent progenitors will be disputed by views that lineage commitment is stochastically initiated (Notta *et al,* 2015).

5. Epigenetic Modification

In the previous chapter, I have discussed the key role that transcription factors play during lineage commitment. However, it is exceptionally noteworthy to discuss the possibly even more important role played by epigenetic modifiers such as chromatin remodelation factors, methylation, and acetylation to name a few. Chromatin is a complex structure comprised of DNA and associated proteins called histones, whose role is to compress DNA into structural units as well as taking part in gene regulation and expression (Lara-Astiaso *et al,* 2014). It is thanks to chromatin's open structure and its modifications that transcription factors can be expressed, and TFs themselves can further regulate gene expression as well as modify chromatin structure by binding to DNA. The current view of chromatin's structure during cell fate decisions is that uncommitted cells display a largely open structure that becomes progressively more restricted through each commitment pathway (Lara-Astiaso *et al,* 2014). However, this view is being challenged as histone modification analyses of stem cells and their differentiated counterparts do not always conform to this observation (Mikkelsen *et al,* 2007). Resolving the controversy requires investigation of the intermediate progenitors on the DNA level.

Epigenetic factors such as chromatin modification and methylation cause long lasting, even permanent changes in HSC gene expression (Müller-Sieburg *et al,* 2002). Through the opening and closing of primed loci, cells become functionally restricted to express only certain genes, while the rest of the potential repertoire is silenced. This provides HSCs with a limited substrate, and essentially directs them to differentiate down a specific transcription program (Akashi *et al,* 2003). Chromatin structure and modification during hematopoiesis and lineage commitment is a very dynamic process, yet very little is known about the molecular mechanisms that compel differentiation.

5.1 Enhancers

Long, non-coding regions in the DNA called enhancers work together with regulatory proteins to control transcription of targeted genes. As the name suggests, enhancers usually faciliate transcription, while on the other hand, silencers inhibit transcription (Lara-Astiaso *et al,* 2014). It was thought that all enhancers are already established at the HSC level, assuming that stem cells have the potential to differentiate

down every lineage pathway, and that they "close" during irreversible fate decisions. This is seemingly not the case as over 17,000 enhancers have been found to be established *de novo* during lineage commitment in the study by Lara-Astiaso et al (2014). Existing studies of chromatin structure changes during development are hindered as single cell epigenetic analysis is not yet possible (Nimmo, May, and Enver, 2015). Currently, the method for profiling structure changes in chromatin is through the use of chromatin immunoprecipitation (ChIP). The method consists of cross-linking DNA to proteins that are thought to be associated with a specific region of the genome and "shearing" using either sound energy called sonication, or through nuclease digestion (Nelson, Denisenko, and Bomsztyk, 2006). Using a specific antibody that binds to the associated protein, the DNA is then selectively immunoprecipitated and purified (Nelson, Denisenko, and Bomsztyk, 2006). Further improvement upon the ChIP method, the iChIP uses bar-coding to mark individual samples and analyze histone modification on as few as hundreds of primary cells (Nimmo, May, and Enver 2015). This study used histone markers such as H3K4me1 (monomethylation of histone lysine 4) to find that progenitors of specific lineages are more closely related to differentiated cells within their particular lineage than to other progenitors of other lineages as can be seen in **Figure 10** (Lara-Astiaso *et al*, 2014).

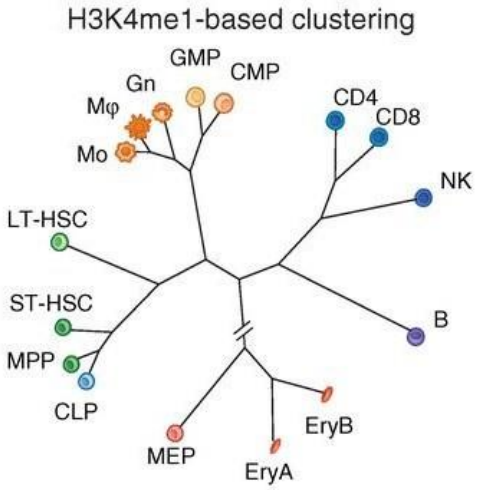

Figure 10 – Clustering of Progenitor Cells by Histone Marker H3K4me1 (Lara-Astiaso *et al*, 2014)

These findings suggest that establishment of enhancers commences earlier during lineage commitment and that the differentiation potential of specified cells can be revealed prior to an expression program (Lara-Astiaso *et al,* 2014). Studied enhancers displayed typical behavior, being established in HSCs, only to become prominent within their own active lineage. Unexpectedly, the study also found that many enhancers became established at the fist point of commitment to a particular lineage progenitor and that silencing of an enhancer is a rather gradual process (Lara-Astiaso *et al,* 2014). **Figure 11** demonstrates the stages and percentages of gained enhancers at each particular phase. In the erythroid lineage, about 65 % of enhancers are actually gained in the MEP stage of commitment, and about 50 % of enhancers are established in the from the MPP-CMP specification in myeloid lineage. Together, in the myeloid lineage, the CMP and GMP are responsible for about 60-80 % of gained enhancers in fully differentiated cells and the largest loss of enhancers is surprisingly in the final differentiation step to mature cells. Finally, the lymphoid linage actually exhibits that the largest gain of enhancers comes from terminally differentiated cells (**Figure 11**) (Lara-Astiaso *et al,* 2014).

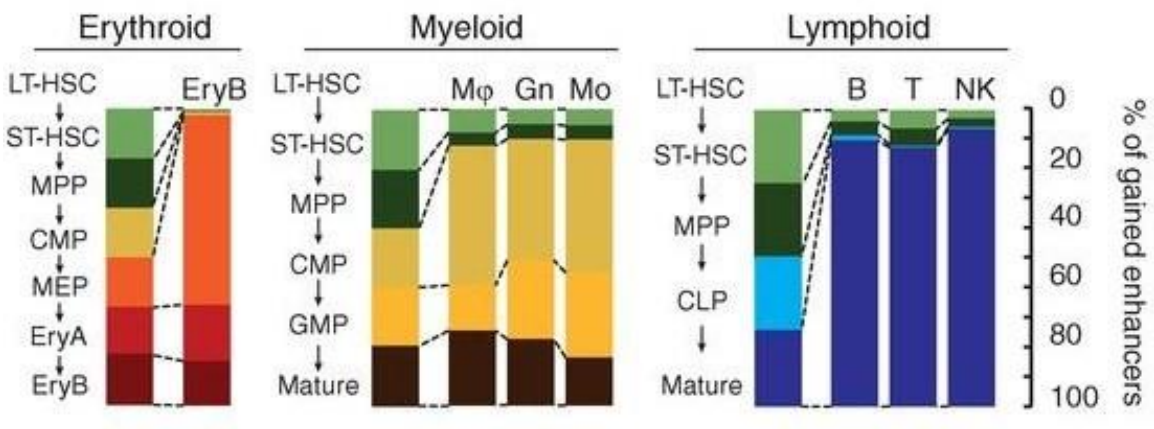

Figure 11 – Percentage of Gained Enhancers During Different Stages of Cell Commitment (Lara-Astiaso *et al,* 2014)

The establishment of enhancers is controlled and promoted by transcription factors, such as HNF3 and GATA-4 which have the ability to access and open chromatin by themselves (Cirillo *et al,* 2002), to previously studied TFs such as PU.1 and GATA-1 that can be classified as regulators of myeloid/erythroid enhancers (Lara-Astiaso *et al,*

2014). However, the mechanisms by which transcription factors bind and alter chromatin structure through histone modifications is unclear and should be further studied as the relationship between TFs, and chromatin modification factors such as enhancers are deterministic in fate choice (Cirillo *et al*, 2002).

The recent studies show that although enhancers gained from LT-HSCs are lost during commitment as was previously predicted, many enhancers are also gained in subsequent progenitors and even terminally differentiated cells prior to their activation and commitment. Furthermore, the vibrant nature of enhancers supports the view that chromatin reorganization during fate choice is a highly dynamic system and suggests that maximum enhancer potential is not found in HSCs as was previously believed, but in multipotent progenitors and subsequent differentiated progeny (Lara-Astiaso *et al*, 2014).

5.2 DNA Methylation

DNA methylation has been found to be an important epigenetic factor in the separation of the myeloid versus lymphoid lineages. DNA methylation typically acts to repress the expression of genes, and both pluripotent cells and multipotent progenitors exhibit methylation patterns represented by active/inactive chromosomal regions that ultimately lead to the suppression of pluripotent transcription factors and the expression of lineage specific genes (Bocker *et al*, 2011). The study of mouse DNA methyltransferase (*Dnmt1*), an enzyme responsible for the regulation and maintenance of DNA methylation explicitly shows the key role that methylation plays (Trowbridge *et al*, 2009). The disruption of *Dnmt1* in HSCs not only hindered their ability to give rise to multipotent progenitors, the myeloid compartment in particular, but also showed disturbance in stem-cell renewal and niche retention (Trowbridge *et al*, 2009). Recent studies attempting to map methylation across hematopoiesis have shown that the quantity of DNA methylation causes cells to become more inclined toward a certain lineage. Lymphoid primed cells have shown to have a larger total methylation than their myeloid counterpart, which are in turn dependent on the loss of methylation (Ji *et al*, 2010).

Distinct patterns and similarity of methylation has also been found within functionally similar cell types, suggesting that modifications of methylation within the HSC compartments point cells toward lymphoid/myeloid lineages prior to lineage commitment (Bocker *et al*, 2011). Furthermore, analysis using CHARM (Comprehensive

High-throughput Array-based Relative Methylation) and real time PCR techniques have demonstrated changes in gene expression relative to differentially methylated regions (DMRs) within distinct populations of MPPs (Ji *et al,* 2010). These analyses illustrate how certain TFs function based on methylation modification. For example, the *Gadd45α* gene, suggested to play a role in myeloid development has been found to be upregulated as well as demethylated in mice, causing the activation of gene expression during the shift from CMP to GMP. Even more importantly, *Gadd45α* has been proposed to cause additional demethylation in other genes promoting hypomethylation in the myeloid lineage, although its role is still disputed (Ji *et al,* 2010). More genes studied in mice such as *Meis1,* and *Hoxa9,* which are necessary for hematopoiesis and megakaryocyte development were also identified to have been hypermethylated, and thus repressed, pointing HSCs toward a myeloid/lymphoid commitment (Ji *et al,* 2010). Chromatin modifiers such as *Hdac7a* and *Dnmt3b* also showed different levels of methylation during fate decisions, thus increasing the variability in epigenetic programs that drive commitment (Ji *et al,* 2010). *Hdac7* is a gene that programs histone deacetylation and suppresses gene expression. In the study by Ji et al (2010) *Hdac7* was found to be demethylated and upregulated in cells that cannot revert to a myeloid lineage, signifying its role for active silencing of genes that are responsible for the maintaining myeloid potential. On the opposite spectrum, *Dnmt3b* is a methyltransferase that was found to be downregulated and methylated in CMPs and GMPs and although the gene has been linked to a role in self-renewal, it's function in lineage commitment remains uncharacterized (Ji *et al,* 2010). Although the genes mentioned above are just few of the many more genes and their interactions affected by methylation, CHARM analysis might prove to be a useful tool to pinpoint still unknown transcription factors affecting lineage commitment.

Multilineage priming previously discussed mirrors epigenetic modification as the extinguishment of alternative differentiation pathways through suppression and activation of enhancers triggers commitment (Nimmo, May, and Enver, 2015). Together, the findings force us to reshape our view of chromatin's role during differentiation and provide groundwork for future studies. Although chromatin remodeling through enhancers and methylation proved to be an integral process driving commitment, it is

unquestionably only one of the many factors among a larger intrinsic/extrinsic network influencing stem cell fate.

6. Extrinsic Cytokine Signaling

In the previous chapters, I have discussed the role of intrinsic factors that affect lineage commitment of HSCs, however we have yet to touch upon extrinsic factors, mainly signaling cytokines that may have a role in the instruction of lineage choice. Cytokines are small signaling proteins utilizing receptor tyrosine kinases to trigger signaling cascades that eventually lead to gene expression affiliated with a specific lineage (Endele, Etzrodt, and Schroeder, 2014).

The role of cytokines during commitment is largely debated upon as it is unclear whether their function is permissive or instructive (Rieger *et al,* 2009; Sarrazin and Sieweke, 2011). Experiments using knockout mice indicated that rather than instructing commitment, the role of cytokines is to allow and promote growth, proliferation and survival of committed progenitors. The permissive model is in an accord with lineage priming as the eventual "win" of one transcription factor also leads to the upregulation of lineage specific cytokines making progenitor cells susceptible to further permissive cytokine signaling promoting expansion of cells (Chang *et al,* 2008). A study by Semerad et al (1999) used targeted mutation of a G-CSF (granulocyte colony stimulating factor) receptor to replace the domain used for cytokine signaling with a domain of an erythropoietin (Epo) receptor. The group found no change in progenitor numbers suggesting that cytokine signaling affects survival and growth but isn't necessary for lineage commitment (Semerad *et al*, 1999). Furthermore, it was found that many cytokines have similar if not interchangeable signaling domains suggesting that the lack of specific cytokine may not compromise a differentiation program as committed cells could be produced by alternate differentiation pathways through redundant cytokines (Semerad *et al,* 1999; Endele, Etzrodt, and Schroeder, 2014). Although the study cited above demonstrates the permissive role that cytokines can play, conversely, cases in which cytokines appear to have an instructive role also exist.

Mossadegh-Keller et al (2013) have shown that M-CSF (macrophage colony stimulating factor) can directly act upon HSCs and activate the promoter region of *PU.1*. The group found that the cell population had an increased number of cells expressing *PU.1* and preference toward myeloid differentiation (Mossadegh-Keller *et al*, 2013). Recent studies of the Epo have shown that it may have an instructive role in the establishment of erythroid lineages (Grover *et al*, 2014). It initiates and reprograms erythroid prone gene expression and even more importantly suppresses alternate differentiation programs especially the GM (granulocyte/macrophage) specification in HSCs and multipotent progenitors (Grover *et al*, 2014). This exemplifies how cytokines can instruct cell fate choices by activating lineage affiliated transcription factors. Although there are many other cases in which cytokines have been shown to play both a permissive and instructive role, the results are inconclusive and the question of cytokines role during lineage commitment remains largely controversial.

7. Conclusion

Lineage commitment of HSCs is influenced by many factors, although none have been identified as having a primary role in definitive differentiation. The molecular mechanisms that regulate stem cell fate decisions remain to be illuminated. The emergence of HSC revealed a broad spectrum of heterogeneity in distinct HSC populations. This heterogeneity is reflected in the various models of lineage commitment that are being continually revised and updated. The role of transcription factors leads to two conversing models that drive lineage commitment. First, a stochastic model in which lineage affiliated transcription factors are co-expressed at low levels, and the fluctuations between gene expressions typically end with one TF prevailing, leading to its upregulation as the other is downregulated, extinguishing alternative differentiation pathways and locking HSCs toward a specific fate. In the stochastic model, the eventual win of one TF over the other is deemed to be random, although specific HSC compartments were shown to be already biased for certain lineages. The opposite assessment views lineage commitment as driven by specific regulation, either by intrinsic factors such as the already mentioned TFs, or through epigenetic modification of DNA, as well as external cues in the form of cytokine signaling that instructs HSCs to express

specific genes, directing them to differentiate. Although there is evidence for both models, neither is definitive. Further research and more sensitive assays will be required to resolve the debate. In conclusion, our views and understanding of HSCs lineage commitment may be due for a change, to see HSCs not as groups of uniform cells, but perhaps more individually, reflecting their dynamic and unique nature.

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