Programming Blood Cell Fates. Insights from Direct Lineage Conversion and Development

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Published: 2018-01-01

Document Version
Publisher's PDF, also known as Version of record

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Insights from Direct Lineage Conversion and Development

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LUND
UNIVERSITY

DOCTORAL DISSERTATION
by due permission of the Faculty of Medicine, Lund University, Sweden.
To be defended at Segerfalksalen, BMC A10, Lund, on January 16th 2018 at 9h00.

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Organization LUND UNIVERSITY	Document name Doctoral Dissertation
Division of Molecular Medicine and Gene Therapy Institution of Laboratory Medicine, Lund	Date of issue December 2017
Author Sandra Capellera Garcia	Sponsoring organization

Title and subtitle: Programming Blood Cell Fates
Insights from Direct Lineage Conversion and Development

Abstract

Red blood cells (RBC) and platelets constitute the non-immune branch of the hematopoietic system and are responsible for the vital functions of transporting oxygen to the tissues and clotting blood vessel injuries, respectively. These cells are produced during embryonic development and throughout life through a process called hematopoiesis which is tightly regulated by extrinsic and intrinsic factors. Essential genes for RBC and/or platelet formation have been identified through targeted gene disruption strategies and studies of human diseases affecting these lineages. However, the minimal set of factors capable of initiating and specifying erythroid (RBC) and megakaryocytic (platelet) cell fate remained elusive. In this thesis, I have explored the potential of direct lineage conversion as a tool to define the master regulators of these lineages with the ultimate goal of recapitulating RBC and platelet development *in vitro*.

In the first paper, we employed a screen for transcription factors allowing direct induction of erythroid cell fate in mammalian fibroblasts. We identified a set of four factors (Gata1, Tal1, Lmo2 and c-Myc, or GTLM) that in eight days converted fibroblasts into induced erythroid progenitors (iEPs). iEPs exhibited properties of *bona fide* erythroid cells, such as morphology, gene expression, and colony-forming capacity; although their transcriptional signature resembled mainly that of primitive erythroid progenitors in the yolk sac.

In the second paper, we sought to identify missing factors and/or pathways necessary to induce adult-like erythropoiesis in fibroblasts. By comparing the transcriptome of iEPs with that of erythroid progenitors from all different layers of erythropoietic ontogeny, we identified several candidate transcription factors that are expressed in definitive erythroid progenitors, but absent in iEPs and primitive erythroid cells. These candidate genes will be tested in a future screening for modulation of developmental programming in iEPs.

In the third paper, we investigated the possibility of skewing the reprogramming process towards the megakaryocytic lineage, given that the four factors identified in the first study are implicated in the development and differentiation of the common megakaryocyte/erythroid progenitor. We found that the addition of Gata2 and Runx1 to the GTLM cocktail efficiently converted mammalian fibroblasts into megakaryocyte-like progenitors. The transdifferentiated cells expressed megakaryocytic markers, displayed polylobulated nuclei, formed megakaryocyte colonies in semisolid media, and gave rise to platelets *in vitro*. Moreover, transplantation of megakaryocyte-like progenitors into NSG mice resulted in engraftment and further maturation *in vivo*.

Overall, the results included in this thesis demonstrate that direct lineage reprogramming is a suitable tool to study erythroid and megakaryocytic cell fate regulation, and could provide a new platform to produce RBCs and platelets for personalized transfusion medicine.

Key words: hematopoietic development, erythropoiesis, megakaryopoiesis, direct lineage conversion

Classification system and/or index terms (if any)

Supplementary bibliographical information		Language: English
ISSN and key title: 1652-8220		ISBN: 978-91-7619-572-7
Recipient's notes	Number of pages	Price
	Security classification	

Signature  Date 2017-12-04

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Coverphoto by Sandra Capellera Garcia

Faculty of Medicine | Department of Laboratory Medicine

ISBN 978-91-7619-572-7
ISSN 1652-8220

Printed in Sweden by Media-Tryck, Lund University
Lund 2017

To my Family

"We have a hunger of the mind which asks for knowledge of all around us, and the more we gain, the more is our desire; the more we see, the more we are capable of seeing"

Maria Mitchell, 1878

Content

Abbreviations

AGM	Aorta-Gonad-Mesonephros
BasoE	Basophilic Erythroblast
BFU-E	Burst Forming Unit Erythroid
Blast-CFC	Blast Colony-Forming Cell
BM	Bone Marrow
BMP	Bone Morphogenetic Protein
CDA	Congenital Dyserythropoietic Anemia
CFC	Colony Forming Cell
CFU-E	Colony Forming Unit Erythroid
CLP	Common Lymphoid Progenitor
CMP	Common Myeloid Progenitor
DBA	Diamond Blackfan Anemia
E	Embryonic Day
EHT	Endothelial-to-Hematopoietic Transition
EMP	Erythro-Myeloid Progenitor
EPO	Erythropoietin
EpoR	Erythropoietin Receptor
EryP-CFC	Primitive Erythroid Colony-Forming Cell
ESC	Embryonic Stem Cell
FA	Fanconi Anemia
FACS	Fluorescence-Activated Cell Sorting
FGF	Fibroblast Growth Factor
FL	Fetal Liver
G-CSF	Granulocyte Colony Stimulating Factor
GC	Glucocorticoid
GCR	Glucocorticoid Receptor
GM-CSF	Granulocyte/Macrophage Colony Stimulating Factor

GMP	Granulocyte/Macrophage Progenitor
GRN	Gene Regulatory Network
HE	Hemogenic Endothelium
HIF	Hypoxia-Inducible Factor
HSC	Hematopoietic Stem Cell
HSPC	Hematopoietic Stem and Progenitor Cell
IGF	Insulin Growth Factor
IL	Interleukin
iPSC	induced Pluripotent Stem Cell
KuO	Kusabira Orange
LMPP	Lymphoid-primed Multipotent Progenitor
LPM	Lateral Plate Mesoderm
Mac-CFC	Macrophage Colony-Forming Cell
Meg-CFC	Megakaryocyte Colony-Forming Cell
MEP	Megakaryocyte/Erythroid Progenitor
MK	Megakaryocyte
MPP	Multipotent Progenitor
NK	Natural Killer
OrthoE	Orthochromatic Erythroblast
PolyE	Polychromatophilic Erythroblast
ProE	Proerythroblast
PS	Primitive Streak
RBC	Red Blood Cell
SCF	Stem Cell Factor
SCNT	Somatic Cell Nuclear Transfer
TGF	Transforming Growth Factor
TPO	Thrombopoietin
WT	Wild Type
YS	Yolk Sac

List of Publications

Papers included in this thesis

Paper I

Capellera-Garcia S, Pulecio J, Dhulipala K, Siva K, Rayon-Estrada V, Singbrant S, Sommarin MN, Walkley CR, Soneji S, Karlsson G, Raya Á, Sankaran VG, Flygare J. Defining the Minimal Factors Required for Erythropoiesis through Direct Lineage Conversion. 2016 Cell Reports; Vol. 15, issue 11, p2550-2562

Paper II

Capellera-Garcia S, Ilsley M, Dhapola P, Flygare J. RNA Sequencing Identifies Potential Missing Factors for Reprogramming of Fibroblasts to Definitive Erythropoiesis. Manuscript in preparation

Paper III

Pulecio J, Alejo-Valle O, **Capellera-Garcia S**, Vitaloni M, Rio P, Mejía-Ramírez E, Caserta I, Bueren JA, Flygare J, Raya A. Direct Conversion of Fibroblasts to Megakaryocyte Progenitors. 2016 Cell Reports; Vol. 17, issue 3, p671-683

Papers not included in this thesis

Capellera-Garcia S, Flygare J. Direct Lineage Reprogramming: a Useful Addition to the Blood Cell Research Toolbox. 2017 Expert Rev Hematol; Vol. 10, issue 2, p107-109

Preface

It has been almost five years since I began the exciting journey of deciphering the molecular instructions for making blood cells. My fascination with stem cells and regenerative medicine dates back to my high school days when I wrote a final paper about stem cells' potential to cure degenerative diseases. At the same time, the discovery that mature cells can be reprogrammed back into a pluripotent state with only four genes was published, which further strengthened my interest in the field. I was, therefore, thrilled to embark on a Ph.D. project that combined stem cells, developmental biology and reprogramming technologies. Today, more than 10 years after the iPSC breakthrough, the field of cellular reprogramming has seen tremendous progress and has provided biologists with new tools to study and treat disease. Moreover, with the rise of direct lineage conversion and the ever-growing list of minimal intrinsic determinants of cell fates, one can envision a not-so-futuristic scenario where any cell type could be produced *à la carte*. In this thesis, I will try to convince you that cellular reprogramming is a powerful tool not only to produce blood cells from skin cells, but also to teach us about how cellular identities are specified. I hope you enjoy reading it as much as I have enjoyed writing it!

Sandra
Lund, 29th November 2017

Background

Blood Cells in the Making

Overview of the hematopoietic system

Blood is an essential liquid tissue in vertebrates. Flowing through an intricate network of blood vessels, it supplies the body with vital nutrients and oxygen, provides defense against pathogens and carries away waste products. Blood is largely composed of fluid plasma, making up 55% of the blood volume. The remainder represents several cellular components with highly specialized functions. These components make up three major groups: red blood cells (RBCs) or erythrocytes, that collect oxygen from the lungs and deliver it to the body's tissues; platelets, which are essential for blood clotting and wound healing; and white blood cells. The latter form the immune system, which can be further divided into the innate and adaptive systems. Cells of the innate immune system include granulocytes, monocyte/macrophages, dendritic cells and Natural Killer (NK) cells, which mediate a rapid defense against pathogens involving phagocytosis and induction of inflammatory responses. In contrast, cells of the adaptive immune system provide a slower and more specific response by killing infected cells (T-lymphocytes) or by producing antibodies against exogenous antigens (B-lymphocytes).

Mature blood cells are predominantly short-lived, and thus need continuous replacement to be maintained in constant numbers. This is possible thanks to a continuous process termed hematopoiesis, from Greek *haîma* (αίμα) for "blood" and *poiēsis* (ποίησις) meaning "to make". Hematopoiesis relies on a small number of bone marrow (BM)-residing hematopoietic stem cells (HSCs) that self-renew – produce additional HSCs through cell division– and give rise to all blood cell lineages (reviewed in Orkin, 2000). Remarkably, healthy adult humans are estimated to produce up to one trillion (10^{12}) blood cells a day (reviewed in Ogawa, 1993). To cope with this enormous proliferative demand, hematopoiesis

occurs in a highly coordinated hierarchical manner where HSCs give rise to many intermediate progenitors with restricted differentiation but extensive proliferation capacity (Figure 1). Historically, evidence supporting this hierarchical model has been largely acquired through the use of *in vitro* colony forming cell (CFC) assays and *in vivo* transplantation. CFC assays identify and quantify lineage-restricted progenitors by their ability to form colonies of maturing cells, such as granulocyte/monocyte, erythroid or megakaryocyte colonies (Gregory et al., 1973; Iscove et al., 1970; Worton et al., 1969; Wu et al., 1968). On the other hand, transplantation provides information about the capacity of a cell to engraft, self-renew and differentiate into the full spectrum of blood cell lineages in an *in vivo* environment (Ford et al., 1956; Jacobson et al., 1951). Because transplantation allows longevity to be measured for as long as the recipient lives, 'true' stem cells can be identified (reviewed in Coulombel, 2004). Thus, HSCs are functionally defined by their ability to provide lifelong reconstitution of the entire blood system of a recipient. However, as new technologies emerge, the classical view on the dynamics with which HSCs contribute to hematopoiesis continues to be revised. Recent studies employing genetic fate mapping and clonal marking suggest that long-lived progenitor cells downstream from HSCs are the main drivers of steady-state hematopoiesis during most of adulthood, and that native hematopoiesis differs from post-transplantation hematopoiesis (Busch et al., 2015; Sawen et al., 2016; Sun et al., 2014).

The blood system has long served as a paradigm to study the biology of adult stem cells. HSCs are arguably the best characterized tissue-specific stem cell and the only stem cells in routine clinical use to date, with HSC-containing grafts being used to treat a wide range of hematological disorders (reviewed in Bryder et al., 2006; Copelan, 2006). The development of fluorescence-activated cell sorting (FACS) technology coupled to *in vitro* and *in vivo* functional assays has enabled the prospective isolation as well as delineation of the hierarchical relationship of HSCs and progenitor cells. This, in turn, has also promoted the study of intrinsic and extrinsic regulators of cell fate in hematopoiesis.

Intrinsic and extrinsic regulation

Central to the intrinsic regulation of cellular phenotypes are transcription factors and their interactions within gene regulatory networks (GRN) (reviewed in Orkin, 2000). Insights into the functions of the critical transcription factors for hematopoiesis have been established by gene-targeting knockout mouse models,

as well as forced expression experiments, complemented by studies in other model organisms, such as zebrafish or *Xenopus* (reviewed in Ciau-Uitz et al., 2010; Orkin and Zon, 2008) (Figure 1). With these methods, one can distinguish between factors required for HSC formation/function and factors controlling lineage-specific differentiation. Examples of "HSC transcription factors" are TAL1 (also known as SCL), Runx1, MLL, Tel/Etv6 and LMO2, whose genes account for the majority of leukemia-associated translocations in patients (reviewed in Orkin, 2000; Proytcheva, 2011). As for lineage-restricted factors, they hold the dual role of promoting their own lineage differentiation while antagonizing another. Examples of this principle are illustrated at several levels. In the common myeloid progenitor (CMP), for instance, PU.1 and GATA1 antagonism leads to the bifurcation into granulocyte/macrophage progenitors (GMP) and megakaryocyte/erythroid progenitors (MEP), respectively (Zhang et al., 1999; Zhang et al., 2000). Similarly, Friend of Gata1 (FOG1) drives CMPs towards the megakaryocyte/erythroid lineage while blocking myeloid differentiation by antagonizing C/EBPα (Mancini et al., 2012). Other examples of cross-regulation include EKLF and Fli-1 for erythroid versus megakaryocytic cell fate (Starck et al., 2003), and Gfi1 and PU.1 for granulocyte versus monocyte/macrophage differentiation (Dahl et al., 2007). Importantly, most of these factors do not act only on one level, but are deployed at multiple stages of blood development. This allows decisions to be inherited from one progenitor to the next, making it possible to re-use a transcription factor in a different network context (reviewed in Graf and Enver, 2009).

Cell fate choices are also dictated by extracellular signals, for example, by hematopoietic cytokines. Cytokines are secreted proteins that affect multiple aspects of every hematopoietic cell type, including survival, proliferation, maturation and functional activation (reviewed in Rieger and Schroeder, 2009). Key cytokines acting on both HSCs and progenitors are depicted in Figure 1. It has so far been difficult to unequivocally demonstrate whether cytokines instruct cell fate changes or merely allow the survival and proliferation of precursors that have already intrinsically committed to one lineage. This is because most, if not all, cytokines exhibit pleiotropy –multiple biological actions– and redundancy –shared biological actions– (reviewed in Rieger and Schroeder, 2009; Zhang and Lodish, 2008). Further studies will be required that analyze the capacity of individual cytokines to instruct lineage choice, and to integrate with other cytokine signals and with intracellular transcription factor networks in specific cell types,

preferably at the single-cell level (reviewed in Rieger and Schroeder, 2012). Cytokines involved in the development of specific blood lineages will be discussed in detail in the following chapters.

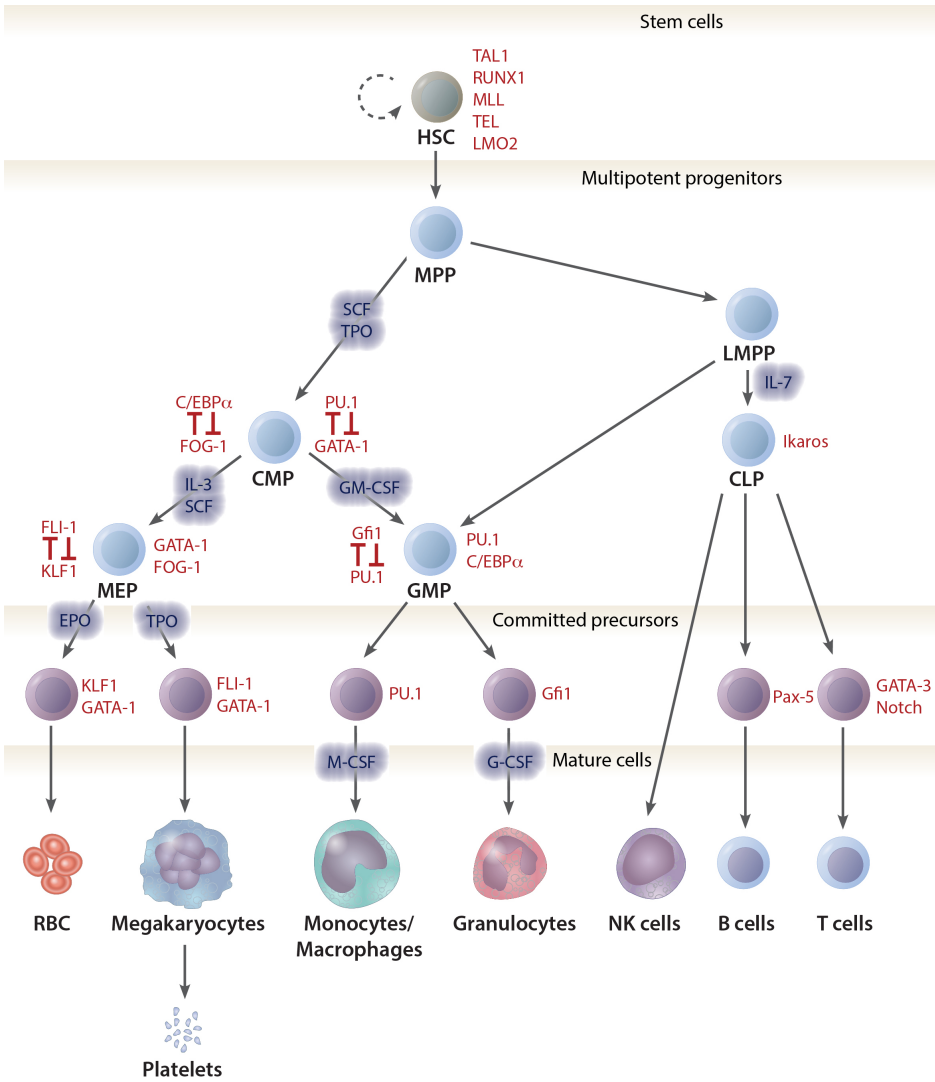

Figure 1. Schematic overview of the adult hematopoietic hierarchy
Hematopoiesis is a hierarchical process in which HSCs give rise to every effector blood cell type through a series of increasingly lineage-restricted progenitor and precursor cells (adapted from Doulatov et al., 2012). Examples of transcription factor antagonism during lineage determination, as well as factors necessary for the development of specific progenitors and precursors are depicted in red. Key cytokines acting at different stages are displayed in blue.

Ontogeny of hematopoiesis

To understand how the different tissues of the adult organism develop, it is important to define their embryonic origins. Tracing the origins of the hematopoietic system is complicated because blood is a mobile tissue and because hematopoiesis in the mammalian conceptus occurs in many sites that are separated both anatomically and temporally (reviewed in Dzierzak and Speck, 2008).

The ontogeny of blood formation has been investigated in multiple model systems from Drosophila to human, including the frog, chick and fish (Dieterlen-Lievre and Martin, 1981; Kau and Turpen, 1983). However, study of the mouse has proven integral to our understanding of hematopoietic development and emerged as an invaluable mammalian counterpart to human to study the embryo. This is mainly because of the ready availability of functional assays for HSCs and progenitor cells and the growing accessibility of mouse genetics to create transgenic models (reviewed in Schmitt et al., 2014). Most findings described in this section will therefore refer to studies made in the murine system.

From mesoderm to hematopoietic fate

During implantation of the blastocyst, the cells of the inner cell mass –which are termed pluripotent because of their ability to generate every cell in the organism– undergo a series of commitment steps, resulting in the formation of the epiblast and the hypoblast. The epiblast subsequently forms the amniotic cavity and the embryonic epiblast (embryo proper), while the hypoblast (also called primitive endoderm) gives rise to the yolk sac (YS). Around embryonic day (E) 6.5 (E6.5), the embryonic epiblast is transformed into the three primordial germ layers (ectoderm, mesoderm and endoderm) in a process termed gastrulation. Each of these three layers gives rise to a different set of tissues: the ectoderm forms the epidermis and the nervous system, the endoderm forms the digestive and respiratory systems, and the mesoderm gives rise to muscle, bone, heart, connective tissue, blood vessels and blood (Schoenwolf and Larsen, 2009). A key feature of gastrulation is the formation of the primitive streak (PS), a structure that changes the embryo from a bundle of cells into an entity with a defined longitudinal axis around which other features can orientate. Cells entering the PS adopt different fates depending on their position relative to the axis.

The first mesoderm cells emerge from the posterior part of the PS and give rise to the extraembryonic mesoderm that forms the allantois and amnion, as well as

the blood and vasculature of the YS (Kinder et al., 1999). As gastrulation proceeds, progenitors for the embryonic mesoderm migrate through more anterior parts of the PS and gradually generate different mesoderm sub-lineages: lateral plate mesoderm (LPM, precursor to the gut wall and intra-embryonic blood and vascular systems), intermediate mesoderm (precursor to the urogenital system), paraxial mesoderm (precursor to the somites) and axial mesoderm (precursor to the notochord) (reviewed in Tam and Loebel, 2007). This patterning is largely influenced by signaling cues from several members of the TGFβ/BMP, Nodal and Wnt families (reviewed in Arnold and Robertson, 2009). Thus, the timing and site of ingression through the PS, as well as exposure to different signaling cues, directly influence mesoderm sub-lineage specification. The hematopoietic and vascular systems are therefore derived from at least two different mesoderm sub-lineages during development: the extra-embryonic mesoderm and the LPM.

As the embryo grows in size and complexity, the demands for oxygen and nutrient supply, as well as immunity and waste disposal increase. These needs are met by the production of successive hematopoietic systems, or so-called "hematopoietic waves", before culminating in the formation of HSCs that will sustain life-long hematopoiesis in the adult. Hematopoietic waves can be broadly classified in "primitive" and "definitive" waves and will be the focus of discussion until the end of this chapter (Figure 2).

Primitive yolk sac hematopoiesis

Primitive hematopoiesis refers to the earliest wave of transient blood formation that occurs in the extra embryonic YS at E7.25 and produces a limited range of hematopoietic sub-lineages: erythroid, megakaryocyte and macrophage progenitors (reviewed in Palis, 2016). Primitive erythroid cells arise from a distinct progenitor called primitive erythroid colony-forming cell (EryP-CFC) that transiently expands in numbers within the YS and gives rise to large nucleated erythroblasts expressing embryonic globins. These macrocytic erythroblasts begin to circulate into the embryo proper with the onset of cardiac contractions at E8.25, undergo terminal maturation and ultimately enucleate between E12.5 and E16.5 (Fraser et al., 2007; Ji et al., 2003; Kingsley et al., 2004). Enucleated primitive erythrocytes are found in the circulation of mouse pups for several days after birth. Similarly, primitive megakaryocytic potential arises from a megakaryocyte colony forming cells (Meg-CFC), which are also first detected in the YS of the mouse conceptus at E7.25. The emergence of Meg-CFC at the same time and location as

EryP-CFC has led to postulate the existence of a common progenitor for the two lineages, as it is the case in the adult with the bipotential MEP. Indeed, experimental evidence for 'primitive' MEPs has been provided using a CFC assay with lineage-specific immunohistochemical stains to identify the megakaryocyte and primitive erythroid cells present within the same colony (Tober et al., 2007). The product of this primitive wave also comprises macrophage potential, with macrophage colony-forming cells (Mac-CFC) emerging in the YS at E7.25. These 'primitive' macrophages are thought to be the source of microglia cells in the brain (reviewed in McGrath et al., 2015b).

The precise cellular origin of these first hematopoietic cells still remains controversial. They were initially thought to arise from a common precursor, the hemangioblast, giving rise to both the endothelial and blood cells that constitute the YS blood islands (Haar and Ackerman, 1971). Evidence for the existence of hemangioblasts was provided in embryonic stem cell (ESC) differentiation cultures with the identification of blast colony-forming cells (Blast-CFC) that generated colonies with hematopoietic, endothelial and smooth muscle potential (Choi et al., 1998). Similar *in vivo* potential was also detected in the primitive streak region of E7.5 mouse embryos (Huber et al., 2004). However, studies involving fate-mapping and clonal labelling of mouse embryos have concluded that the earliest blood and endothelial lineages within the YS are not clonally related, suggesting that if hemangioblasts indeed exist, they are an extremely infrequent and/or transient population (Padron-Barthe et al., 2014; Ueno and Weissman, 2006).

Definitive erythro-myeloid progenitor-derived hematopoiesis

Studies in the mouse have shown that primitive hematopoiesis is not sufficient to support embryonic survival until HSCs are functional. At E8.25, just prior to the onset of circulation, a second wave of YS-derived hematopoiesis takes place, producing erythro-myeloid progenitors (EMP) with a broader differentiation capacity than primitive hematopoietic progenitors. EMPs are then released into the circulation and seed the fetal liver (FL), where they differentiate into definitive erythroid, megakaryocyte, macrophage, neutrophil and mast cell lineages by E11.5 (reviewed in Frame et al., 2013; McGrath et al., 2015a). This second wave is generally considered "definitive" because it generates erythroid cells with adult characteristics (smaller in size and expressing mostly adult globins), although this designation is still controversial because the term "definitive" has been used by

several authors to refer exclusively to adult-repopulating HSC activity (reviewed in Frame et al., 2013). In addition, EMPs are the source of tissue-resident macrophage populations that persist in multiple organs throughout adulthood (Gomez Perdiguero et al., 2015). Altogether, EMP-derived and primitive hematopoiesis are sufficient to support the survival until birth of mouse embryos lacking HSCs (Chen et al., 2011).

Recently, a unique cell surface phenotype for EMPs has been identified (ckit$^+$ CD41$^+$ CD16/32$^+$) that facilitated their isolation and analysis. While CD41 and CD16/32 also mark maturing megakaryocytes and macrophages, respectively, ckit has been found to unambiguously label emerging EMPs in the YS (Frame et al., 2016). EMPs also express endothelial markers, suggesting an endothelial ancestry (McGrath et al., 2015a). In line with this, endothelial cell clusters containing ckit$^+$ Runx1$^+$ cells are found within the vascular plexus of the YS as EMP first emerge (Frame et al., 2016; Li et al., 2005). Moreover, in Runx1-null mouse YS there is a complete lack of ckit$^+$ CD41$^+$ CD16/32$^+$ EMPs, while maturing primitive erythroid, megakaryocyte and macrophage populations remain intact (Frame et al., 2016). These studies support the notion that EMP emerge from endothelial cells in a Runx1-dependent manner, a process called endothelial-to-hematopoietic transition (EHT).

Multipotent progenitors with lympho-myeloid potential

Several studies have recently demonstrated that lymphoid and lymphomyeloid multipotent progenitors, including B-1 cell progenitors, also arise before HSCs, challenging the long-lasting belief that lymphoid potential is an exclusive feature of HSC-derived hematopoiesis (Boiers et al., 2013; Inlay et al., 2014; Kobayashi et al., 2014; Yoshimoto et al., 2011; Yoshimoto et al., 2012). These progenitors, devoid of long-term repopulation ability, have been detected in the mouse YS as early as E9.5, as well as in the para-aortic splanchnopleura (the precursor of the aorta-gonad-mesonephros (AGM) region). It is still unclear if they all emerge from a common lymphomyeloid progenitor wave or independently from each other, and what their relationship with EMPs is.

Definitive hematopoietic stem cell-derived hematopoiesis

The presence of transplantable HSCs in cord blood indicated that HSCs emerge during embryogenesis. It was previously thought that HSCs arise in the YS (Moore and Metcalf, 1970), however, later studies involving transplantation of

cells from various regions of the E8-E12 mouse embryo unequivocally demonstrated that HSCs capable of engrafting and repopulating myeloablated adult recipients first arise at E10.5 in the AGM region of the dorsal aorta (de Bruijn et al., 2000; Medvinsky and Dzierzak, 1996; Muller et al., 1994). Other sites are known to generate HSCs *de novo* shortly after, including the placental labyrinth, vitelline and umbilical arteries and YS (de Bruijn et al., 2000; Gekas et al., 2005; Gordon-Keylock et al., 2013; Kumaravelu et al., 2002; Robin et al., 2009). After emergence, HSCs migrate through the circulation and colonize the FL, which provides the environment for their successful expansion and maturation (Ema and Nakauchi, 2000). They subsequently colonize the BM, where they ultimately reside, mostly quiescent, during the lifetime of the organism. HSC's long-term repopulating ability has been associated temporally and spatially with clusters of round hematopoietic cells budding from endothelium via Runx1-dependent EHT (Chen et al., 2009b; de Bruijn et al., 2002).

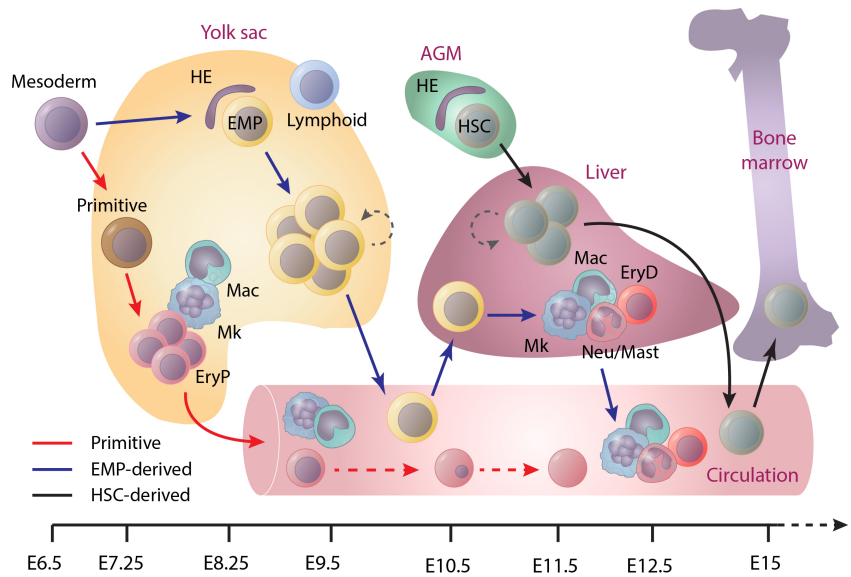

Figure 2. Current model of hematopoietic ontogeny in the murine embryo over time
During development, several waves of hematopoietic progenitors with distinct multilineage differentiation capacity emerge to support survival and growth of the embryo. The first, primitive wave (red arrows), emerges in the yolk sac at E7.25 and consists of primitive erythroid (EryP), megakaryocyte (Mk) and macrophage (Mac) lineages. EryP mature semi-synchronously in the bloodstream between E9.5 and E12.5. The second wave (blue arrows) consists of erythro-myeloid progenitors (EMP) which emerge in the yolk sac beginning at E8.25, seed the newly formed fetal liver and give rise to definitive erythroid (EryD), Mk, Mac, neutrophil and mast cell (Neu/Mast) lineages. Lymphomyeloid potential is also detected in the yolk sac and embryo proper before HSCs emerge. The final wave (black arrows) involves HSCs, which first arise at E10.5 in large arterial vessels of the embryo and subsequently seed the fetal liver, where they undergo expansion and maturation until around birth. HSCs eventually colonize the bone marrow, where they reside after birth and throughout post-natal life. The timeline at the bottom illustrates the developmental time points (in days *post-coitum*) (adapted from Palis, 2016).

From Stem Cells to Red Blood Cells

Red blood cells (RBC), otherwise known as erythrocytes, constitute the most common cell type in the blood. They are pivotal to the survival of all vertebrate organisms, fulfilling the essential functions of transporting oxygen and facilitating gas exchange in the lungs and peripheral tissues. Oxygen is bound and transported by hemoglobin molecules, which are composed of four globular protein subunits and a heme group, and take up 98% of the cytoplasmic protein content in RBCs (D'Alessandro et al., 2010). RBCs have a limited lifespan, approximately 40 days in adult mice and 120 days in adult humans, so they constantly need to be generated to maintain the red cell mass. Healthy human adults produce approximately two million RBCs every second in a process called erythropoiesis. Erythropoiesis is required in all stages of life and occurs at multiple anatomical sites during ontogeny, leading to the formation of two distinct types of RBCs: primitive (embryonic) and definitive (adult) (Dzierzak and Philipsen, 2013). The presence of two developmentally and morphologically distinct populations of erythroid cells was first reported more than a century ago and has been the subject of intense research over the last decades (Maximow, 1909; reviewed in Palis, 2014). This chapter will summarize advances in our understanding of how the erythroid lineage develops and is regulated.

The definitive erythroid compartment

The classical model of HSC-derived, definitive erythropoiesis begins with multipotent HSCs and progresses through a series of lineage-committed erythroid progenitors and precursors, which terminally differentiate to enucleated erythrocytes. This process takes place in the FL during development and postnatally in the BM. Importantly, as explained in the previous chapter, definitive erythropoiesis also occurs in the YS beginning at E8.25, as part of EMP-derived hematopoiesis (reviewed in Palis, 2016).

As depicted in Figure 3, the earliest committed definitive progenitor, the slowly proliferating burst forming unit erythroid (BFU-E), gives rise to the rapidly dividing colony forming unit erythroid (CFU-E). These progenitors are defined by their capacity to form colonies of mature erythroid cells in semisolid media: BFU-E-derived colonies require 7 and 14 days to develop in mouse and human systems, respectively, and typically contain a thousand erythroid cells; while CFU-E-

derived colonies require only 2 and 7 days in mouse and human systems, respectively, and consist of 16-32 cells. In turn, CFU-E differentiates into morphologically distinct, nucleated precursors that undergo a stepwise maturation from proerythroblast (ProE) to basophilic (BasoE), polychromatophilic (PolyE), and orthochromatic (OrthoE) erythroblast forms. This maturation is characterized by progressive (1) decrease in cell size, (2) nuclear condensation, (3) decrease in RNA content, (4) erythroblast expansion through a limited set of symmetric cell divisions and (5) massive accumulation of hemoglobin (reviewed in Palis, 2014). Lastly, OrthoEs exit the cell cycle and form reticulocytes by extruding their nuclei. Enucleation takes place in the FL and BM within erythroblastic islands, structures composed of erythroblasts physically attached to macrophages (reviewed in Chasis and Mohandas, 2008). Soon after OrthoEs enucleate, pyrenocytes (extruded nuclei) provide an "eat me" signal so they can be ingested by macrophages. The reticulocyte is then released into the blood stream where it matures into an erythrocyte. Reticulocyte maturation results in a 20% loss of plasma membrane surface area, decreased cell volume, a tighter association of the cytoskeleton to the plasma membrane and the loss of all residual cytoplasmic organelles, including mitochondria and ribosomes (Waugh et al., 2001). All these changes convert the reticulocyte into a biconcave disc with a diameter of 6-8 μm and increased viscoelasticity, i.e. the mature RBC. These features enable RBCs to flow through the smallest capillaries in the tissues and maximize the surface area for gas exchange.

Cell membrane proteins have been identified that are selectively expressed or repressed during erythroid maturation and can be tracked by FACS to isolate and analyze the different erythroid compartments. For example, the transferrin receptor (CD71) is upregulated in the transition from murine BFU-E to CFU-E. Likewise, ckit is expressed from the HSC-level until the early ProE stage. When CFU-Es differentiate into erythroblasts, erythroid specific protein Ter119 is upregulated while expression of CD71 is progressively lost (Figure 3) (Kina et al., 2000; Koulnis et al., 2011; Liu et al., 2013a). One study showed that CD44 is a more effective surface marker to distinguish between erythroblasts at different stages of maturation than CD71 (Chen et al., 2009a). As for globin genes, definitive erythroid cells express "adult" β1-, β2- and α-globin genes in the mouse, and "fetal" γ-, "adult" β-, and α-globin genes in the human.

The primitive erythroid compartment

Primitive erythropoiesis emerges in the YS of the mouse conceptus from a transient wave of progenitors, the EryP-CFC, that first emerge at E7.25, peaks in numbers at E8.25 and are no longer detectable at E9.0 (Palis et al., 1999). EryP-CFC form colonies *in vitro* that require 5 days to develop and contain several hundred mature primitive erythroid cells, a potential that is intermediate to that of the definitive BFU-E and CFU-E progenitors. Primitive erythroid cell maturation is also characterized by the progression of cells through progenitor, precursor and mature RBC compartments. However, unlike definitive erythropoiesis, primitive precursor maturation happens semi-synchronously in the bloodstream between E9.5 and E12.5. By E12.5, the primitive erythroblasts have reached the orthochromatic stage and cell division terminates.

Primitive erythroid cells express a unique set of globin genes that has historically facilitated their identification and study. In mouse, the embryonic εγ-, βH1-, ζ- and α-globin genes are expressed, while the ε-, γ-, ζ- and α-globin genes are expressed in human. Embryonic globins have also been used as a marker to unequivocally identify primitive erythroid cells in the mouse conceptus and demonstrate that these enucleate in the bloodstream between E12.5 and E16.5 (Fraser et al., 2007; Kingsley et al., 2004; Palis, 2014).

Not so much is known about primitive erythroblasts maturation and the components of their membrane cytoskeleton during this process. One study showed that primitive erythroid cells lose 35% of their surface area and 50% of their volume between E14.5 and E17.5, independently of whether or not the cells enucleate. This suggested that, unlike definitive erythropoiesis, membrane remodeling and enucleation may be uncoupled processes in terminally mature primitive erythroid cells (Waugh et al., 2013).

Extrinsic regulation of erythropoiesis

Erythropoiesis is a dynamic process that responds to oxygen tension in the body and is tightly regulated through both intrinsic and extrinsic factors. Extrinsic factors, such as cell adhesion molecules, cytokines and growth factors, are mostly provided by the microenvironment (or niche) where erythroid cells develop.

As for definitive erythropoiesis, BFU-Es are responsive to cytokines including insulin like growth factor 1 (IGF-1), glucocorticoids (GC), the stress hormone

cortisol, interleukin 3 (IL-3), interleukin 6 (IL-6) and stem cell factor (SCF). The latter binds to its receptor ckit, whose expression is high in both BFU-Es and CFU-Es and declines at the ProE stage. SCF is required for proliferation of erythroid progenitors and disruption of its signaling cascade impairs erythroid recovery following stress (Broudy et al., 1996). Also, SCF and hypoxia have been shown to synergize with BMP4 to promote the expansion and differentiation of BFU-E during the recovery from acute anemia (Perry et al., 2007). At subsequent stages, the major extrinsic regulator is the clinically relevant erythropoietin (Epo). Epo is produced in the FL and adult kidney in response to hypoxia and it interacts with cells expressing its receptor, EpoR. CFU-Es are exquisitely dependent on EPO for their survival and differentiation, but not for their generation (Koury and Bondurant, 1990; Wu et al., 1995). Epo signaling is no longer required at the late phases of erythroid maturation. Consistent with this, EpoR expression peaks at the CFU-E and ProE stages, but is downregulated as erythroid progenitors undergo terminal differentiation (Broudy et al., 1991; Zhang et al., 2003). Epo binding triggers the homodimerization of EpoR and initiates a signaling cascade through Jak2/Stat5, PI3K/AKT, and MAPK pathways (reviewed in Richmond et al., 2005). The Jak2/Stat5 pathway appears to mediate the anti-apoptotic effect of Epo via induction of Bcl-xL (Socolovsky et al., 1999). Moreover, exogenous expression of Bcl-xL in primary murine erythroblasts allows these cells to undergo terminal maturation without the presence of cytokines (Dolznig et al., 2002). GCs, in turn, are lipophilic hormones that act through binding and regulating the transcriptional activity of glucocorticoid receptors (GCR). GCs have been shown to cooperate with Epo and SCF to induce proliferation of erythroid progenitors *in vitro*, and mice deficient for GCRs exhibit normal erythropoiesis but fail to increase erythrocyte production upon stress (Bauer et al., 1999; von Lindern et al., 1999). More recently, Flygare et al. demonstrated that GCs induce limited self-renewal of BFU-Es, but not of CFU-Es or erythroblasts. Additionally, they showed that many of the genes induced by GCs in BFU-Es contain binding sites for hypoxia-induced factor 1α (HIF1α), denoting a synergistic effect between these factors on promoting BFU-E self-renewal (Flygare et al., 2011). TGF-β signaling has also been implicated in regulating early steps of erythropoiesis. Specifically, blocking TGF-β signaling by receptor kinase inhibitor increases BFU-E self-renewal and total erythroblast production (Gao et al., 2016).

As for primitive erythropoiesis, Epo also seems to be playing a critical role. EpoR is expressed in YS blood islands between E7.5-E8.5 and in the YS of E9.5-E11.5

mouse embryos (Makita et al., 2001; McGann et al., 1997). Addition of Epo in E7.5 mouse YS explants was shown to increase erythroid cell numbers and embryonic globin expression (Palis et al., 1995). Moreover, disruption of EpoR caused severe retardation in the proliferation of mouse primitive erythroblasts after E9.5 (Lin et al., 1996). Recently, Suzuki *et al* demonstrated that neuroepithelial cells of E8.5-E11.5 mouse embryos express Epo and are likely the source of this hormone sustaining primitive erythropoiesis (Suzuki et al., 2013). Furthermore, primitive erythroid progenitors, unlike their definitive counterparts, do not self-renew when cultured *ex vivo* in the presence of EPO, SCF and dexamethasone (a synthetic GC hormone), a difference connected to the differential expression of the receptors for the latter two factors (England et al., 2011; Palis, 2014).

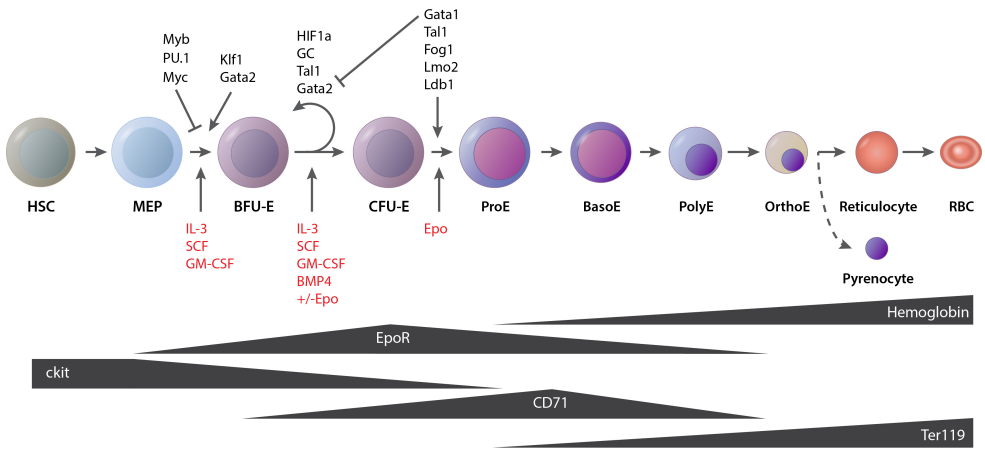

Figure 3. Schematic overview of adult erythropoiesis
Formation of RBCs from HSCs progresses through a series of lineage-committed erythroid progenitors and precursors, which terminally differentiate to enucleated erythrocytes. The earliest committed progenitor, the slowly proliferating burst forming unit erythroid (BFU-E), gives rise to the rapidly dividing colony forming unit erythroid (CFU-E). In turn, CFU-E differentiates into morphologically distinct precursors that undergo a stepwise maturation characterized by a progressive decrease in cell size, reduced proliferative capacity, nuclear condensation and massive accumulation of hemoglobin. Cytokines and transcription factors that influence this process are depicted in red and black, respectively. Bottom panels represent expression levels of key erythroid markers (adapted from Hattangadi et al., 2011).

Intrinsic regulation of erythropoiesis

Downstream of these cytokines, intracellular signal transduction proteins interact with a relatively small number of transcription factors, including GATA-1, SCL/TAL1, LMO2, LDB1 and KLF-1, to produce mRNAs essential for erythropoiesis (reviewed in Cantor and Orkin, 2002; Hattangadi et al., 2011).

These transcription factors are present in diverse multiprotein complexes (Figure 4), and their functions have been established by gene-targeting knockout mouse models, cell-based *ex vivo* assays and studies of diseases of ineffective erythropoiesis. Although intrinsic regulation of erythropoiesis occurs at several levels –micro RNAs, chromatin modifiers, etc.–, this section will only focus on describing key transcriptional regulators.

GATA-1

GATA-1 is a member of the GATA family of X-linked zinc-finger transcription factors and arguably the most studied erythroid transcription factor. It is expressed in erythroid, megakaryocytic, eosinophilic, mast and multipotential hematopoietic precursors (Evans and Felsenfeld, 1989; Tsai et al., 1989). Gata1 plays a central role in the regulation of both primitive and definitive erythroid cells, i.e. disruption of Gata1 leads to the maturational arrest of both primitive and definitive erythroid lineages at the ProE stage (Fujiwara et al., 1996; Pevny et al., 1995). It was first identified by its ability to bind DNA regulatory sequences found in globin genes (Evans and Felsenfeld, 1989; Tsai et al., 1989). Since then, GATA-binding motifs (A/T)GATA(A/G) have been identified in numerous promoters and enhancers of virtually all erythroid and megakaryocytic-specific genes (Weiss and Orkin, 1995b). However, it has recently been discovered that GATA-1 does not always occupy these sites during erythropoiesis *in vivo*. Genome-wide analysis of GATA factor chromatin occupancy have shown that the majority of GATA-1 binding occurs at distal regulatory elements such as enhancers, with very few (10-15%) in the proximal promoter regions (Fujiwara et al., 2009; Yu et al., 2009). Lastly, different functional domains of GATA-1 are required to activate gene expression in primitive vs. definitive erythroid cells, suggesting that different GATA-1-containing transcriptional complexes may function different in these lineages (Shimizu et al., 2001).

Alterations in GATA-1 cause a diverse range of RBC and platelet disorders whose precise characteristics relate to specific structure-function properties of the protein (reviewed in Crispino and Weiss, 2014). Mutations in GATA-1 N- and C-terminal zinc fingers disrupt DNA binding or associations with essential cofactors, which cause a variety of inherited anemias and/or thrombocytopenias, such as congenital dyserythropoietic anemia (CDA) or Gray platelet syndrome (Del Vecchio et al., 2005; Kratz et al., 2008; Phillips et al., 2007; Tubman et al., 2007). Also, distinct mutations within exon 2 lead to predominant expression of a

shortened isoform of GATA-1, named GATA-1s, which retains both N-terminal and C-terminal zinc fingers, but is missing the N-terminal activation domain. Germ-line GATA-1s mutations are associated with congenital hypoplastic anemia, including Diamond Blackfan anemia (DBA) (Parrella et al., 2014; Sankaran et al., 2012).

FOG-1

Friend of GATA (FOG-1) is a very close interacting partner of GATA-1 and a zing finger-containing protein that does not directly bind DNA (Fox et al., 1999). FOG-1 is highly expressed in erythroid and megakaryocytic cells and its interaction with GATA-1 is indispensable for erythroid differentiation (Tsang et al., 1997). ProEs expressing GATA-1 with a point mutation that disrupts the interaction with FOG-1 (but not DNA binding) fail to undergo erythroid maturation (Crispino et al., 1999).

SCL/TAL1-LMO2-LDB1-E2A complex

The basic helix-loop-helix (bHLH) transcription factor SCL/TAL1 binds to a short consensus DNA motif (CANNTG) called the E-box. SCL/TAL1 expression largely mirrors that of GATA-1, as it is expressed in erythroid cells, megakaryocytes and mast cells (reviewed in Cantor and Orkin, 2002). The SCL/TAL1 gene knockout results in the absence of hematopoiesis in the YS, while the conditional knockout of the same gene in adult hematopoiesis leads to a failure in erythropoiesis (Mikkola et al., 2003; Robb et al., 1995). In erythroid cells, SCL/TAL1 forms a complex with the ubiquitous E47/E2A and with the LIM domain containing cofactors LMO2 and LDB1. This complex interacts with GATA-1 to form a pentameric complex that binds to composite E-box/GATA-1 DNA motifs spaced 9-11 nucleotides apart (Wadman et al., 1997). These motifs are found in many erythroid genes and in the regulatory elements of key transcription factor genes including Gata1, Scl/Tal1 and Klf1 (Anderson et al., 1998; Cohen-Kaminsky et al., 1998; Wadman et al., 1997) (Figure 4A). LMO2, GATA-1, SCL/TAL1 and LDB1 are all required for erythropoiesis in mice (Li et al., 2010; Robb et al., 1995; Shivdasani et al., 1995; Warren et al., 1994). SCL/TAL1 complex can also mediate gene repression by recruitment of the corepressors ETO2 and Mtgr1 (Fujiwara et al., 2009; Soler et al., 2010; Tripic et al., 2009) (Figure 4B).

KLF1

KLF1 (also called EKLF) is a zinc finger transcription factor, whose expression is largely restricted to the erythroid cell lineage (Miller and Bieker, 1993; Southwood et al., 1996). It regulates the expression of several erythroid-specific genes, including the adult and embryonic globins, heme biosynthetic enzymes, several transcription factors, cytoskeletal proteins and blood group antigens (Basu et al., 2007; Hodge et al., 2006; Nilson et al., 2006). Mice heterozygous for the KLF1 gene appear completely healthy, however, in the complete loss of KLF1, the fetuses develop severe anemia and die around E14 (Nuez et al., 1995). Consistent with role of Klf1 in cytoskeletal gene regulation, Klf1-null primitive erythroblasts display markedly abnormal cell membranes and ruffled cell surfaces (Isern et al., 2010). Since KLF1 has a broad impact on erythroid-specific gene expression, the array of human erythroid phenotypes associated to KLF1 mutations is large. To name a few, the first loss-of-function mutations in human *KLF1* gene were discovered in individuals with the Lutheran (a-b-) blood group phenotype, and always occurred in the presence of a normal KLF1 allele (Singleton et al., 2008). These individuals did not exhibit anemia or RBC abnormalities, indicating that one allele of KLF1 appears to suffice for normal erythropoiesis, just like in mice. On the other hand, two unrelated patients with CDA were found to carry missense mutations of critical residues in the DNA binding domain of KLF1 and displayed very high levels of fetal hemoglobin (HbF, $\alpha 2\gamma 2$) and high levels of circulating nucleated RBCs (Arnaud et al., 2010).

Developmental regulators

While primitive and definitive erythropoiesis share key transcription factors essential for erythropoiesis, there are several that are only expressed in one lineage. The transcription factor c-MYB is expressed highest in CFU-Es and early erythroblasts and acts as an inhibitor of terminal erythroid differentiation (Emilia et al., 1986; Vegiopoulos et al., 2006). c-Myb null mouse embryos do not contain definitive erythrocytes and die of anemia around E15, but primitive erythropoiesis remains intact (Mucenski et al., 1991; Tober et al., 2008). In humans with Trisomy 13, dysregulation of the c-Myb gene and upstream microRNAs is associated with the persistence of embryonic and fetal hemoglobin expression (Sankaran et al., 2011). SOX6 and BCL11A are also differentially expressed transcriptional regulators, being exclusively expressed in definitive erythropoiesis. BCL11A, initially shown to be crucial for B cell development, has been associated with the

"fetal hemoglobin switch", that is, the replacement of γ-globin by adult β-globin after birth (Uda et al., 2008). Sankaran et al. demonstrated that BCL11A acts as a stage-specific repressor, silencing γ-globin gene expression by occupying several sites within the β-globin gene cluster (Sankaran et al., 2008). Also, BCL11A has been identified as a direct target of KLF1 in human and mouse erythroid cells (Borg et al., 2010; Zhou et al., 2010). Haploinsufficiency for KLF1, and thus the failure to activate BCL11A, was found to be the cause of hereditary persistence of fetal hemoglobin in a in a Maltese family (Borg et al., 2010). In addition, BCL11A and SOX6 have been shown to interact physically and co-occupy the human β-globin cluster along with GATA-1, cooperating in the silencing γ-globin transcription in adult human erythroid progenitors (Xu et al., 2010) (Figure 4C). Taking into account that downregulation of Bcl11a would reactivate fetal globin expression, this gene has emerged as a valuable molecular target to treat patients suffering from β-hemoglobin disorders, such as sickle cell disease and β-thalassemia (reviewed in Sankaran, 2011).

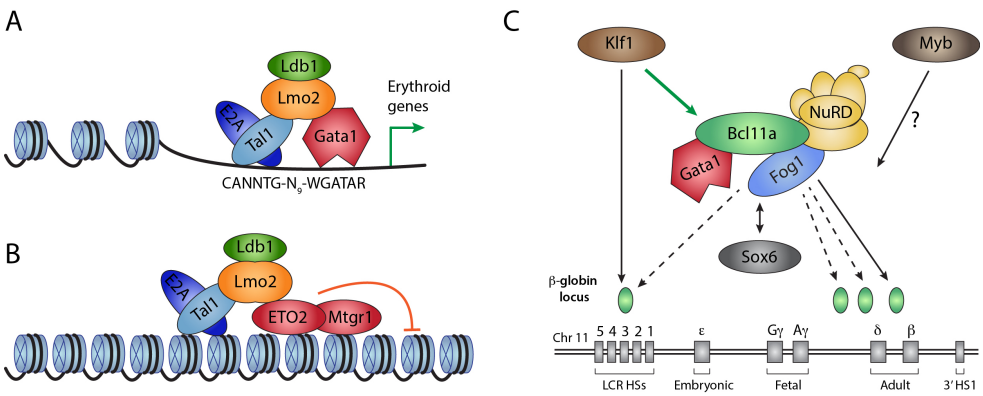

Figure 4. Selected erythroid transcription factor complexes
(A) Gata1/Tal1/Lmo2/E2A/Ldb1 pentameric complex has been shown to facilitate transcriptional activation of crucial erythroid genes, such as glycophorin A, the α-globin locus and Klf1. (B) The Tal1 complex can also recruit the corepressors ETO2 and Mtgr1 and mediate gene silencing. (C) Model of BCL11A-mediated silencing of γ-globin genes. Depicted at the bottom is the human β-globin gene locus, located on chromosome 11. BCL11A and its interaction partners GATA1, FOG1 and NuRD complex bind to sequences within the globin locus and repress the expression of the γ–globin genes. KLF1 reinforces this process by activating transcription of Bcl11a (green arrow) and also by directly binding to and promoting transcription of adult β-globin gene. Mechanistic studies have shown that BCL11A-mediated silencing of γ-globin involves long-range interactions and cooperation with SOX6. Bcl11a expression varies between humans and mice, but its role in globin gene switching is conserved. Mice lacking BCL11A have normal erythropoiesis, but fail to downregulate the embryonic globin genes in definitive erythroid cells. MYB has also been implicated in the regulation of fetal hemoglobin expression, although the precise mechanisms are not fully understood. HSs indicates DNase I–hypersensitive sites; LCR, locus control region (adapted from Sankaran, 2011).

From Stem Cells to Platelets

Platelets are small blood components that play an essential role in repairing vascular damage and initiating thrombus formation following blood vessel injury. They circulate in the blood stream in a quiescent form, and upon stimulation, activate to release their granule contents and spread on the affected tissue to create a physical barrier that prevents blood loss (reviewed in Machlus et al., 2014). Platelets have a short lifespan of only 7-8 days in humans and 3-5 days in mice, and possess no cell nucleus in mammals –they are fragments of cytoplasm (2-3 μm diameter) derived from large progenitor cells, megakaryocytes (~50-100 μm diameter), in a process called megakaryopoiesis. In healthy human adults, each megakaryocyte can generate up to 3 x 10^3 platelets, resulting in a total production (and removal) of 1 x 10^{11} platelets every day (Thon et al., 2010). Akin to erythroid cells, platelets and megakaryocytes are detected before HSCs emerge, implying several waves of megakaryopoiesis throughout development. This chapter will review the current knowledge on the process of platelet formation, with emphasis on the intrinsic and extrinsic regulators.

The megakaryocytic compartment

The classical model for HSC-derived megakaryopoiesis involves the commitment of HSCs to CMPs, which in turn generate MEPs that ultimately differentiate into megakaryocytes (Figure 5). However, some studies have hinted that alternative routes towards the megakaryocytic lineage exist. It has been shown that progenitors that have surface markers similar to HSCs but have become $Flt3^+$, termed lymphoid-primed multipotent progenitors (LMPPs), do not have the capacity (or very limited) to produce megakaryocytes or erythrocytes *in vivo* or *in vitro*, but retain lymphoid and granulocyte-monocyte potential; questioning the classical CMP-CLP model as an obligatory route for lineage commitment (Figure 1) (Adolfsson et al., 2005; Mansson et al., 2007). In addition, another study revealed that the HSC compartment itself contains stem-like megakaryocyte-committed progenitors that are transcriptionally primed towards the megakaryocytic lineage and can rapidly replenish the platelet pool in case of acute inflammation (Haas et al., 2015). All of these findings suggest that megakaryocytic lineage commitment may be a more plastic and context-dependent process than previously thought.

The first cells fully committed to the megakaryocytic lineage, termed Meg-CFC, form a small cluster of megakaryocytes *in vitro*. Meg-CFC give rise to 2N megakaryocytes, which, in turn, go through an endomitotic cell cycle –they replicate DNA but do not undergo cytokinesis– and an expansion of the cytoplasmic mass, resulting in the formation of a pool of mature megakaryocytes with a DNA content up to 128N per cell (reviewed in Machlus et al., 2014; Pang et al., 2005). These large megakaryocytes then undergo a maturation process involving the generation of a demarcation membrane system (which will form the plasma membrane of future platelets), the expression of GP1bα/GP1bβ/GPIX/GPV surface markers, the generation of distinctive platelet organelles such as the α- and dense granules, and the synthesis of organelle granular proteins such as platelet factor 4 and Von Willebrand factor (reviewed in Deutsch and Tomer, 2006). Subsequently, fully mature megakaryocytes extend long, branching pseudopods called proplatelets, which are composed of platelet-sized swellings in tandem arrays that are connected by thin cytoplasmic bridges (Italiano et al., 1999). The entire megakaryocyte cytoplasm is converted into a mass of proplatelets, and the nucleus of the megakaryocyte is eventually extruded. Individual platelets are then released from proplatelet ends and into the bloodstream (reviewed in Patel et al., 2005).

As discussed in the first chapter, megakaryocyte potential is also present before the onset of HSC-derived hematopoiesis. It is first detected in the YS of mouse embryos at E7.25, with the emergence of Meg-CFC capable of generating small colonies of megakaryocytes *in vitro* (Tober et al., 2007; Xu et al., 2001). A second wave of megakaryocyte progenitors is then found in the YS between E8.5 and E10.5, at which point they are also present in the blood stream and in the FL (Tober et al., 2007). Since the emergence of these two waves coincide in time and space with the emergence of primitive and EMP-derived hematopoiesis, the existence of 'primitive' and 'definitive' megakaryopoiesis has also been postulated (Tober et al., 2007; Tober et al., 2008; Xu et al., 2001). However, unlike primitive and definitive erythroid cells, which can be mainly distinguished by their morphology and globin gene expression; little is known about the differences between the 'waves' of megakaryocytic activity. It has been demonstrated that megakaryocytes derived from the E7.5 YS form colonies more rapidly than those derived from the adult BM, arise independently from Runx1 and have limited capacity to endoreplicate (Potts et al., 2014; Tober et al., 2007; Xu et al., 2001). Also, the initial emergence of megakaryocytes and platelets in the early YS is

independent of the cytokine thrombopoietin (TPO), which is later indispensable for FL and BM megakaryocytes (Gurney et al., 1994; Potts et al., 2015; Xie et al., 2003). While the role of embryonic platelets is still elusive, fetal platelets have now been recognized to play a critical role in the closure of blood and lymphatic vasculatures (Bertozzi et al., 2010; Carramolino et al., 2010).

Extrinsic regulation of megakaryopoiesis

Numerous hematopoietic growth factors regulate different aspects of megakaryopoiesis. Many of these cytokines have broad effects on all hematopoietic lineages, such as granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-3, IL-6, IL-11 and IL-12 (reviewed in Pang et al., 2005). More than 20 years ago, TPO was identified to be the ligand of the c-Mpl receptor and a potent promoter of megakaryocyte progenitor expansion and differentiation (de Sauvage et al., 1994; Kaushansky et al., 1994; Lok et al., 1994; Wendling et al., 1994). In mice, deletion of either c-Mpl or TPO decreases megakaryocyte numbers in the BM and circulating platelets by 85% (Alexander et al., 1996; Gurney et al., 1994; Murone et al., 1998). TPO has also been shown to function in early hematopoietic progenitors, promoting the expansion of CD34+ progenitor cells (Young et al., 1996). TPO is predominantly produced in the liver by hepatocytes, and subsequently secreted into the blood. Recently, it was discovered that levels of circulating TPO are regulated by binding of aged platelets to the Ashwell-Morell receptor in hepatocytes, thereby controlling the expression of TPO mRNA and protein, and thus, platelet production (Grozovsky et al., 2015). TPO is highly homologous to EPO in its N-terminus, indicating a close evolutionary relationship between the two signaling pathways. Defects in TPO:c-Mpl signaling are present in several human disorders. For example, c-Mpl mutations causing frameshifts occur in congenital amegakaryocytic thrombocytopenia, a rare megakaryocyte deficiency in infancy (Ballmaier et al., 2001; Ihara et al., 1999), and activating mutations in the TPO gene promoter and the c-Mpl protein are found in some patients with familial essential thrombocythemia (Ding et al., 2004; Ghilardi and Skoda, 1999).

Stromal cell-derived factor-1 (SDF-1), on the other hand, has an important TPO-independent effect on megakaryopoiesis. SDF-1 binds to the CXCR4 receptors in megakaryocyte progenitors and enhances their chemotactic activity,

possibly regulating their movement from the “osteoblastic niche” to the “vascular niche” for platelet formation (Avecilla et al., 2004).

Intrinsic regulation of megakaryopoiesis

During megakaryopoiesis, a series of transcription factors form complexes that coordinately activate megakaryocyte-specific genes and/or simultaneously repress gene expression that supports other cell types. Most of these factors have been identified in loss-of-function studies in mice and analysis of human diseases. Important players are discussed below.

GATA-1, GATA-2 and FOG-1

GATA-1 is a central transcription factor directing megakaryocyte development. While targeted disruption of Gata1 in mice causes embryonic lethality due to anemia, a megakaryocyte-specific knock down of GATA-1 expression leads to thrombocytopenia and increased numbers of immature megakaryocytes (Fujiwara et al., 1996; Shivdasani et al., 1997). GATA-2, a closely related transcription factor, is expressed earlier in the hematopoietic hierarchy and is believed to have some overlapping functions with GATA-1 (Fujiwara et al., 2004; Weiss and Orkin, 1995a). GATA-2 is also expressed during early megakaryopoiesis, which may explain the partial ability for platelet formation in the GATA-1 knockdown mouse. Similarly, GATA-2 knockdown in wild-type (WT) BM progenitors causes a reduction in the CFU-Meg activity (Huang et al., 2009).

The association of GATA-1 with its cofactor FOG-1 is critical for embryonic hematopoiesis and megakaryocytic development. Targeted disruption of Fog-1 in mice markedly inhibits erythroid development and causes an early block to megakaryocytic development with no identifiable precursors (Tsang et al., 1997). Also, GATA-1 and FOG-1 synergistically enhance the expression of the megakaryocyte-specific α_{IIb} gene (Gaines et al., 2000). GATA-1 N-terminal zinc finger mutations that impair binding to FOG-1 cause CDA and/or thrombocytopenia, highlighting the importance of this association (Campbell et al., 2013; Mehaffey et al., 2001; Nichols et al., 2000; Yu et al., 2002).

An important role for GATA-1 in controlling megakaryopoiesis is further evidenced by the discovery that expression of GATA1s contributes to Down syndrome-associated transient myeloproliferative disorder or acute megakaryoblastic leukemia (Greene et al., 2003; Wechsler et al., 2002).

FLI-1

The Ets family of transcription factors reside in close proximity to GATA sequences in megakaryocyte-specific promoters, suggesting functional interactions between these two classes of factors (Lemarchandel et al., 1993). This is the case for Fli-1, an Ets transcription factor that binds to the proximal promoter of megakaryocyte-specific genes together with GATA-1/FOG-1 (Wang et al., 2002). Overexpression of Fli-1 induces megakaryocytic features in undifferentiated hematopoietic cell lines (Athanasiou et al., 1996), while homozygous loss of functional Fli-1 alleles in mice leads to embryonic lethality because of severe defects in fetal megakaryopoiesis and embryonic hemorrhaging (Spyropoulos et al., 2000). Hemizygous deficiency of Fli-1 expression causes thrombocytopenia in patients with Paris-Trousseau syndrome (Raslova et al., 2004).

NF-E2

Nuclear factor erythroid 2 (NF-E2) is a leucine zipper transcription factor that controls terminal megakaryocyte maturation, proplatelet formation and platelet release by regulating a battery of genes that are crucial in the process of platelet production (Lecine and Shivdasani, 1998). NF-E2 deficient mice have profound thrombocytopenia with megakaryocyte maturation arrest, disorganized internal membranes and reduced granule numbers, among others (Shiraga et al., 1999).

RUNX-1

Acute myeloid leukemia/runt-related transcription factor 1 (RUNX-1) is a hematopoietic/vasculogenic-specific protein mostly known for its involvement in several leukemic chromosomal translocations, particularly t(8;21), which generates the AML1-ETO fusion protein (Nucifora and Rowley, 1995). RUNX-1 is expressed in MEPs, but is lost during erythroid differentiation (Lorsbach et al., 2004; North et al., 2004). RUNX-1 has been shown to participate in the programming of megakaryocytic lineage commitment through functional and physical interactions with GATA-1 (Elagib et al., 2003). Also, it was recently reported that RUNX-1 epigenetically represses KLF1 and shifts the KLF1:FLI-1 ratio toward FLI-1. Thus, RUNX-1 represses the erythroid gene expression program during megakaryocytic differentiation (Kuvardina et al., 2015). Haploinsufficiency of CBFA2, the DNA-binding subunit of RUNX-1, causes a rare, dominantly inherited thrombocytopenia associated with an increased risk of developing acute myeloblastic leukemia (Michaud et al., 2002; Song et al., 1999).

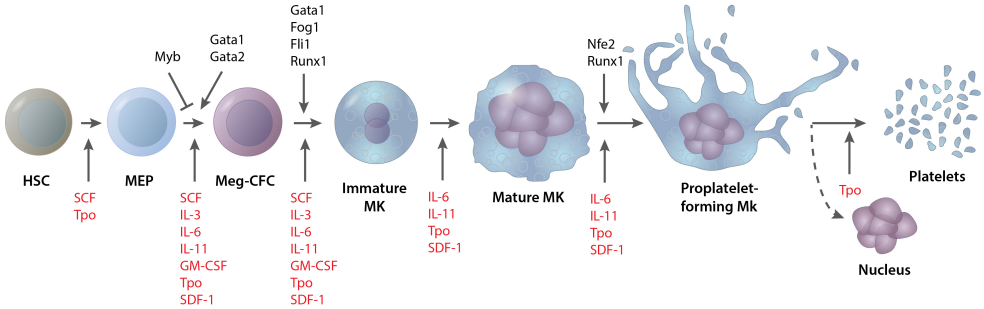

Figure 5. Schematic overview of adult megakaryopoiesis
Formation of platelets from HSCs comprises a series of steps that are regulated at multiple levels. Bipotential megakaryocyte/erythroid progenitors (MEP) give rise to unipotential megakaryocyte progenitors (Meg-CFC), which in turn form immature megakaryocytes (MK). MK maturation involves nuclear endoreplication and cytoplasmic expansion. Large mature MKs eventually form thick pseudopods called proplatelets, from which individual platelets are released. The nucleus is eventually extruded. Cytokines and transcription factors that influence this process are depicted in red and black, respectively.

Cellular Reprogramming

During development, cells progress through a path of decreasing potential and increasing specialization. It was long thought that the acquisition of specialized cellular functions was coupled to a permanent exhaustion of developmental potency and a permanent inactivation of genes irrelevant for terminal differentiation, like Waddington depicted in his famous epigenetic landscape (Waddington, 1957). However, over the past 60 years, several discoveries employing somatic cell nuclear transfer (SCNT) (Figure 6A), cell fusion (Figure 6B) and transcription factor-mediated nuclear reprogramming (Figure 6C), have firmly established that differentiated cells are not irreversibly committed to their fate. This chapter will summarize the critical discoveries to date and highlight the progress made in reprogramming cell types into blood.

Historical outlook

In the late 1950s, John Gurdon and colleagues made the groundbreaking discovery that the nucleus of a differentiated tadpole cell, when transferred into an enucleated oocyte, could be reprogrammed back to the totipotency of a zygote and then give rise to a mature fertile frog (Gurdon et al., 1958). This technique, termed SCNT, had successfully been developed six years before by Briggs and King using *Rana pipiens* (Briggs and King, 1952). Gurdon's findings demonstrated for the first time that differentiated cells retain the genetic information necessary to support the generation of whole new organism, in this case an entire tadpole. Moreover, they indicated that the oocyte contains trans-acting reprogramming factors that can erase epigenetic marks in differentiated cells and return them to a pluripotent state. It took however thirty years more and lots of failed experiments to make the leap from *Xenopus* to mammals. In 1997, Ian Wilmut and colleagues reported the birth of Dolly the sheep, cloned from the nuclei of epithelial cells (Wilmut et al., 1997), and a year later, the first mice were cloned (Wakayama et al., 1998). It is now known that the key to success was to use an unfertilized recipient egg at the meiotic stage II or a zygote at the mitotic stage of metaphase, so that the nuclear membrane would be disrupted and the nuclear factors would be available in the cytoplasm to enable nuclear reprogramming (reviewed in Egli et al., 2007). A gnawing doubt persisted, however, that the reprogramming event was due to contaminating stem cell-like cells among the donor cells. This speculation

was eventually ruled out when Rudolf Jaenisch and Konrad Hochedlinger reported the generation of mouse blastocysts and ESCs derived from the nuclei of adult B and T cells, yielding viable mice containing immunoglobulin or T cell receptor rearrangements in all tissues (Hochedlinger and Jaenisch, 2002).

It was initially unclear whether reprogramming was due to the unique molecular features of the oocyte or to the inherent developmental plasticity of somatic cells. Pioneering studies by Ernst Hadorn in *Drosophila* showed that when imaginal discs –larval structures meant to become appendages in the adult, such as wings, legs, genitals or antennae– were dissociated into single cells and transplanted to ectopic sites in larvae, they could change their fate; i.e. a leg disc could then form a wing (Hadorn, 1968). This suggested that "committed" cells of the embryo are plastic, because they are susceptible to environmental cues that can alter their fate. Early attempts to examine the developmental plasticity of somatic cells also included cell fusion, i.e. the union of two cells to form a single mononucleated entity (synkaryon) or multinucleated entity (heterokaryon). Helen Blau found that in heterokaryons formed by fusing human amniocytes and mouse muscle cells, the expression of several human muscle-specific genes was reactivated without DNA replication (Blau et al., 1983). She subsequently showed that the direction of differentiation, i.e. whether nuclear genes were silenced or activated, was determined by the nuclear ratio of the fused cells (Blau et al., 1985). Altogether, these observations provided conclusive evidence that reprogramming activity was not unique to the oocyte, and that the terminally differentiated state was dictated and maintained through the balance of trans-acting factors.

In 1987, Weintraub and colleagues showed that the bHLH transcription factor MyoD was sufficient to convert dermal fibroblasts into contracting myocytes (Davis et al., 1987), demonstrating that a single transcription factor is sufficient to instruct cell fate changes. The concept of transcription factor-directed cell fate conversion, coupled to the establishment of mouse and human ESC lines (Evans and Kaufman, 1981; Martin, 1981; Thomson et al., 1998), encouraged Yamanaka to postulate that specific pluripotency-inducing factors could be identified. Indeed, they showed that ectopic expression of a combination of four transcription factors (Oct4, Sox2, Klf4 and c-Myc, collectively referred to as OSKM) was sufficient to reprogram mouse and human fibroblasts to pluripotent stem cells, termed induced pluripotent stem cells (iPSC) (Takahashi et al., 2007; Takahashi and Yamanaka, 2006). This was a major breakthrough that opened up a wide range of new research lines and raised great hope for regenerative medicine, with applications

including cell replacement, drug screening and disease modeling (reviewed in Yamanaka and Blau, 2010). The discovery of iPSCs won Yamanaka the 2012 Nobel Prize in Physiology and Medicine, which was also awarded to John Gurdon for his SCNT experiments in *Xenopus* (Jaenisch, 2012). Furthermore, iPSCs reignited the field of reprogramming and prompted many scientists to start screening for factor combinations that could instruct lineage conversion without reaching pluripotency first. This approach, called direct lineage reprogramming, or transdifferentiation, has been shown to yield a wide variety of medically relevant cell types, such as cardiomyocytes, hepatocytes and neurons (Huang et al., 2011; Ieda et al., 2010; Vierbuchen et al., 2010), and has emerged as a promising approach for obtaining functional cell types for therapy.

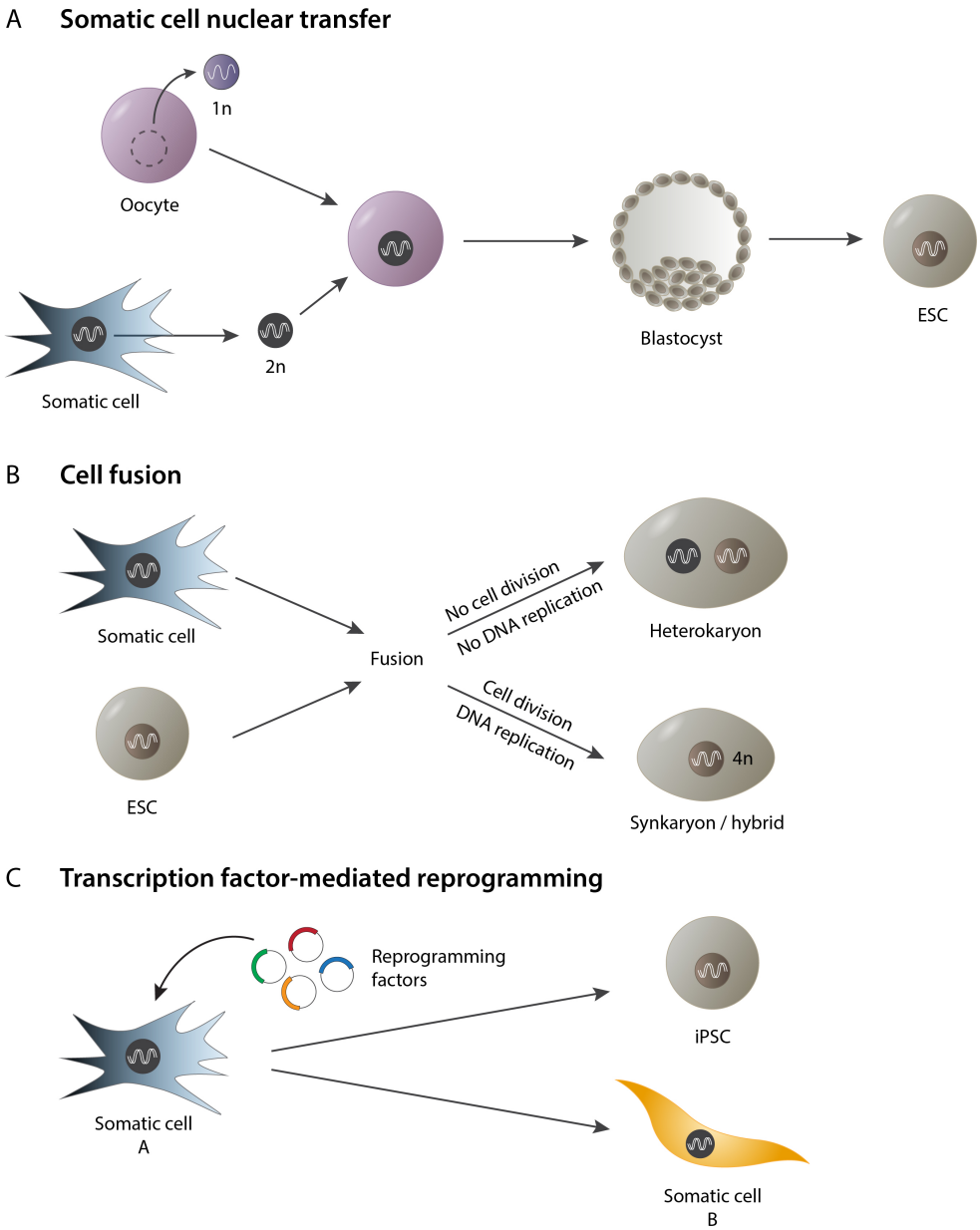

Figure 6. Approaches to nuclear reprogramming
(A) Somatic cell nuclear transfer consists in transplanting the nucleus of a somatic cell (2*n*, diploid) into an enucleated oocyte. Trans-acting factors present in the cytoplasm of the oocyte reprogram the nucleus of the somatic cell, so that the resulting cells are pluripotent. A blastocyst is then generated, from which embryonic stem cell (ESC) lines, or an entire organism, can be derived. (B) Cell fusion involves the combination of two distinct cell types into a single entity. The resulting cells can be heterokaryons (multinucleated) or synkaryons/hybrids (single nucleus), depending on whether cell division and DNA replication occurs. (C) Nuclear reprogramming can also be achieved by ectopic expression of transcription factors in somatic cells, resulting in induced pluripotent stem cells (iPSC) or other somatic cell types depending on the factors introduced (adapted from Yamanaka and Blau, 2010).

Induced pluripotent stem cells

Takahashi and Yamanaka sought to find specific factors that, when expressed in fibroblasts, could convert them back to pluripotent stem cells. They performed a systematic screening approach using a retroviral library expressing 24 candidate genes expressed in ESCs. Pluripotency was assessed by examining the activation of reporter genes into the promoter of *Fbx15*, a ESC-specific gene (Tokuzawa et al., 2003). Co-expression of these 24 factors in mouse fibroblasts activated *Fbx15´*s expression and induced the formation of colonies with characteristic ESC morphology. They then used a reductive "leave one out" strategy to determine the minimal set of factors required for iPSC formation, and the four-factor OSKM cocktail was defined (Takahashi and Yamanaka, 2006). Importantly, iPSCs formed teratomas –tumors that include cells of all three germ layers– following subcutaneous transplantation into immune deficient nude mice, but initially failed to produce adult chimeric mice, thus raising doubts about their ESC-like properties. However, within a year, two independent groups showed that iPSCs could indeed form adult chimaeras and functional germ cells (Okita et al., 2007; Wernig et al., 2007). Subsequent studies demonstrated that pluripotency could be induced from human fibroblasts (Takahashi et al., 2007), without c-Myc (Nakagawa et al., 2008), with a different cocktail of factors (Yu et al., 2007) and even with a combined genetic and chemical approach (Huangfu et al., 2008; Shi et al., 2008). Also, a wide variety of starting cell types and species have been successfully reprogrammed and non-integrating factor delivery methods have been employed (Li et al., 2009; Liu et al., 2008; Singh et al., 2015).

Extensive investigation has also been carried out on the underlying mechanisms of reprogramming. It has been described that the OSKM reprogramming can be subdivided in three phases termed initiation, maturation and stabilization (reviewed in Buganim et al., 2013). The initiation phase is characterized by increased proliferation, downregulation of somatic genes, initiation of mesenchymal-to-epithelial transition and activation of RNA processing and DNA repair (Buganim et al., 2013; Samavarchi-Tehrani et al., 2010). The reprogrammed cells then enter an intermediate or maturation phase, during which cells stochastically activate pluripotency markers and activate glycolysis (Buganim et al., 2012; Hansson et al., 2012). This second phase is the rate-limiting step that is responsible for the low efficiency of the reprogramming process (from 0.001% to 4.40% depending on the delivery method) (reviewed in Singh et al., 2015). In the

final phase, the cells stabilize into the pluripotent state, in which transgenes are silenced, the cytoskeleton remodeled to an ESC-like state, the epigenome reset and the endogenous pluripotency network activated, yielding fully reprogrammed iPSCs (Buganim et al., 2012; Golipour et al., 2012; Hansson et al., 2012; Polo et al., 2012). Apart from transcriptional changes, the epigenetic signature of the somatic cell needs to be erased during reprogramming in order to acquire a stem-cell-like epigenome. These changes include chromatin reorganization, DNA demethylation of promoter regions of pluripotency genes such as Oct4, Sox2 and Nanog, global histone modifications and X chromosome reactivation (Maherali et al., 2007; Wernig et al., 2007).

Applications of induced pluripotent stem cells in blood research

The ability to generate iPSCs from patient-specific cells offers great potential for regenerative medicine and disease modeling. These cells can be expanded indefinitely *in vitro* while still maintaining the potential to give rise to any cell type in the body, and unlike ESCs, offer hope for a truly personalized therapy (reviewed in Robinton and Daley, 2012). Since the number of reports on applications is too broad to summarize here, only a few examples in the field of hematology are described.

In 2007, a groundbreaking study by Jaenisch and colleagues provided proof-of-principle for the therapeutic use of iPSCs. They used homologous recombination to repair the genetic defect in iPSCs derived from a humanized mouse model of sickle-cell anemia. They then differentiated the repaired iPSCs into hematopoietic progenitors and transplanted them into affected mice, rescuing the disease phenotype (Hanna et al., 2007). Similarly, another study by Raya et al. showed that iPSCs can be derived from Fanconi anemia-corrected somatic cells, and they can subsequently give rise to disease-free hematopoietic progenitors (Raya et al., 2009).

Significant effort has also been made in differentiating iPSCs towards the hematopoietic lineage. To date, nearly all mature blood cell types have been generated from either ESCs or iPSCs, such as erythrocytes (Lapillonne et al., 2010; Ma et al., 2008), platelets (Nakamura et al., 2014), dendritic cells (Vodyanik and Slukvin, 2007), osteoclasts (Grigoriadis et al., 2010), T cells (Kennedy et al., 2012; Timmermans et al., 2009), B cells (Carpenter et al., 2011), NK cells (Woll et al., 2005) and myeloid cells (Choi et al., 2011). Despite this success, the biggest and still unfulfilled quest in this field is to differentiate pluripotent stem cells into

HSCs capable of long-term reconstitution of a patient's blood system. iPSCs could provide an unlimited source of patient-specific HSCs, circumventing graft-versus-host disease and shortages of certain human leukocyte antigen-matched donors for stem cell transplants.

Most attempts for HSC derivation via directed differentiation of pluripotent stem cells have sought to recapitulate hematopoietic development using morphogens and/or stromal cell co-cultures (reviewed in Rowe et al., 2016). Since HSCs only derive from a definitive hematopoietic program, some studies have devised strategies to distinguish the emergence of primitive-like from definitive-like hematopoietic cells in differentiating pluripotent stem cell cultures (Kennedy et al., 2012; Sturgeon et al., 2014). Lymphoid differentiation capacity and an EHT-like process have been proposed as key criteria to mark the onset of definitive hematopoiesis in these culture systems. Although hematopoietic progenitors have been obtained that have the potential to express adult-like T cell markers and polyclonal TCRαβ rearrangements, and acquire definitive globin expression, they have yet not yielded cells capable of engrafting and reconstituting the blood system *in vivo* (Kennedy et al., 2012; Sturgeon et al., 2014). A radically different approach was taken by Tenen's group directly injecting human iPSCs to immunocompromised mice, which gave rise to teratomas containing transplantable hematopoietic stem/progenitor cells (HSPC) with multilineage potential (Amabile et al., 2013). This study highlighted the importance of the *in vivo* environment in specifying true HSCs.

Direct lineage reprogramming

Since the advent of iPSCs, the field of direct lineage reprogramming has seen tremendous progress. The number of different cell types derived using this strategy in both mice and humans has substantially increased, alternative reprogramming factors have been identified and assays to characterize induced cells have been developed (reviewed in Xu et al., 2015a). This section will summarize the major advances in this field.

Novel reprogramming strategies

In addition to lineage-specific transcription factors, recent findings have demonstrated that epigenetic regulators, microRNA and small molecules can also induce lineage conversion (Figure 7). Since lineage reprogramming fundamentally

involves the transition between different epigenetic states, it is not surprising that epigenetic modifiers participate in this process. For example, the cardiac-specific subunit of BAF chromatin remodeling complexes, *Baf60c*, permits the binding of Gata4 to cardiac genes and enables the transdifferentiation of non-cardiac mesoderm in mouse embryos into cardiomyocytes (Takeuchi and Bruneau, 2009). On the other hand, microRNAs have been shown to drive neural and cardiac lineage conversion, among others (Ambasudhan et al., 2011; Jayawardena et al., 2012; Yoo et al., 2011), although the efficiency is not as high as with transcription factors. Small molecules have also been reported to promote the efficiency of lineage conversion, to replace the requirement for exogenous factors or to directly induce fate changes, mainly in neural conversion (Cheng et al., 2014; Ladewig et al., 2012; Liu et al., 2013b; Sayed et al., 2015). The latter is potentially a very promising approach for the clinical application of lineage reprogramming, since it would not raise safety concerns related to genetic manipulation. Moreover, small molecules are cell permeable and cost-effective, and their effects can be fine-tuned by varying concentrations.

Another strategy that has lately gained a lot of interest is the use of pluripotency factors for indirect lineage reprogramming. This is based on the notion that pluripotency-factor-driven lineage conversion is dependent on the presence of an epigenetically unstable population during early and intermediate stages of reprogramming, which can then be directed towards the desired cell fate under the proper signaling conditions (reviewed in Ma et al., 2013). Several cell types have been induced using this strategy, including hepatocytes (Zhu et al., 2014), pancreatic cells (Li et al., 2014) and cardiomyocytes (Efe et al., 2011). This is also the case for one of the first studies on direct conversion by Szabo et al., reporting that hematopoietic progenitors can be directly induced from human fibroblasts by ectopic expression of Oct4 and a specific cytokine treatment (Szabo et al., 2010).

Molecular mechanisms

Elucidating how the GRN of the target cell type is reactivated in reprogrammed cells has also been subject of intense study. Perhaps the most in-depth exploration in this regard has been done in neuron induction from fibroblasts by Ascl1, Brn2, and Mytl1. In this setting, Ascl1 acts as a pioneer factor, i.e. it binds to its target genomic sites in closed chromatin at the early phase of induction and enables the recruitment of other factors, such as Brn2, to their binding sites in later stages

(Wapinski et al., 2013). This study also showed that even though reprogramming factors are overexpressed simultaneously, they function in a hierarchical manner.

Several reports have also pointed out that reprogramming factor stoichiometry greatly affects the reprogramming process and the quality of the converted cells. For example, higher levels of Mef2c with lower levels of Gata4 and Tbx5 significantly boosts the efficiency of cardiac reprogramming (Wang et al., 2015a). Similarly, fine-tuning the levels of the different OSKM factors results in the generation of high-quality iPSCs with the ability of generating mice through tetraploid complementation (Carey et al., 2011).

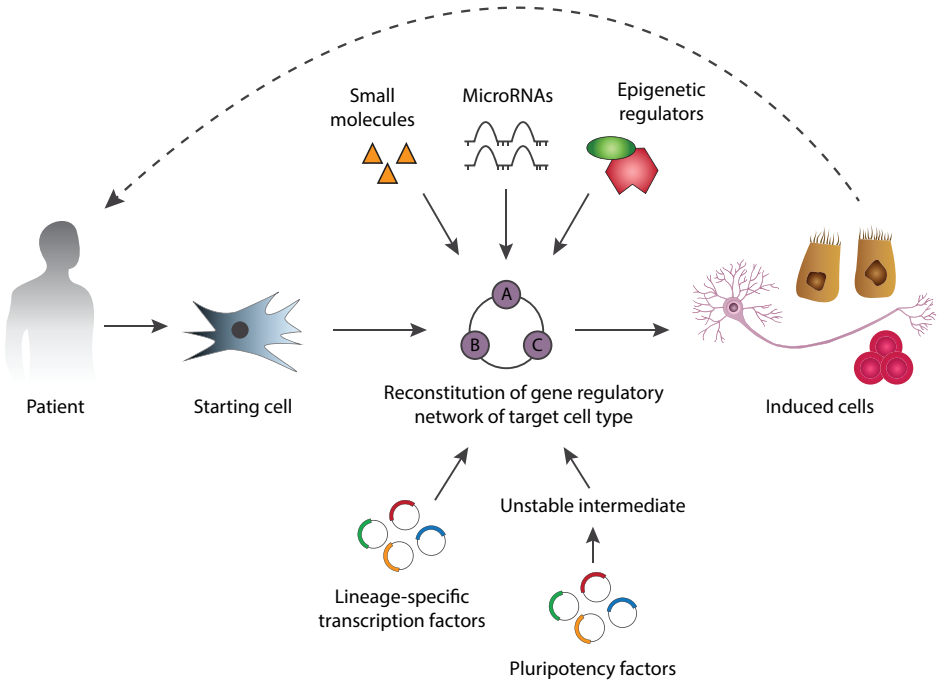

Figure 7. Lineage reprogramming strategies
Lineage reprogramming can be induced by several means, including lineage-specific transcription factors, small molecules, epigenetic regulators, microRNAs and pluripotency factors. Downstream of these factors, the gene regulatory network that specifies the target cell type is established (adapted from Xu et al., 2015b).

Functional maturation

Even though direct lineage reprogramming bypasses the multiple steps of lineage specification that occur during development, the generation of fully functional mature cells remains a major challenge. In many cases, the converted cells fail to silence the expression program of the starting cell type, or display a fetal-like immature phenotype (Cahan et al., 2014). It has been postulated that cell fate determination factors used to activate the GRN of the target cell type may not be sufficient for inducing fully functional maturation. Therefore, additional factors may be required during lineage conversion. One approach to identify maturation factors is to compare global gene expression profiles in immature embryonic/fetal cells and mature adult cells (reviewed in Xu et al., 2015a). Du et al. were able to identify CEBPA, ATF5 and PROX1 as hepatocyte maturation factors by comparing expression patterns of induced hepatocytes, immature fetal hepatocytes and freshly isolated primary hepatocytes. The combination of these maturation factors with cell fate determination factors resulted in the induction of fully functional human induced hepatocytes (Du et al., 2014).

Other strategies to induce functional maturation have sought to mimic the *in vivo* environment where the target cells develop. Some have attempted to co-culture the reprogrammed cells with supporting niche cells (Sandler et al., 2014), while others have showed that converting the cells directly in their *in vivo* environment could be the most effective way to promote functional maturation (Qian et al., 2012). Actually, the fact that lineage reprogramming can be conducted *in vivo* is an obvious advantage over directed differentiation from pluripotent stem cells. An increasing number of reports are published using this strategy, including conversions in the brain (Guo et al., 2014; Niu et al., 2013; Torper et al., 2013) and in the heart (Qian et al., 2012; Song et al., 2012). Despite rapid progress, major hurdles need to be overcome such as off-target effects and the risk of cellular heterogeneity within the reprogrammed population.

Large scale production for therapeutic purposes

A major limitation of direct lineage reprogramming is that the converted cells have poor proliferative capacity, posing a major barrier for biomedical applications that require large cell numbers. A possible solution is to reprogram somatic cells to stem cells or progenitors with engraftment and proliferation capacity. Several groups have recently succeeded in inducing neural stem cells or progenitors (Han et al., 2012; Kim et al., 2011; Lujan et al., 2012; Ring et al., 2012), hepatic stem

cells (Yu et al., 2013), HSCs (Riddell et al., 2014) and oligodendrocyte precursor cells (Najm et al., 2013; Yang et al., 2013) by direct lineage reprogramming, proving that this is a feasible strategy.

Another possible solution is to overcome proliferation arrest by overexpressing factors such as c-Myc or downregulating p53 in combination with reprogramming factors, creating transient intermediate states with proliferation capacity. Once expansion is achieved, exogenous factors can be silenced, thus allowing the intermediates to fully mature. This has been proven a useful strategy to expand human induced hepatocyte progenitors (Du et al., 2014).

Transcription factor-mediated conversion strategies for blood derivation

Transcription-factor based conversion strategies have been thoroughly explored during the past decade as means to derive blood products *in vitro*. This powerful technique has been applied to pluripotent stem cells as well as somatic cells. Below, key examples of published methods are discussed (Figure 8).

From pluripotent stem cells

In one of the earliest studies, it was shown that overexpression of HoxB4 in murine ESCs can specify differentiation and generate HSPCs with multilineage engraftment potential in primary and secondary recipient mice (Kyba et al., 2002). This gene was later found not to have the same effects in human cells (Wang et al., 2005) and researchers focused on other transcription factors.

A more recent approach from George Daley's lab incorporated aspects of morphogen-directed differentiation of human iPSCs with transcription factor-mediated reprogramming. They showed that hematopoietic progenitors derived from directed differentiation can be respecified to definitive progenitors with short-term engraftment of myeloid and erythroid lineages by forced expression of HoxA9, Erg, Rora, Sox4 and Myb (Doulatov et al., 2013). Another study from Slukvin and colleagues demonstrated that overexpression of Gata2 and Etv2 or Gata2 and Tal1 specifies endothelial fate in human ESCs and iPSCs. With Gata2 and Etv2, hemogenic endothelium (HE) develops with subsequent generation of myeloid-biased hematopoietic cells. With Gata2 and Tal1, on the other hand, HE gives rise to hematopoietic cells with erythroid-megakaryocytic potential (Elcheva et al., 2014). Similarly, another group reported that Gata2, Lmo2, Mycn, Pitx2,

Sox17 and Tal1 overexpression converts ESCs (also fetal liver cells) to expandable hemangioblasts. Once released from the control of ectopic factors and cultured with fibroblast growth factor (FGF), these hemangioblasts give rise to endothelial cells, smooth muscle and leukocytes, but not erythrocytes (Vereide et al., 2014). Still, neither this report nor the one by Elcheva and colleagues demonstrated the production of cells capable of multilineage engraftment.

In 2017, another study from Daley's lab was published reporting the production of HSCs from human pluripotent stem cells (Sugimura et al., 2017). They employed a previously published protocol to derive HE from pluripotent stem cells (Ditadi et al., 2015), and they subsequently identified seven transcription factors (Erg, HoxA5, HoxA9, HoxA10, Lcor, Runx1 and Spi1) that were sufficient to convert HE into immature HSCs. These HSCs were then transplanted into adult mice, where they engrafted and produced myeloid, B and T cells in primary and secondary recipients. Interestingly, two of these seven factors (Erg and HoxA9) were also used in their 2013 study.

Finally, a report focusing on the molecular mechanisms controlling the progression from hematopoiesis to erythropoiesis found that five transcription factors (Scl, Lmo2, Gata2, Ldb1 and E2A), together with inhibition of the FGF pathway, convert pluripotent epiblast cells from the chicken embryo to YS-like erythrocytes, highlighting the role of these factors in the specification of primitive erythropoiesis (Weng and Sheng, 2014).

From somatic cells

Multiple efforts have also been made to derive blood cells directly from other somatic cells without going through a pluripotent state. As previously mentioned, a study by Bhatia's lab showed that ectopic expression of Oct4 and cytokine treatment generated $CD45^{+}$ hematopoietic cells from human fibroblasts, giving rise to cells with very limited self-renewal and with myeloid, but no lymphoid differentiation potential (Szabo et al., 2010).

More recently, the Moore lab used a screening strategy to identify transcription factors that could activate a CD34 reporter in mouse fibroblasts. Gata2, Gfi1b, cFos and Etv6 were found to activate the reporter and reprogram fibroblasts to HE, with the subsequent appearance of definitive hematopoietic cells (Pereira et al., 2013). Similarly, Lacaud and colleagues reported that hematopoietic progenitor cells can be induced from mouse fibroblasts by overexpressing Gata2, Scl, Runx1c, Lmo2 and Erg via an HE-like intermediate. These cells expressed a

mixture of primitive and definitive globins, underwent rearrangement of TCRβ and immunoglobulin loci, and displayed short-term engraftment *in vivo* (Batta et al., 2014).

Other groups attempted to use starting cell types that would be developmentally closer to hematopoietic cells than fibroblasts. Rossi's lab demonstrated that murine adult pre-/pro-B cells and myeloid progenitors could be directly converted into serially transplantable, multilineage-reconstituting HSCs by ectopic expression of eight transcription factors, namely Runx1t1, Hlf, Lmo2, Pbx1, Zfp37, Prdm5, Mycn and Meis1. Importantly, the expression of the exogenous factors was turned on after the cells had been transplanted into mice, allowing the *in vivo* niche to perform the reprogramming (Riddell et al., 2014). Rafii and colleagues have instead attempted the feat of producing HSCs *in vitro* starting from their developmental precursors, endothelial cells. Taking into account their previous findings that Fosb, Gfi1, Runx1 and Spi1 can directly convert human endothelial cells to MPP-like cells (Sandler et al., 2014), they recently showed that these factors can also induce HSC-like cells from adult mouse endothelial cells. These HSCs are though initially 'immature' and need to be grown on a layer of supportive endothelial cells in order to fully mature and provide serial primary and secondary multilineage reconstitution (Lis et al., 2017).

Lastly, two studies have described methods for direct induction of erythroid cells and megakaryocytes (Ono et al., 2012; Sadahira et al., 2012). Sadahira et al. demonstrated that differentiated murine B cells can be reprogrammed to erythroid-like cells by forced expression of Gata1, Scl and C/EBPα. Gata1 and Scl were sufficient for reprogramming, while C/EBPα boosted the efficiency by inhibiting Pax6, a critical transcription factor for B cell differentiation (Sadahira et al., 2012). On the other hand, direct transdifferentiation of mouse and human fibroblasts to mature megakaryocyte-like cells was reported through overexpression of only three transcription factors, p45NF-E2, Maf-G, and Maf-K (Ono et al., 2012).

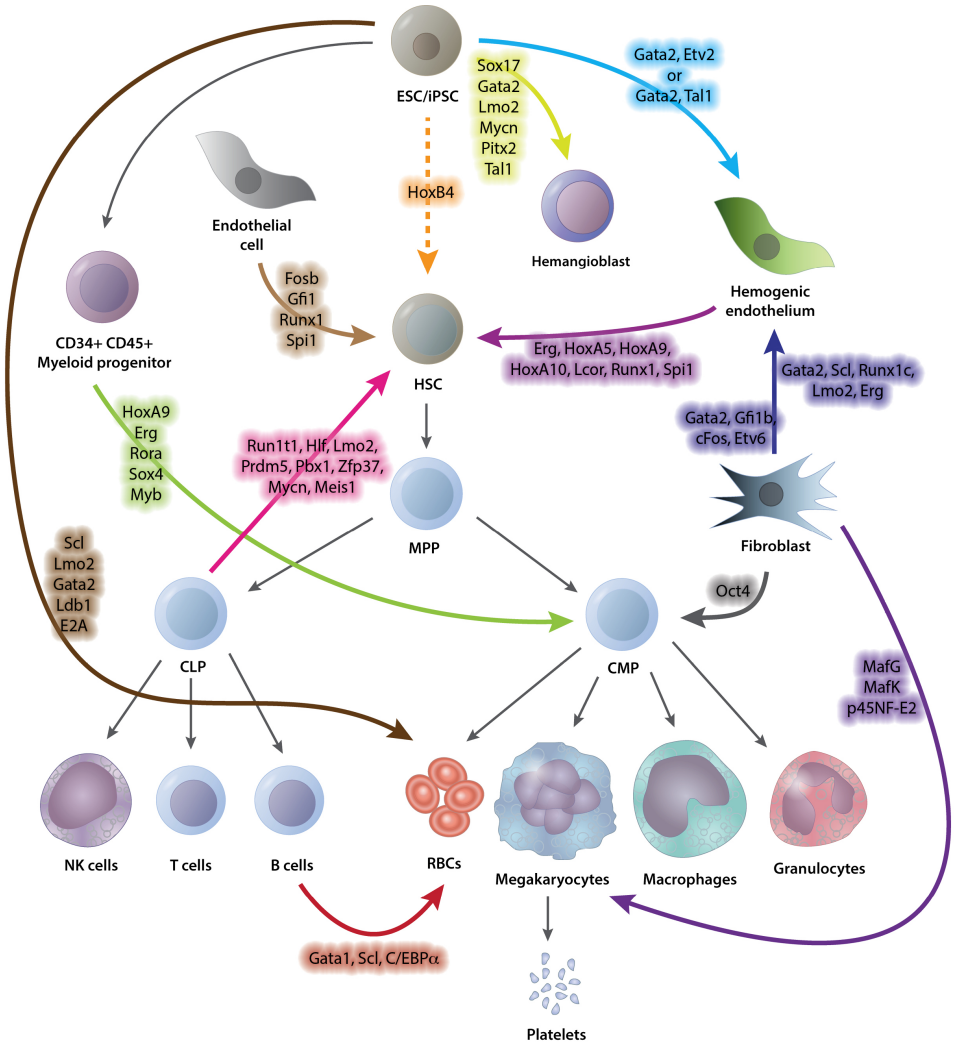

Figure 8. Transcription factor-based reprogramming in hematopoiesis
Summary of published strategies to derive hematopoietic cells using transcription factor overexpression, as described in the main text. Transcription factor cocktails are color-coded to the arrows that indicate the outcome of reprogramming (adapted from Daniel et al., 2016).

Red blood cells and platelets in the clinic

Transfusion medicine

RBCs and platelets from blood donors are used in the clinic as transfusion products. Transfusions serve as an essential component in emergency medicine, major surgical procedures and chemotherapy, and are one of the major treatment options for individuals with inherited disorders, such as DBA and Bernard-Soulier bleeding syndrome (reviewed in Bouhassira, 2012; Wang and Zheng, 2016). On average, one unit of platelet concentrate contains 3 x 10^{11} platelets, while one unit of packed RBCs contains 2.5 x 10^{12} cells. Transfusions are, at present, totally donor dependent. The system is currently sufficient to cover most transfusion needs in high-income countries, but supply problems remain in many parts of the developing world due to inefficient collection procedures (WHO 2017). Moreover, the reliance on blood donors is associated with infectious risks, high costs of screening, and supply bottlenecks for rare blood types and for alloimmunized patients requiring chronic transfusions (reviewed in Migliaccio et al., 2012; Wang and Zheng, 2016). Supply problems are expected to worsen over the next 20 to 30 years due to an increasing proportion of aged people (Ali et al., 2010). A solution to this problem would be to manufacture RBCs and platelets *in vitro* perfectly matched for all blood groups. If successful, this would be a major clinical breakthrough providing transfusion dependent patients with non-immunizing safe transfusion products.

In vitro production of RBCs and platelets: current progress

Considerable progress has been made towards the *in vitro* generation of RBCs and platelets for transfusion. Different sources currently under investigation are discussed below.

Hematopoietic stem and progenitor cell sources

The idea of using primary stem cell sources to generate RBCs *in vitro* arose when it was realized that discarded umbilical cord blood units have the potential to generate sufficient erythrocytes for several transfusions (Neildez-Nguyen et al., 2002). Since then, primary $CD34^+$ HSPCs derived from umbilical cord blood have

been used to study *in vitro* culture conditions that can efficiently generate enucleated mature erythrocytes (Giarratana et al., 2005; Miharada et al., 2006; Neildez-Nguyen et al., 2002). Current protocols produce sufficient RBCs for their functional evaluation in vivo (10^7). In 2011, Douay's lab provided evidence that autologous RBCs generated from mobilized $CD34^+$ cells survived *in vivo* in man as long as their natural counterparts (Giarratana et al., 2011). This study provided proof-of-principle that transfusion of *in vitro*-derived RBCs is a safe procedure, but the therapeutic application is still unrealistic based on the protocol described.

Regarding platelet production, several studies have described protocols to generate $CD41^+$ $CD42b^+$ polyploid megakaryocytic cells *in vitro* from $CD34^+$ HSPCs derived from BM, umbilical cord blood or peripheral blood (Ivetic et al., 2016; Matsunaga et al., 2006; Mattia et al., 2002). These megakaryocytes are capable of generating platelets *in vitro* that get activated upon stimulation with specific agonists, such as thrombin or fibrinogen. However, these protocols demand further improvements before they can be standardized, such as xeno-free culture conditions and functional tests for platelets.

Despite promising advances, the limited availability and restricted expansion potential of HSPCs make them a suboptimal source to provide sufficient numbers of RBCs and platelets for transfusion at a reasonable cost.

Pluripotent stem cell sources

Stem cell sources with unlimited expansion potential, such as ESCs and iPSCs, could be an attractive alternative source for large-scale production of transfusion products (reviewed in Migliaccio et al., 2012). The potential genomic instability and tumorigenicity of these stem cell sources poses a reduced safety concern in this context since the final product, RBCs and platelets, do not contain a nucleus. Moreover, iPSCs have an additional value, as they can be generated from patients with rare blood types.

In this regard, methods have been established for producing RBCs from ESCs (Lapillonne et al., 2010; Olivier et al., 2006; Qiu et al., 2008) and iPSCs (Kobari et al., 2012). However, the yields of fully mature cells are still too low and the duration of the culture is too long, making the process costly and inefficient (reviewed in Rousseau et al., 2014).

Megakaryocytes have also been successfully produced from human ESCs (Gaur et al., 2006; Lu et al., 2011; Takayama et al., 2008) and iPSCs (Feng et al., 2014; Liu et al., 2015; Moreau et al., 2016), however, they show a restricted capacity to

generate platelets *in vitro*. Physiologically, one megakaryocyte releases thousands of platelets into the circulation. In contrast, these protocols only allow the production of up to hundreds of platelets per megakaryocyte (reviewed in Baigger et al., 2017).

Immortalized cell lines

Ongoing efforts focus also on establishing immortalized cell lines able to produce large amounts of RBCs and platelets *in vitro*. These cell lines would combine the desired features of erythroid and megakaryocytic lineage commitment and unlimited growth capacity.

A few years ago, Kurita et al. reported the establishment of immortalized erythroid progenitor cell lines derived from human umbilical cord blood and iPSCs by overexpressing the human papilloma virus E6/E7 gene. These cell lines have infinite growth capacity, express erythroid specific cell surface markers and produce erythrocytes with functional fetal hemoglobin. Moreover, they only need minimal culture conditions to be maintained (Kurita et al., 2013). Another two groups established self-renewing erythroblast cell lines by overexpressing Sox2, c-Myc and shRNA against TP53 in umbilical cord blood cells (Huang et al., 2014), and c-Myc and Bcl-xL in iPSCs (Hirose et al., 2013). Despite producing enucleated hemoglobin-containing erythrocytes, the use of these cell lines is limited by very low enucleation efficiency and a high rate of cell death upon induction of differentiation, hurdles that are currently being addressed in the laboratory (Trakarnsanga et al., 2017).

In the platelet field, Nakamura et al. reported the generation of immortalized megakaryocyte progenitor cell lines by stepwise overexpression of c-Myc, Bmi-1 and Bcl-xL in human ESCs and iPSCs (Nakamura et al., 2014). These lines are able to produce functional $CD42b^+$ platelets *in vitro* once the exogenous factors are downregulated. However, these platelets do not perform as well *in vivo* as human endogenous platelets, and further refinements to the protocol need to be made. Also, the authors detected that several clones showed karyotypic abnormalities after long-term cultivation, leading to leukemogenesis upon infusion into immunodeficient mice. This highlights the importance of transplantation studies for clone selection.

Novel sources

Revolutionary sources have been proposed as alternatives to the classical procedures of platelet and RBC transfusion therapies. For example, two studies provided evidence that megakaryocyte progenitors can be directly infused *in vivo* and release functional platelets into the circulation (Fuentes et al., 2010; Wang et al., 2015b). Wang et al. also demonstrated that the platelets released by infused megakaryocytes closely resembled donor platelets and appeared more physiologic in nature than platelet-like particles obtained during *ex vivo* cultures (Wang et al., 2015b). On the other hand, erythroblasts have also been suggested as an innovative transfusion product. In fact, transfusions in developing countries have been successfully carried out with 40-80 mL of matched cord blood containing 4-8 x 10^{10} RBCs plus 4-8 x 10^{7} erythroblasts (Migliaccio et al., 2009). Thus, using progenitor populations as transfusion products would reduce the cell numbers required for transfusion and the costs of production.

Aims of the Thesis

The ultimate goal of the work presented in this thesis is to understand how the erythroid and megakaryocytic lineages develop and to translate this knowledge into approaches that recapitulate erythropoiesis and megakaryopoiesis *in vitro* for medical purposes. To this end, several specific aims were defined:

- To identify the minimal set of transcription factors capable of instructing erythroid cell fate in fibroblasts and characterize the reprogrammed cells (**Paper I**)

- To identify transcriptional cues and/or pathways that are missing in Paper I to induce a definitive erythroid program in fibroblasts (**Paper II**)

- To identify the minimal set of transcription factors capable of instructing megakaryocytic cell fate in fibroblasts and characterize the reprogrammed cells (**Paper III**)

Summary of Results

Paper I

Defining the Minimal Factors Required for Erythropoiesis through Direct Lineage Conversion

Direct lineage reprogramming as a tool to identify erythroid master regulators

GRNs controlling erythroid lineage commitment and differentiation have been well studied (Cantor and Orkin, 2002; Kim and Bresnick, 2007; Swiers et al., 2006). Essential genes for erythropoiesis have been identified through targeted gene disruption strategies, as well as studies of diseases of ineffective erythropoiesis. However, the minimal set of factors capable of initiating and specifying erythroid cell fate remained elusive. We postulated that direct lineage reprogramming could be a good strategy to reveal the master regulators of the erythroid lineage for two reasons: (1) it allows genes to be tested in different cellular contexts beyond their physiological role, thus enabling the characterization of common transcription factor networks, and (2) in contrast to loss-of-function studies, where the effects of gene disruption can be masked by redundancy, it facilitates the distinction of essential cell fate-inducing factors from permissive factors (Capellera-Garcia and Flygare, 2017; Vierbuchen and Wernig, 2011). This study was therefore conceived to identify the minimal set of transcription factors that could convert somatic cells, in this case, fibroblasts, directly into erythroid cells.

A retroviral library was created expressing 63 potential reprogramming factors, selected because of their involvement in erythroid and blood development. For the screening, we employed an erythroid lineage tracing mouse model, in which the Cre recombinase is knocked into one allele of the endogenous EpoR promoter and the yellow fluorescent protein (eYFP) is expressed from the Rosa26 (R26) locus (*Epor*-Cre *R26*-eYFP). When EpoR is expressed, the Cre recombinase excises the STOP codon in front of the eYFP coding region, resulting in YFP labeling of all

cells that have once expressed the EpoR gene at any time of their development. We overexpressed combinations of transcription factors in adult mouse tail-tip fibroblasts and cultured them in the presence of murine SCF, murine IL-3, human EPO and dexamethasone. We identified seven transcription factors (Gata1, Tal1, Lmo2, c-Myc, Klf1, Myb and Nfe2) that could convert adult mouse tail-tip fibroblasts into clusters of eYFP+ (EpoR+) round cells that emerged 5 to 8 days after transduction and displayed an erythroid progenitor-like morphology. After performing a reductive "leave one out" strategy, we found that only Gata1, Tal1, Lmo2 and c-Myc (GTLM) were necessary and sufficient for reprogramming. We called these cells iEPs, termed for induced erythroid progenitors/precursors. Importantly, iEPs could also be obtained from murine embryonic fibroblasts and human foreskin fibroblasts.

Characterization of induced erythroid progenitors

We observed that iEPs exhibited several properties of erythroid cells: they accumulated hemoglobin –confirmed by detection of globin transcripts and benzidine staining–, they expressed erythroid-specific genes and erythroid cell surface markers such as CD71 and Ter119, and formed visibly red colonies in semisolid media. Global gene expression analyses by microarray revealed that iEP red colonies were transcriptionally more similar to BFU-Es from E14.5 FL and adult BM than to the starting fibroblasts. When analyzing the differences between iEP red colonies and *bona fide* BFU-Es closely, we observed that genes with lower expression in iEP red colonies had, on average, lower expression in primitive erythroid cells than definitive erythroid cells in data retrieved from the publicly available Erythron database (Kingsley et al., 2013). This observation, coupled to the facts that iEPs expressed mainly embryonic globins and did not enucleate efficiently, suggested that iEPs were more similar to primitive than definitive erythroid cells.

Towards adult-like erythropoieisis

We hypothesized that while GTLM factors were sufficient to induce erythroid cell fate in fibroblasts, additional factors were necessary to instruct an adult-like program. We tested transcription factors Sox6, Bcl11a, Klf1 and Myb, all previously identified to directly or indirectly downregulate the expression of embryonic and fetal globin genes. Moreover, Sox6, Bcl11a and Myb are uniquely expressed in definitive erythroid cells (Palis, 2014). We found that only the

addition of Klf1 or Myb to the GTLM cocktail increased the expression ratio of adult *Hbb-b1* over embryonic *Hbb-y* compared to the GTLM factors alone.

We finally sought to examine whether iEPs were clonally heterogeneous, i.e. some clones were more similar to primitive erythroid cells, while others were more similar to definitive erythroid cells; and what the impact of overexpressing Klf1 and Myb was at the single-cell level. Thus, we performed qRT-PCR on single eYFP+ Ter119+ iEPs and analyzed the expression levels of 64 genes, including globin, primitive-specific and definitive-specific genes. We found that: (1) single iEPs expressed both embryonic and adult globins, as well as primitive-specific and definitive-specific genes, reflecting a mixture between primitive and definitive erythroid programs at the single-cell level; and (2) Klf1 and Myb overexpression increased the frequency of single cells with adult-like globin expression pattern, but did not change the expression of selected primitive and definitive-specific genes.

Paper II

RNA Sequencing Identifies Potential Missing Factors for Reprogramming of Fibroblasts to Definitive Erythropoiesis

A comprehensive gene expression study

After Paper I, the question remained what factors and/or pathways need to be activated to instruct a fully definitive erythroid program in fibroblasts. We proposed that an unbiased, direct comparison between iEPs and *bona fide* erythroid cells from different stages of development was necessary to unequivocally assess the transcriptional status of iEPs and identify missing reprogramming factors. Moreover, no study has been published to date comparing global gene expression in early erythroid progenitors from all three waves across development (primitive, EMP-derived and HSC-derived waves).

We therefore decided to perform RNA sequencing on iEPs and on erythroid progenitors obtained from four different sites and time points across development: the E9-9.5 YS, where primitive erythropoiesis occurs and the first EMPs emerge; the E11-11.5 FL, where EMPs expand and differentiate, the E14.5 FL, where HSCs expand and differentiate, and the adult BM, where HSC-derived hematopoiesis takes place. We hypothesized that the erythroid lineage tracing

mouse *Epor*-Cre *R26*-eYFP would be a good tool to afford the capture of phenotypically equivalent erythroid progenitors, since *Epor* is continuously expressed throughout development (Kingsley et al., 2013). Indeed, $eYFP^+$ ($EpoR^+$) cells were found in all time points analyzed. Early erythroid progenitors ($eYFP^+$ $Ter119^-$) were isolated by excluding late erythroid progenitors and precursors ($eYFP^+$ $Ter119^+$) and further purified using ckit. cKit has been established to be the earliest and most specific marker of definitive hematopoietic commitment, distinguishing Runx1-dependent EMPs ($ckit^+$) from maturing hematopoietic lineages ($ckit^-$) in the E8.5 and E9.5 YS (Frame et al., 2016). Consistent with these findings, we detected and isolated two populations, $ckit^-$ and $ckit^+$, within the $eYFP^+$ $Ter119^-$ subset in the E9-9.5 YS (YS9$ckit^-$ and YS9$ckit^+$). As for the FL and BM samples, the majority of cells expressed ckit (FL11$ckit^+$, FL14$ckit^+$ and BM$ckit^+$), while all iEPs were $ckit^-$ (iEP$ckit^-$).

Potential missing reprogramming factors identified

Principal-component analysis revealed that the iEP$ckit^-$ was the most transcriptionally distinct group, as it clustered farther away from the *bona fide* samples. A clear distance was also observed between YS9 samples and FL and BM samples, denoting major differences among *bona fide* cell sources. We identified previously recognized definitive-specific genes Bcl11a, Myb and ckit to be expressed in EMP-derived and HSC-derived (YS9$ckit^+$, FL11$ckit^+$, FL14$ckit^+$ and BM$ckit^+$) but not iEP (iEP$ckit^-$) and primitive (YS9$ckit^-$) erythroid cells, validating our isolation strategy. Following a similar expression pattern, we identified transcription factors Runx3 and Ikzf1, which have previously been implicated in the establishment of definitive hematopoiesis in zebrafish and in the regulation of γ globin gene expression, respectively (Bottardi et al., 2009; Bottardi et al., 2011; Kalev-Zylinska et al., 2003; Landry et al., 2008). Similarly, expression of IL-17 and IFN-γ receptors was at least five times higher in EMP-derived and HSC-derived cells than in primitive cells and iEPs, consistent with the molecular signature for inflammatory signaling regulating definitive, but not primitive, erythropoiesis (Greenfest-Allen et al., 2013). In contrast, hedgehog pathway modulator Gli3 and Hippo pathway effectors Yap1 and Tead1 displayed an inverse expression pattern, being mostly expressed in iEP $ckit^-$ and primitive YS9 $ckit^-$.

These findings set the ground for a future screening of candidate genes that can modulate developmental programming in iEPs. For this purpose, it will be

fundamental to define a method to check the developmental status of iEP after the secondary screening. One possibility would be to use cell surface markers that are uniquely expressed in definitive cells, such as ckit, CD43, CD44 or Cxcr4, some of which have been identified in the present study. Another option would be to employ a Runx1 reporter system, assuming that reprogramming of fibroblasts to definitive erythropoiesis would involve EHT. In addition, it will be important to determine the lineage potential of each of the sorted populations to check if they are truly erythroid-restricted, as well as increase the number of biological replicates for some sample sets.

Paper III

Direct Conversion of Fibroblasts to Megakaryocyte Progenitors

Skewing transdifferentiation towards the megakaryocytic lineage

Considering Paper I's findings in the context of transcription factors known to be important for differentiation of MEPs, we hypothesized that the transdifferentiation process could be skewed to favor the megakaryocytic lineage given the appropriate culture conditions. We started by transducing human fibroblasts with the GTLM cocktail and cultured them in media containing both EPO and TPO. This resulted in the emergence of both erythroid and megakaryocytic cells, marked by the expression of CD235 and CD41, respectively, starting at day 4 post-transduction. Importantly, removal of any of the four factors was enough to completely block the generation of reprogrammed cells. This encouraged us to search for additional transcription factors that could enhance the megakaryocytic output. We decided to test Gata2 and Runx1, since they play an essential role in both the progression of MEPs towards the megakaryocytic lineage and subsequent megakaryocyte maturation (Kuvardina et al., 2015; Tijssen et al., 2011). Indeed, addition of both Gata2 and Runx1 to the GTLM cocktail doubled the percentage of $CD41^+$ cells by day 12 post-transduction. We could also generate $CD41^+$ megakaryocyte-like progenitors from mouse embryonic fibroblasts (MEF), both with the GTLM and the GTLM+Gata2/Runx1 cocktails. Remarkably, only the $CD41^+$ population produced by overexpression of the six-factor cocktail could be kept and expanded in culture for at least 2 weeks.

Characterization of induced megakaryocyte progenitors

Human $CD41^+$ cells obtained from GTLM+Gata2/Runx1 reprogramming resembled *in vitro*-derived MK progenitors from cord blood $CD34^+$ cells in terms of morphology, gene expression and megakaryocyte colony-forming ability. Specifically, they expressed megakaryocyte-specific marker tubulin beta 1 class VI, displayed polylobulated nuclei (with ploidies reaching 8N), and formed proplatelet-like structures in culture. We could also detect vital platelet-like particles in the supernatant of these cultures, confirmed by electron microscopy and calcein staining. Importantly, $CD41^+$ cells obtained from reprogramming human fibroblasts with only GTLM factors failed to form megakaryocyte colonies in semisolid media and express tubulin beta 1 class VI.

To evaluate the *in vivo* functionality of the megakaryocyte-like progenitors, we added a GFP retrovirus to the reprogramming factor cocktail and intravenously injected 1 x 10^5 $CD41^+/GFP^+/CD42^-$ cells into sub-lethally irradiated NOD.Cg-$Prkdc^{scid}$IL-$2R^{tm1Wjl}$ (NSG) mice. After a week, we detected a $CD41^+$/ $GFP^+/CD42^+$ population in the peripheral blood of 75% of transplanted mice (16.7% of the total $CD41^+$ population). After two weeks, a cluster of $CD41^+/GFP^+$ cells was detected in the BM and lungs (2.2% and 7.2% of total $CD41^+$ population, respectively), and some of these cells also expressed CD42, indicating that the megakaryocyte-like progenitors are able to differentiate into megakaryocytes *in vivo*. Notably, these engraftment percentages decreased over time, and were almost undetectable from three weeks post-transplantation.

Clinical relevance

Lastly, we provided proof-of-principle that our transdifferentiation protocol could be applied in a clinical setting by reprogramming fibroblasts from Fanconi anemia (FA) patients, which suffer from thrombocytopenia and require frequent platelet transfusions. We used both uncorrected and gene-corrected FA fibroblasts, and evaluated their reprogramming ability. Both fibroblast sources could be reprogrammed and generate CD41+ cells, but the conversion efficiency was significantly higher in gene-corrected sources. Also, only fibroblasts that had been gene-corrected gave rise to large cells with polylobulated nuclei, proplatelets, CFU-MKs and platelet-like particles upon reprogramming.

General Discussion and Future Perspectives

1. Activating erythro-megakaryocytic gene regulatory networks – roles of reprogramming factors

Although the difference in global gene expression between erythroid cells and megakaryocytes involves hundreds of genes, the core GRN that determines a specific cell fate is comprised by relatively few transcription factors. Some factors are specifically expressed in either erythroid cells or megakaryocytes, such as KLF1 and FLI-1, respectively; but most factors (GATA-1, GATA-2, TAL1, GFI1b and FOG-1) are shared between the two lineages with unique roles in each (Hu et al., 1997; Kerenyi and Orkin, 2010; Miyamoto et al., 2002; Ng et al., 2009; Novershtern et al., 2011; Zandi et al., 2010). Combinatorial interactions between these master regulators ultimately controls cell type-specific chromatin binding and gene expression.

In HSCs, GATA-2 functions in large part within the context of LDB1-complexes –comprising LMO2, TAL1 and E2A– to control expression of genes responsible for HSC maintenance (Li et al., 2011). As HSCs differentiate and commit to the erythro-megakaryocytic lineage, GATA-1 is induced and gradually replaces GATA-2 at GATA occupancy sites, in a process referred to as a "GATA switch" (Bresnick et al., 2010; Dore et al., 2012; Kaneko et al., 2010; Snow et al., 2011; Weiss et al., 1994). In megakaryocytes, GATA-2 expression persists and most of these sites continue to be occupied by either GATA-1 or GATA-2. In contrast, in erythroid cells, GATA-1 completely replaces GATA-2 and only a minority of the sites originally occupied by GATA-2 in HSCs are bound by GATA-1 in erythroblasts (Pimkin et al., 2014; Visvader and Adams, 1993). These complexes function as primary mediators of global erythroid and megakaryocytic gene activation.

Considering these data, it is not surprising that induction of erythroid and megakaryocytic cell fate in fibroblasts (**Paper I** and **Paper III**) occurs through overexpression of several members of the aforementioned complexes. Our results are also in accordance with the extensive role of GATA-2 in megakaryopoiesis, but not in erythropoiesis. In line with that, it has recently been shown that GATA-1 and GATA-2 regulate distinct gene sets in megakaryocytes –GATA-1 tends to activate megakaryocyte/platelet-specific genes, while GATA-2 tends to repress genes expressed by HSCs and alternate lineages (Pimkin et al., 2014). Pimkin et al also showed that an HSPC-expressed transcription factor heptad involving GATA-2, RUNX1, LYL1, TAL1, FLI1, ERG and LMO2, occupies an extensive set of megakaryocyte-specific genes in HSPCs. Binding of this heptad is associated with low-level gene expression in HSPCs, and subsequent further induction in committed megakaryocytes, indicating the existence of a robust mechanism of megakaryocytic lineage priming. Furthermore, the fact that RUNX-1 promotes megakaryocytic reprogramming is consistent with the finding that RUNX-1 represses the erythroid gene expression program during megakaryocytic differentiation by inhibiting KLF1 (Kuvardina et al., 2015).

The role of oncogene c-Myc in our reprogramming strategies is less obvious. c-Myc is involved in a variety of cell behaviors, including cell-cycle progression, proliferation and differentiation, and has been found deregulated in many cancers (Hoffman et al., 2002). As one of the four Yamanaka factors, c-Myc has been shown to enhance early steps of reprogramming to iPSC by repressing fibroblast-specific genes and up-regulating the metabolic program of the embryonic state (Sridharan et al., 2009). Additionally, c-Myc is a universal amplifier of any given transcriptional state a cell finds itself in at the time of c-Myc activation (Nie et al., 2012). Although c-Myc was found to be dispensable for iPSC generation (Nakagawa et al., 2008), it was always required in our reprogramming strategies. Several lines of evidence have emerged indicating that c-Myc has a role beyond that of promoting cell proliferation and enhancing reprogramming efficiency. For example, epiblast-restricted c-Myc disruption causes apoptosis of primitive erythroblasts and severely impairs definitive hematopoiesis, leading to embryo demise at E12 due to severe anemia (Dubois et al., 2008). It has also been demonstrated that MEPs from c-Myc$^{-/-}$ mice can differentiate to both megakaryocyte and erythroid progenitors; however, erythroid differentiation is blocked at the progenitor stage, and thus more MEPs differentiate to megakaryocyte progenitors and form platelets (Guo et al., 2009). Still, these

megakaryocytes are significantly smaller in size and lower in ploidy than their WT counterparts (Guo et al., 2009). All these data support the notion that c-Myc is likely to have more than just a cell proliferation function during erythro-megakaryocytic reprogramming. Future studies on c-Myc's chromatin occupancy during reprogramming will be needed to determine its precise role in this setting.

2. Inducing a primitive or a definitive program?

One of the remaining concerns regarding the use of pluripotent stem cell-derived blood cells is the failure to recapitulate the final wave of hematopoiesis involving transplantable HSCs and production of adult-like blood cells (reviewed in Peters et al., 2010). Most culture systems appear to recapitulate YS hematopoiesis, consisting of a wave of primitive hematopoiesis followed by the emergence of distinct EMP-derived populations (McGrath et al., 2015a). Our results in **Paper I** and **II** suggest that this is also the case in GTLM reprogramming –iEPs predominantly express embryonic globins, form small EryP-CFC-like colonies, are unable to expand and enucleate in culture, and retain a primitive-like expression signature. Although single iEPs express a mixture of primitive- and definitive-specific genes, the process and molecular mechanisms by which they emerge are unclear. Unpublished data show that ckit is not expressed at any time during GTLM reprogramming (Figure 9A). Likewise, RNA sequencing data reveals that Runx1 is expressed at very low levels in iEPckit$^-$ compared to all other *bona fide* sources (Figure 9B), suggesting that GTLM reprogramming does not occur via an EHT and therefore, does not result in definitive hematopoiesis. Another interesting observation is that a CD41$^+$ population is detected already at day 2 post-transduction and peaks at day 4, preceding the peak of erythroid output (Ter119$^+$) (Figure 9A). Apart from being a marker for the megakaryocytic lineage, CD41 has been claimed to serve as the earliest marker of primitive erythroid progenitors cells in the E7.0 YS (Ferkowicz, 2003). Ferkowicz et al. also showed that high-level expression of this integrin identifies essentially all E8.25 YS definitive hematopoietic progenitors, indicating that differing levels of CD41 distinguish between primitive and definitive hematopoiesis in the YS. This also fits with the recent findings from the Palis' lab, where they characterize distinct sources of hematopoietic progenitors in the early mouse embryo using CD41 (McGrath et al., 2015a). Similarly, the CD41^{dim} but not CD41^{bright} population was

identified as the immediate precursor of primitive erythroid cells in ESC differentiation cultures (Otani et al., 2005). Although we do not seem to distinguish $CD41^{dim}$ and $CD41^{bright}$ populations in our cultures, the fact that ckit is not expressed at any time during reprogramming suggests that the CD41 population could represent an immediate precursor of primitive erythropoiesis (Figure 9A). Future colony assays and morphological inspection of sorted CD41 populations during reprogramming will be required to answer this question.

This discussion also has important implications in the context of megakaryocytic reprogramming. In **Paper III**, we found that $CD41^{+}$ cells could be induced from fibroblasts by overexpressing both four factors (GTLM) and six factors (GTLM+Gata2+Runx1), but only the cells generated with the six-factor cocktail could be expanded in culture for at least two weeks and possessed features of megakaryocyte function. This raises the question of whether these two cocktails induce fundamentally different genetic programs, or whether Gata2 and Runx1 are only required to promote functional maturation of megakaryocyte progenitors. Since clusters of $ckit^{+}$ EMPs emerge in the YS in a Runx1-dependent manner (Frame et al., 2016), it is tantalizing to think that overexpression of Runx1 may induce ckit expression, and thus a definitive program, in fibroblasts. A closer examination of the chromatin binding profiles of these factors and their impact on global gene expression in both overexpression contexts will shed light on this question.

Elucidating the molecular mechanisms behind blood formation during ontogeny is critical if we wish to faithfully recapitulate HSC-derived hematopoiesis *in vitro* and manufacture transfusion products. To recapitulate the final wave of hematopoiesis is important because embryonic blood cells possess different properties than adult cells –i.e. primitive RBCs are substantially larger in size and have altered cell surface protein expression, which may result in an impaired ability to circulate in the adult body (Van Handel et al., 2010). Also, fetal megakaryocytes have lower ploidy and generate fewer platelets compared to adult megakaryocytes (Ferrer-Marin et al., 2013; Ma et al., 1996; Sola-Visner, 2012). Therefore, it is imperative to identify discriminatory surface markers or gene expression signatures for primitive and definitive blood cells. In **Paper II**, side-by-side comparisons of early committed erythroid progenitors across ontogeny and iEPs allowed us to identify transcription factors that could potentially induce a definitive program in fibroblasts, as well as cell surface markers that distinguish the different hematopoietic waves. Although these candidates still need to be

validated, this study provides a good foundation to elucidate ontogenic differences in erythropoiesis and translate this knowledge into *in vitro* production of blood cells.

Finally, we will need to determine whether the timing of factor overexpression impacts the reprogramming outcome. Should these "definitive"-specific transcription factors be induced before, together or after GTLM factor overexpression? Are erythroid programs (primitive, EMP-derived and HSC-derived) intrinsically different from each other, or GRNs are conserved and additional factors can be overexpressed to reprogram primitive erythroblasts to definitive erythroblasts? iEPs provide a very good platform to answer these questions.

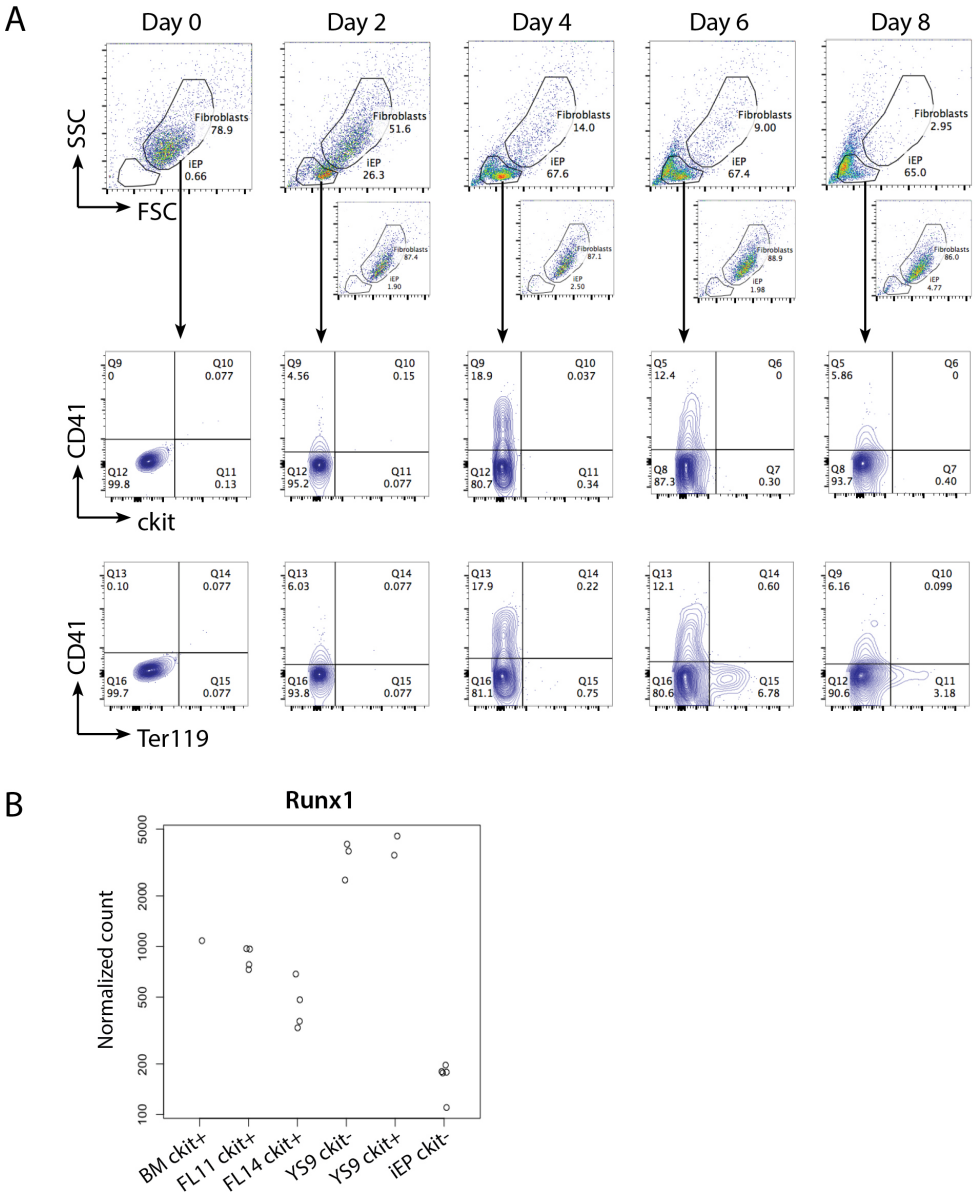

Figure 9. Unpublished data
(A) Related to Paper I, time-course flow cytometry analysis of untransduced mouse adult fibroblasts (day 0) and bulk GTLM-transduced mouse adult fibroblasts harvested at day 2, 4, 6 and 8 showing CD41, ckit and Ter119 expression. (B) Related to Paper II, normalized read counts for Runx1 in the different populations examined.

3. Functional assessment of induced erythroid progenitors

Although phenotypic traits such as morphology and lineage-specific gene expression are valid indicators of cellular identity, they may not be directly linked to the function of converted cells. Thus, an effective functional assay is required and clearly missing in **Paper I**. In the context of RBCs, functionality can be evaluated by several assays measuring parameters such as oxygen carrying and releasing ability, membrane deformability and hemoglobin content. However, the gold standard assay to evaluate functionality is to transplant cells into recipients and check how they perform *in vivo*. It is important to consider that iEPs are progenitor/precursor cells, therefore, they do not possess stem cell properties (i.e. they cannot be assessed in a long-term reconstitution assay) and they are not fully mature RBCs (i.e. cell number for a transfusion assay is limited). Moreover, erythroid cells lose CD45 expression upon maturation, so the use of the CD45.1/CD45.2 congenic system to track the contribution of donor cells in the recipient (Spangrude et al., 1988) is not possible. We attempted several *in vivo* experiments reprogramming fibroblasts from Kusabira Orange (KuO) mouse, which stably expresses the KuO fluorescent protein throughout the body, including erythrocytes and platelets (Hamanaka et al., 2013). In one set of experiments, we induced acute anemia in WT mice by injecting phenylhydrazine (60 mg/kg body weight), a chemical compound that induces hemolysis, and intravenously transplanted 2 million bulk day 5 KuO-iEPs a day after. We did not detect any KuO-derived cells in spleen, BM or peripheral blood, 3 or 8 days after transplantation. There could be several mutually non-exclusive explanations for this observation: (1) cells get eaten up by both circulating and tissue-resident macrophages; (2) iEPs do not engraft; and (3) it is a suboptimal *in vivo* assay. Also, we observed that several mice suffered sudden death right after intravenous injection, probably because transplanted cells were sticky and formed clots in the lungs. To circumvent all these issues, we attempted a second set of experiments, where we used sub-lethally irradiated NSG mice pretreated with clodronate liposomes, a drug encapsulated in liposomes that induces macrophage apoptosis (Su and Van Rooijen, 1989). We then transplanted 1 million bulk day 5 KuO-iEPs directly into the femurs of these mice. Three days after transplantation, we detected a small KuO+ Ter119+ population (0,00047% of total live cells) in the BM of mice injected with iEPs, and no cells in mice injected with KuO fibroblasts.

Although this does not unequivocally demonstrate iEP's functionality, it shows that they can survive *in vivo*. Further experiments are required to assess if iEPs can mature into reticulocytes *in vivo* and survive as long as their *bona fide* counterparts.

4. Towards therapeutic implementation of induced progenitors

One major hurdle of direct lineage reprogramming strategies is that converted cells typically have poor proliferative capacity, posing a major barrier for applications that require large cell numbers, such as cell replacement therapies. Also, it is necessary that these protocols achieve therapeutic-scale production at a reasonable cost.

One possibility would be to couple our transcription factor-mediated reprogramming approaches with immortalization methods to generate erythroid and megakaryocyte progenitor cell lines that could be expanded at high density and be synchronously induced to differentiate. This could provide an infinite source of "universal" donor RBCs and platelets, as well as simplify culture conditions to reduce production costs (Capellera-Garcia and Flygare, 2017).

A major limitation of our current reprogramming approaches is the use of non-selectable single-factor retroviral vectors with constitutive expression of transgenes. This results in genetically heterogeneous cell populations and high variability in reprogramming efficiency, which limits the experiments that can be performed afterwards. Additionally, the constitutive expression of reprogramming factors, such as c-Myc, is likely to be an impediment for erythroid and megakaryocyte progenitors to undergo terminal maturation (Brewer, 2000; Jayapal et al., 2010; Kirsch et al., 1986). The development of a robust inducible system will thus be necessary to improve scalability and maturation.

We are currently designing new reprogramming vectors with doxycycline-inducible polycistronic configurations, which have been shown to increase reprogramming efficiency in other studies (Riddell et al., 2014). By including two or three reprogramming genes in each vector, we could reduce the number of final vectors to only two. Upon doxycycline administration, exogenous factors would be induced and reprogramming achieved. iEPs or induced megakaryocyte progenitors could be then immortalized by overexpression of a doxycycline-

inducible human papilloma virus 16 (HPV16)-E6/E7 gene, which has been previously used to transform erythroid progenitor cells (Kurita et al., 2013; Wong et al., 2010). Established cell lines would then be grown and maintained in the presence of doxycycline, which could be removed from the culture media when differentiation is desired. We envision that this method can establish erythroid and megakaryocyte progenitor cell lines that grow infinitely and can be timely pushed to differentiate. Likewise, it would offer the scalability we need to test these cells *in vivo*.

Concluding Remarks

Towards the goal of defining and inducing the genetic programs that instruct erythro-megakaryocytic cell fate, the work presented in this thesis contributes with the following:

- the identification of the minimal sets of transcription factors capable of directly converting mammalian fibroblasts into erythroid (**Paper I**) and megakaryocyte progenitors (**Paper III**), and

- the interrogation of gene expression changes during erythroid ontogeny to identify master regulators of adult erythropoiesis and improve the reprogramming outcome (**Paper II**)

We envision that the transcription factor cocktails identified here can constitute the foundation of future protocols to manufacture erythrocytes and platelets *in vitro* for personalized transfusion medicine.

Populärvetenskaplig sammanfattning på svenska

Blodet, en livsnödvändig vävnad i ryggradsdjur, består av flytande plasma och tre huvudsakliga celltyper: vita blodkroppar, röda blodkroppar och blodplättar. De vita blodkropparna utgör kroppens immunförsvar mot patogener, röda blodkroppar transporterar syre samt underlättar gasutbytet i lungor och i perifer vävnad, och blodplättar reparerar skadad vävnad för att förhindra blodförlust. Eftersom alla mogna celltyper, såsom de i blodet, har en begränsad livslängd behöver de konstant ersättas av nya celler, vilka bildas i en dynamisk process som kallas hematopoes. Denna påbörjas från de sällsynta blodstamcellerna som finns i benmärgen hos vuxna människor. Blodstamceller har förmågan att göra ett obegränsat antal kopior av sig själva och kan dessutom bilda alla olika typer av blodceller, en process som är minutiöst reglerad av både kroppens yttre faktorer såsom hormoner och cellens egna inre faktorer exempelvis proteiner som binder till DNA (transkriptionsfaktorer). Hur blodstamceller förbinder sig till att bilda en specifik celltyp är ett ämne som det intensivt forskas på. Inom mina doktorandprojekt har vi haft som mål att besvara följande frågor: Vilka gener behövs för att initiera den process som leder till bildning av röda blodkroppar? Och till blodplättsbildning?

Frågorna ovan är också av medicinsk relevans. Röda blodkroppar och blodplättar från donatorer används regelbundet i kliniken som transfusionsprodukter inom akutsjukvården och för att behandla patienter med olika typer av blodsjukdomar, inklusive kroniska anemier och benmärgsbristsjukdomar. I industrialiserade länder beräknas en enhet blod användas per 20 personer per år. Trots dess prevalens och standardiserade förfarande är det donatorbaserade insamlingssystemet associerat med risk för infektioner samt problematik med låg tillgång av ovanliga blodtyper. Dessutom kan transfusionspatienter komma att utveckla immunreaktioner mot transfunderat blod. Med en ökande äldre population förväntas dessa problem att förvärras de

kommande 20 till 30 åren. Därför finns ett behov av alternativa källor till blodprodukter för transfusion. En lösning som har föreslagits är produktion i labmiljö av röda blodkroppar som är perfekt matchade för alla olika blodtyper.

För att uppnå detta mål har vi använt oss av en metod som kallas cellulär reprogrammering, genom vilken en cells identitet kan ändras till en annan via artificiell tillförsel av gener (vanligtvis transkriptionsfaktorer) till cellens genomiska DNA. Med denna teknologi kan man förändra i princip vilken celltyp som helst, exempelvis hudceller, till en annan celltyp såsom en embryonal stamcell eller en nervcell. Utöver möjligheten att generera olika celltyper för regenerativa behandlingar är cellreprogrammering också användbart för att utröna vilka gener som är essentiella för en cells identitet. I den första artikeln har vi identifierat fyra gener – Gata1, Tal1, Lmo2 och c-Myc (från de över 20 000 gener som utgör det mänskliga genomet) – som har förmågan att konvertera mammaliska hudceller direkt till celler som är föregångare till röda blodkroppar. Vi observerade att dessa artificiellt genererade celler uppvisade kännetecken för äkta röda blodkroppar: de hade liknande utseende, uttryckte gener som har att göra med röd blodkroppsfunktion och cellerna ackumulerade också hemoglobin, det viktigaste proteinet för syretransporten. När det gällde genttryck märkte vi dock att de reprogrammerade cellerna jämfört med vuxna röda blodkroppar istället var mer lika röda blodkroppar som bildas under den embryonala utvecklingen i gulesäcken. I den andra artikeln utförde vi därför en så kallad screen för att identifiera ytterligare gener som skulle behövas för att reprogrammera en hudcell till en röd blodkropp mer lik vuxna sådana. Vi har hittat ett flertal kandidater som ska testas i framtiden. Slutligen, i den tredje artikeln, har vi visat att två transkriptionsfaktorer, Gata2 och Runx1, tillsammans med de fyra faktorerna ovan konverterar hudceller till celler som är föregångare till megakaryoyter (dvs de celler som bildar blodplättar). Dessutom har vi visat att dessa megakaryocytföregångare kan transplanteras till möss och där bilda funktionella blodplättar.

Sammanfattningsvis tillhandahåller våra resultat en ny plattform för att studera de genetiska program som styr utvecklingen av röda blodkroppar och blodplättar. Vi tror dessutom att våra resultat i framtiden kan utgöra grunden till protokoll för laboratorisk produktion av röda blodkroppar och blodplättar i syfte att användas till individualiserad transfusionsbehandling.

Resum en Català

La sang és un teixit líquid essencial per la vida dels animals vertebrats. Està formada per una part líquida, anomenada plasma, i tres tipus cel•lulars: els glòbuls blancs, els glòbuls vermells (també anomenats eritròcits) i les plaquetes. Els glòbuls blancs intervenen en la resposta immunitària contra patògens; els glòbuls vermells s'encarreguen de transportar oxigen des dels pulmons fins als teixits perifèrics; i les plaquetes participen en el procés de coagulació per evitar la pèrdua excessiva de sang en cas d'hemorràgia. Com que les diferents cèl•lules de la sang tenen una vida útil limitada, s'han de renovar constantment a través d'un procés dinàmic anomenat hematopoesi. Aquest procés de formació comença a partir d'un precursor cel•lular comú i no especialitzat conegut com a cèl•lula mare hematopoètica, que en humans adults es troba en la medul•la òssia. Aquestes cèl•lules mare tenen la doble capacitat de multiplicar-se per formar més cèl•lules mare i de diferenciar-se per donar lloc a tots els altres tipus cel•lulars de la sang. Aquests dos processos estan regulats per factors extrínsecs a la cèl·lula, com ara hormones; i factors intrínsecs a la cèl·lula, com ara proteïnes que s'uneixen a l'ADN (factors de transcripció). Els mecanismes que les cèl•lules mare hematopoètiques fan servir per donar lloc a un tipus cel•lular especialitzat, per exemple un glòbul vermell, és una pregunta que ha intrigat els científics durant dècades. Durant el meu doctorat, hem treballat per resoldre dues preguntes: quin són els gens necessaris per iniciar el procés de producció dels glòbuls vermells? I per iniciar el procés de formació de les plaquetes?

Aquestes preguntes també tenen una rellevància clínica. Els glòbuls vermells i plaquetes de donants es fan servir diàriament als hospitals per a transfusions de sang en casos d'emergències mèdiques i per tractar pacients amb malalties com ara l'anèmia o la plaquetopènia. Es calcula que en països industrialitzats, de mitjana, 1 unitat de sang és transfosa per 20 persones per any. Malgrat ser una pràctica habitual i protocol·litzada, el sistema de donants està associat al risc d'infeccions i a la manca de donants per grups sanguinis poc freqüents o per pacients que han desenvolupat immunitat contra la sang transfosa. A més a més,

s'estima que aquests problemes s'agreujaran en les pròximes dècades a causa de l'envelliment general de la població. Per tant, esdevé una necessitat imperativa trobar fonts alternatives de productes sanguinis per transfusions. Una de les solucions que es plantegen és produir, al laboratori, glòbuls vermells i plaquetes que siguin perfectament compatibles amb tots els grups sanguinis.

Amb aquesta fita en ment, vam decidir utilitzar un mètode anomenat reprogramació cel•lular, que consisteix en canviar la identitat d'una cèl•lula mitjançant la introducció artificial de gens exògens en el genoma. Utilitzant aquesta tecnologia, els científics poden convertir qualsevol tipus cel•lular, com ara una cèl•lula de la pell, en un altre, com ara una cèl•lula mare embrionària o una neurona. A part de proporcionar un mètode per generar cèl•lules a la carta amb finalitats terapèutiques, la reprogramació cel•lular també és útil per identificar els gens essencials que estableixen la identitat d'una cèl•lula especialitzada. En el primer estudi, vam identificar quatre gens, Gata1, Tal1, Lmo2 i c-Myc, que podien convertir cèl•lules de la pell directament en cèl•lules precursores dels glòbuls vermells. Les cèl•lules reprogramades posseïen característiques típiques de cèl•lules eritroides: tenien una mida i forma similar, expressaven gens relacionats amb les funcions del glòbuls vermells i acumulaven hemoglobina, la proteïna responsable de transportar oxigen. No obstant això, pel que fa a l'expressió gènica, les cèl•lules reprogramades s'assemblaven més als eritròcits que apareixen durant el desenvolupament embrionari que no pas als eritròcits que es troben en la sang del cos adult. Per aquesta raó, en el segon estudi vam decidir portar a terme un cribratge genètic per trobar gens addicionals que poguéssim introduir en el genoma de les cèl•lules de la pell perquè es convertissin en glòbuls vermells "adults". Vam trobar diversos gens candidats que seran posats a prova en futurs experiments. Finalment, en el tercer estudi, vam identificar dos gens, Gata2 i Runx1, que en combinació amb els quatre gens definits en el primer estudi, podien convertir cèl•lules de la pell en progenitors megacariocítics, els precursors cel•lulars de les plaquetes. A més a més, vam demostrar que aquests progenitors megacariocítics poden ser trasplantats per via intravenosa en ratolins, on es diferencien i donen lloc a plaquetes.

Per concloure, els resultats presentats en aquesta tesi proporcionen una nova eina per estudiar els gens que governen el desenvolupament dels glòbuls vermells i les plaquetes. A més, anticipem que serviran de fonament per a futurs protocols encarats a produir glòbuls vermells i plaquetes en el laboratori per oferir una medicina de transfusió personalitzada.

Acknowledgements

"If I have seen further it is by standing on the shoulders of Giants"
Isaac Newton, 1675

This thesis is the result of four years and eight months of hard work, long hours of commuting and some sleepless nights. But most importantly, it is the product of priceless contributions by many people from close and far who really need to be acknowledged.

First and foremost, **Johan Flygare**, my supervisor. Thank you for taking me in as a student in your newly established group and for your trust and generosity along the way. You inspire me to better myself and think big. I am truly grateful for the opportunities you have given me during my studies and proud of what we have achieved together.

Malin Parmar, my co-supervisor. Thank you for providing valuable input on my work and progress. You were always there when needed, it is only my fault that we have not interacted more often.

Sofie Singbrant, thank you for so many things! You are a role model of perseverance and leadership, I am sure you are going to rock it as a principal investigator. Thank you for encouraging me, providing constructive feedback in our meetings and for helping me in those crazy day-long *in vivo* experiments.

Kenichi Miharada, my external advisor in the now dissolved Thesis Advisory Committee. Thank you for always stimulating discussions! Your commitment and passion for science are admirable.

Jonas Larsson and **Stefan Karlsson**, thank you for creating an exceptional working and learning environment at A12 where I could thrive as an early-career scientist. Jonas, thank you also for your valuable input during meetings.

Christine Karlsson, thank you for your continuous support over the last couple of years and for helping me navigate the stressful thesis writing period. It is so good to have someone like you around!

I am also deeply thankful to the past and present colleagues in the Flygare group. I will never forget our adventurous and ambitious excursions, carefully planned by our boss – get ready future members! **Kishori**, thank you for all your hard work in the early days that paved the way for my main project. **Kavitha**, thank you for guiding me in the lab and for always being willing to help! **Sara**, thank you for your kindness and help in many moments, and for always having the right words to say. **Abdul**, there is not enough space here to thank you for all the times you have helped me (even saved me) in the lab and outside. Thank you from the bottom of my heart! **Alex**, thank you for your friendship, your always wise advice and for sharing a little bit of your family with me. **Melissa**, thank you for taking over the reprogramming project and for helping me out with rather stressful RNA-seq analysis. I would also like to thank **Jun**, **Isabel**, **Alban** and the Singbrant group members **Taha** and **Mayur**. You have been a great bunch to work with!

Johan, Alban, Melissa, Alex and Maria, thank you also for critical reading of this thesis.

I cannot forget to mention the people that brought me to A12 in the first place. Thank you, **Niels-Bjarne Woods** and **Carolina Guibentif**, for taking me in as a master student and teaching me the first things about hematopoiesis. Carolina, your lab supervision did not traumatize me, rather the opposite :)

Karin Olsson, **Silja Andradottir**, **Ineke de Jong**, **Natsumi Miharada** and **Xiaojie Xian**, thank you for your hard work and dedication in ensuring that the lab run smoothly during my time as a PhD student. I would like to extend my appreciation to the staff at the animal facilities for doing a great job. Special thanks to **Matilda Sjöstrand** at the conventional and **Linda Aspegren** at CRC for helping me with mice breedings and checking for plugs early morning. I should also mention and thank the FACS core staff **Teia Roschupkina** and **Zhi Ma** for helping me sort in numerous occasions, and **Beata Lindqvist** for virus production.

David Yudovich, thank you for teaching me some of your cloning skills last year. I really enjoyed working with you and I hope that the vectors we created are put into use in the near future.

To the people I have shared office with: **Hooi Min**, **Roksana**, **Matilda**, **Maria**, **Roger**, **Abdul**, **Alex**, **Taha**, **Melissa**, **Els** and **Mayur**. Thank you for the good times!

Thank you also to present and past members of the Yuan group, **Joan**, **Trine**, **Sebak**, **Karin**, **Stijn** and **Hugo**, for sharing lab space with us in the early days.

Shamit Soneji and **Stefan Lang**, thank you for putting up with my (sometimes too frequent) bioinformatics requests, and for doing so with great humor.

Göran Karlsson and **Mikael Sommarin**, thank you for jumping on board our project during the hectic revision period and helping us with your single-cell qPCR expertise.

I would specially like to express my gratitude to our collaborators abroad. **Julian Pulecio** and **Ángel Raya** in Barcelona, without whose help I would have probably not graduated as fast. Julian, thank you for your hard work and commitment to the projects, I was really lucky to meet you in San Francisco and I truly enjoyed working with you! Thank you Ángel for inviting me to talk at the EHA pre-congress meeting, it was a wonderful experience! **Vijay Sankaran** in Boston, thank you for your enthusiastic input on the reprogramming project and your assistance in many aspects!

Thank you to all past and present members at the Molmed and Molecular Hematology departments for creating a collaborative and friendly atmosphere to work in. Special thanks to my book club buddies, **Leal**, **Ludwig**, **Svetlana**, **Simon** and **Alex**, for our evenings of good food and interesting discussions. We still need to decide on our next book! **Mehrnaz**, you are such a kind soul, thank you for your support! **Sarah**, I love that you are motivated about almost everything, never lose that enthusiasm! **Svetlana**, you are the friend everyone wishes to have and I am so happy to count you as mine. Thank you also for our adventures in sunny California. To be continued...:) A warm thank you also to **Talía**, **Dani**, **Tania** and **all the Spanish-speaking crew** at BMC and beyond with whom I shared some really fun times over the years.

I cannot forget to thank my friends back home for always being available to spend some time with me and making me feel like I never left. Gràcies **Estefania**, **Agnès**, **Ariadna**, **Maria**, **Sònia**, **Núria**, **Raquel**, **Carla**, **Marta** i **Vanessa**. Especialment gràcies a tu, Vanessa, per la nostra amistat tan única amb la que sé que sempre podré comptar.

And finally, thank you to my biggest inspiration, my family. Gràcies **avi** i **iaia** per cuidar-me sempre. Especialment gràcies pels teus dinars, iaia, que m'han alimentat tan bé durant aquests últims mesos quan més ho he necessitat. **Laura**, gràcies per tantes coses... però per sobre de tot per ser una inspiració constant de la força amb què tots hauríem de viure cada dia. I finalment, gràcies a vosaltres, **mama**, **papa** i **Marc**, pel vostre amor i suport incondicional. Tot és gràcies a vosaltres! Us estimo i us he trobat a faltar!

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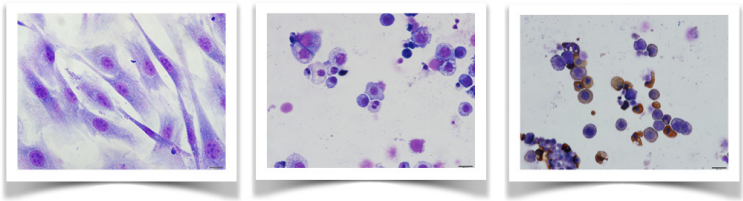
Printed by Media-Tryck, Lund 2017 NORDIC ECOLABEL, 3041 0903



Switching identities I-III

These pictures, illustrating the direct reprogramming of spindle-shaped fibroblasts into round hemoglobin-containing erythroid progenitors, were part of the exhibition "The invisible body" at Sven-Harry's art museum in Stockholm during Autumn 2017.

LUND UNIVERSITY
Faculty of Medicine

Department of Laboratory Medicine

Lund University, Faculty of Medicine
Doctoral Dissertation Series 2018:5
ISBN 978-91-7619-572-7
ISSN 1652-8220