Deciphering Metabolic Regulation of Hematopoietic Stem Cell Fate

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Dedicated to my parents for their unconditional love and support…

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SUMMARY

Hematopoietic stem cells (HSCs) are responsible for life-long production of all mature blood cells. This unique characteristic makes them an ideal candidate for cell-based therapies to treat various hematological malignancies. Their extensive use in the clinic is often hampered due to insufficient number of cells obtained from donors. Countless attempts to expand HSCs *in vitro* have failed, primarily due to our inability to recapitulate key features of the native bone marrow microenvironment, termed niche, in a dish. The absence of important niche signals *in vitro* results in rapid proliferation of HSCs with a concomitant loss of their long-term multi lineage blood reconstitution potential. The niche in the bone marrow involves a highly complex network of physical and biochemical signals that, in concert with cell-intrinsic mechanisms, is believed to control HSC fate choices. Moreover, the hypoxic conditions in the niche present an extreme metabolic environment, imposing HSCs to attain a distinct metabolic identity as compared to their differentiated progeny. However, despite decades of research and new insights on HSC niche composition and their metabolic regulation, it is currently very poorly understood how HSCs take the decision to either undergo self-renewal or differentiation. Insights into the mechanisms regulating HSC fate choices are key to design better strategies for HSC maintenance and expansion *in vitro* for use in clinical transplantation.

The overall goal of this thesis is to employ innovative experimental tools to explore the role of metabolism in regulating HSC fate choices. In the first part of this thesis a versatile cell-tracking assay was developed to follow HSC divisions *in vitro*. A combination of cell tracking and immunostaining was used to systematically map phenotypic changes in HSCs up to four divisions, under defined culture conditions imposing specific fates. Our preliminary analysis showed that the proportion of cells maintaining an HSC phenotype decreased with increasing number of cell divisions, supporting the notion that faster cycling results in HSC exhaustion.

In the second part of this thesis, we for the first time identify a causative link between mitochondrial metabolism and HSC fate decision. Using flow cytometry and long-term blood reconstitution assays, low mitochondrial activity was established as a reliable marker of functional HSCs, independent of their cell cycle state. Consequently, we could use this marker to reliably identify self-renewing HSCs from heterogeneous *in vitro* cultures. Strikingly, we found that HSC fate could be altered by artificially modulating their mitochondrial activity *in vitro*. These results suggest that mitochondrial activity is a determinant of HSC fate.

The last part of this thesis describes an experimental paradigm to analyze *in vivo* niche-instructed fate choices in paired HSC daughter cells. Live single cell imaging revealed a significant increase in asynchronous divisions in niche activated HSCs compared to control cells, suggesting a possible involvement of niche-instructed asymmetric cell division program. Indeed, a significantly higher level of asymmetric

gene expression was found in paired daughter cells arising from niche-instructed HSCs. This analysis led to the identification of 12 asymmetrically expressed genes, among them were key enzymes belonging to glycolytic and mitochondrial TCA cycle metabolic pathways.

Altogether, this thesis successfully employed unique experimental strategies to provide an intriguing link between metabolism and HSC fate choices. As many adult stem cell populations reside in similar metabolic microenvironments this knowledge can potentially be used to identify and study stem cells from other systems.

Keywords: asymmetric cell division (ACD), cell tracking, differentiation, hematopoietic stem cells (HSCs), niche, metabolic regulation, microenvironment, mitochondrial activity, paired daughter cells (PDCs), self-renewal, single cell analysis, stem cell fate, transplantation

RÉSUMÉ

Les cellules souches hématopoïétiques (CSHs) sont à l'origine de toutes les cellules sanguines matures, assurant ainsi la régénération du sang durant toute la vie d'un être humain. Grâce à cette particularité unique, ces cellules sont considérées comme des candidates idéales pour la thérapie cellulaire afin de soigner les différentes maladies sanguines. Cependant, l'utilisation systématique des CSHs en clinique est limitée par leur faible nombre, principalement à cause de l'insuffisance de donation et de l'inefficacité des méthodes d'expansion des ces cellules *in-vitro*. En effet, les méthodes d'expansion actuelles, souvent basées sur l'utilisation de boîtes de Petri, reproduisent pauvrement l'environnement spécifique de la moelle osseuse, aussi appelé « niche ». L'absence des signaux biologiques importants *in vitro* rapidement induit les CSHs à proliférer rapidement et perdre leur potentiel à produire les multiples lignées descendantes sur le long terme. Un réseau complexe de signaux physiques, chimiques et métaboliques régule le choix du devenir des CSHs. De plus, l'hypoxie de la niche représente un environnement métabolique extrême permettant aux CSHs de maintenir une identité métabolique unique et différente de leur descendance. Ainsi, la compréhension des mécanismes qui contrôlent le devenir des CSHs est la clé de la standardisation des transplantations de CSHs artificiellement générées *in vitro*.

L'objectif de ce travail de doctorat est de comprendre la relation en la régulation métabolique et le choix du devenir des CSHs en utilisant des techniques expérimentales innovatives. Dans la première partie de cette étude, nous avons mis en place une méthode de surveillance de cellules permettant de suivre en temps réel la division cellulaire des CSHs *in vitro*. Ainsi, nous avons pu suivre au moins cinq divisions successives et comprendre la dynamique de la division cellulaire sous différentes conditions de culture. De plus, nous avons pu observer que la fraction des cellules qui conservent le phénotype des CSHs est petite dans la population qui se multiplie rapidement comparé à leurs homologues se divisant lentement. Dans la deuxième partie, nous identifions pour la première fois le lien entre l'activité mitochondriale et le choix du devenir des CSHs. En utilisant la cytométrie en flux, et l'analyse de la reconstitution sanguine à long terme, nous avons défini qu'une faible activité mitochondriale apparaît comme une marque fiable pour identifier les CSHs fonctionnelles dans la moelle osseuse. En particulier, ce caractère peut être utilisé pour identifier et isoler les CSHs ayant la capacité de s'auto-renouveler à partir d'une population cultivée *in vitro*, souvent très hétérogène. De plus, nous avons réussi à moduler le comportement des CSHs en artificiellement altérant leur activité mitochondriale *in vitro*. Ensemble, ces données indiquent une relation de causalité importante entre l'activité mitochondriale et la décision du devenir des CSHs.

La dernière partie de cette étude se focalise sur l'élucidation des signaux impliqués dans la régulation d'un phénomène important de la biologie des CSHs, à savoir la division cellulaire asymétrique (DCA). Nous avons mis en place un paradigme expérimental unique qui combine les techniques d'imagerie des cellules vivantes et d'analyse d'expression génétique à l'échelle des cellules individuelles. Ceci a permis l'identification de gênes clés impliqués dans le métabolisme qui sont exprimés différemment entre les deux cellules filles, suggérant leur rôle indispensable dans la

DCA. Spécifiquement, les cellules maintenues *in vivo* présentent présentent un haut niveau d'asymétrie dans l'expression génique comparées à leur homologues générés *in vitro,* confirmant encore le rôle critique de la niche dans le control du comportement des CSHs dans la DCA.

Cette étude révèle de nouveaux médiateurs métaboliques contrôlant le choix du devenir des CSHs. Une meilleure compréhension dans la régulation des CSHs va contribuer à la mise en place des nouvelles stratégies pour conserver et amplifier les CSHs *in vitro* en vue de leur transplantation. De plus, étant donné que la majorité des cellules souches adultes se trouvent dans des microenvironnements métaboliques similaires, nous suggérons que ces résultats peuvent être utiles pour identifier et étudier les cellules souches dans d'autres tissus.

Mots clés : division cellulaire asymétrique (DCA), suivi des cellules, cellule souche hématopoïétique (CSH), niche *in vivo*, régulation métabolique, micro-environnement, activité mitochondriale, auto-renouvellement, expression génique de cellule individuelle, devenir de cellule souche, transplantation

सारांश

हिमेटोपोयटिक स्टेम कोशिकाओ (**हसक**) सभी रक्त कोशिकाओ के जीवन भर के उत्पादन के लिए उत्तरदायी हैं। यह विशेष गुण उन्हें विभिन्न हिमेटोलोजिकल कैंसर के इलाज के लिए और सेल आधारित चिकित्सा के लिए एक आदर्श उम्मीदवार बनाता है। क्लिनिक में उनका व्यापक उपयोग अक्सर दानदाताओ से प्राप्त कोशिकाओ की अपर्याप्त संख्या के आड़े आती है। परन्तु इनमें एक कमी है कि इन **हसक** को प्रयोगशाला में उत्पन नही किया जा सकता क्योकि प्रयोगशाला की परिस्थितियाँ इन **हसक** की अस्थि मज्जा की परिस्थितियो से अलग है। इसके अलावा अस्थि मज्जा में ऑक्सीजन की कमी होने के कारण **हसक** एक अलग metabolic पहचान प्राप्त कर लेते हैं। परन्तु यह metabolic पहचान कैसे **हसक** को विनियमित करती है कि वो और अधिक **हसक** बनाये या फिर और रक्त कोशिका बनाये (fate choice), अनेक दशको की खोज इस प्रक्रिया को समझ नही पायी है।

इस थीसिस का मुख्य उदेश्य है **हसक** का नई तरह के प्रयोगिक उपकरण के रुप में उपयोग करना जिनमें उनके metabolism को नियंत्रित करके ऊपर लिखी प्रक्रिया को बेहतर समझना। थीसिस के प्रथाम भाग में **हसक** के विभाजन पर नजर रखने का एक नया तरीका विकसित किया गया जो **हसक** के विभागो का प्रयोगशाला में अनुसरण कर सके। इस तरीके को हमने immunostaining के साथ जोड़ के यह प्रमाणित किया कि अगर **हसक** तेज़ी से विभाजित होते हैं तो उनका phenotype जल्दी नष्ट हो जता है।

थीसिस के द्वितीय भाग में हुमने पहली बार माइटोकॉन्ड्रियल metabolism और **हसक** की fate choice के बीच सम्बन्ध पाया। इस सम्बन्ध को स्थापित करने के लिए flow cytometry और रख्त बनाने की शमता का उपयोग किया, तथा कम माइटोकॉन्ड्रियल गतिविधि की **हसक** के विशवसनीय marker के रुप में पहचान हुई। साथ ही हमने यह पाया कि अगर माइटोकॉन्ड्रिया की गतिविधि बदली जये तो **हसक** का भाग्य (fate choice) कुछ हद तक बदला जा सकता है। परिणामतः माइटोकॉन्ड्रियल गतिविधि **हसक** के भाग्य का एक महत्वपूर्ण निर्धारक है।

थीसिस के अन्तिम भाग में हमने अस्थि मज्जा में तयार **हसक** के विभाजन के बाद उत्पन होने वाली कोशिकाओ को प्रयोगशाला में समझने का प्रयतन किया। Gene expression के माध्यम से हमने बारह ऐसे genes की पेहचन करी जो **हसक** के विभाजन के बाद उत्पन होने वाली कोशिकाओ में अलग स्तर पर व्यकत होती हैं। इन में से कयी genes glycolytic और माइटोकॉन्ड्रियल TCA cycle enzymes की हैं।

कुल मिलाकर इस metabolism और **हसक** fate choice के मध्य सम्बन्ध का ज्ञान अन्य स्टेम कोशिकाओ का भी अध्ययन कर सकते हैं।

कीवर्ड: हिमेटोपोयटिक स्टेम कोशिकाओ (हसक), अस्थि मज्जा, metabolic पहचान, कोशिका विभाजन, माइटोकॉन्ड्रियल गतिविधि, हसक fate choice

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Chapter 1
Introduction

Motivation

A hallmark of adult stem cells is their ability to maintain and to some extent regenerate a particular tissue throughout the life of the organism. This unique trait makes them ideal for cell-based therapies to treat many life-threatening diseases. The hematopoietic system, responsible for life-long blood production, has one of the best characterized adult stem cell populations. With more than 30,000 bone marrow transplantations being carried out every year in Europe alone, hematopoietic stem cells (HSCs) hold immense potential in the clinic (Passweg et al., 2014). Although the number of transplants being performed has gone up consistently over the past few decades (Tan et al., 2007), the widespread clinical use of HSCs is often limited by the amount of cells obtained from donors. Even though bone marrow and peripheral blood are the primary sources of HSCs isolated from donors today (Passweg et al., 2013), umbilical cord blood (UCB) has become an attractive source of HSCs in recent years, due to its widespread availability, non-invasive collection and easy long-term cryo preservation (Ballen et al., 2013). However, due to low cell numbers, UCB use has been restricted to pediatric patients; double cord blood transplantation did not show encouraging survival rates in adult patients (Ballen et al., 2013).

Expansion of HSCs *in vitro* therefore holds great potential and will allow overcoming limitations due to low cell number. However, despite several decades of research, a reliable clinically approved *in vitro* culture method does not exist for expanding HSCs without a concomitant loss of their multi-potential capacity (Walasek et al., 2012). Moreover, *in vitro* culture of purified HSCs results in an extremely heterogeneous population of cells, consisting primarily of progenitors, after few rounds of division. Additionally, post *in vitro* culture, the cell surface marker repertoire does not reliably identify functional HSCs, hindering their isolation from these heterogeneous populations (Dorrell et al., 2000).

The rapid loss of HSC multi-potency in culture is primarily due to our inability to fully recapitulate the complex HSC microenvironment, termed niche, in an *in vitro* culture setting. Indeed, the *in vivo* niche, instrumental in regulating HSC fate choices, presents a complex cocktail of chemical, physical and metabolic cues to ensure life long HSC maintenance and, therefore, blood production (Lutolf and Blau, 2009; Morrison and Spradling, 2008; Nakamura-Ishizu and Suda, 2013; Wang and Wagers, 2011; Wilson and Trumpp, 2006). Importantly, the hypoxic state of the niche exposes HSCs to an extreme metabolic environment that is believed to impose a unique metabolic identity as compared to their differentiated progeny (Ito and Suda, 2014; Suda et al., 2011; Zhang and Sadek, 2014).

Therefore, the maintenance and expansion of HSCs *in vitro* without genetic manipulation would constitute a major advance in bone marrow regenerative medicine. To successfully overcome this obstacle, a better understanding of the

mechanisms that control HSC fate and, in particular the choice of the cell to self-renew or differentiate is crucial.

Objectives and Overview

The overall goal of this thesis is to employ innovative experimental tools to explore the role of metabolism in regulating HSC fate choices. *Mus musculus* is used as an experimental model system, as it provides powerful tools for HSC isolation from the bone marrow and for performing *in vivo* functional assays to assess long-term HSC function.

Chapter 1 of this thesis summarizes the current knowledge of the hematopoietic system, focusing primarily on the HSC niche and its role in regulating HSC fate choices.

Chapter 2 describes the development of a cell-tracking assay to follow divisional dynamics of HSCs in various *in vitro* culture conditions. Combining HSC surface marker analyses with division tracking allowed precise estimation of progeny retaining HSC phenotype in different divisional states.

In **Chapter 3** of the thesis, the role of mitochondrial activity in determining HSC fate decisions is explored. Using flow cytometry analysis and long-term *in vivo* blood reconstitution assays, we establish low mitochondrial activity as a reliable marker to identify functional HSCs from the bone marrow and, from heterogeneous *in vitro* cultures. Intriguingly, we could alter HSC fate by artificially modulating mitochondrial activity, suggesting that the metabolic state is a key determinant of HSC fate choices.

Chapter 4 presents a novel experimental paradigm to analyze the disparate cell fates in paired HSC daughters. Live-cell imaging combined with single-cell gene expression analysis performed on paired daughter cells arising from *in vivo*-instructed HSCs led to the identification of a set of asymmetrically expressed genes. This set included some key metabolic genes involved in glycolysis and mitochondrial TCA cycle. Moreover, *in vivo*-instructed cells showed higher levels of asymmetric gene expression, suggesting execution of niche instructed asymmetric fate choice program.

Chapter 5 summarizes the work described in this thesis and discusses its relevance in our current understanding of the hematopoietic system.

Background

Hematopoietic stem cells

The bone marrow hematopoietic stem cells (HSCs) are crucial for lifelong production of all mature blood cells. They are characterized by their dual ability to simultaneously generate identical copies of themselves (self-renewal) and give rise to differentiated progeny (commitment) to replenish the blood system (Figure 1.1). Although the majority of HSCs are present in the quiescent cell cycle state (Cheshier et al., 1999; Wilson et al., 2008) the rapid turnover of blood production is ensured by highly proliferative transient progenitors downstream of HSCs that lack self-renewal ability yet possess full lineage differentiation potential (Morrison and Weissman, 1994). These multi potent progenitors (MPPs) produce mature blood cells via a multi-step commitment process by first giving rise to lineage restricted progenitors that further divide to produce only one mature cell type (Christensen and Weissman, 2001). Hematopoiesis occurs in anatomically distinct locations through mammalian development. Before birth HSCs are present in the fetal liver and the spleen, making them the major sites for hematopoiesis. Eventually, hematopoiesis shifts primarily to the bone marrow, where blood formation is maintained throughout postnatal life (Wang and Wagers, 2011).

Classically, adult HSCs were identified by their ability to efflux the Hoechst dye through membrane transport pumps and were shown to be enriched in the "side population" from the bone marrow (Goodell et al., 1996). Recent advances in the field have allowed for the use of cell surface markers for the identification and isolation of HSCs by flow cytometry. They were initially shown to be contained in the Lin- cKit+ Sca1+ (LKS) population from the bone marrow, which was identified by negative selection for markers for mature hematopoietic cell lineages (Lin-) and positive selection for the cKit and Sca1 cell surface markers (Okada et al., 1992). Further enrichment of the HSC population was achieved with the use of CD34 negativity (Osawa et al., 1996). However, long-term blood reconstitution assays demonstrated that only 30-50% of the LKS CD34- cells had stem potential (Osawa et al., 1996). Further purification of the HSC fraction could be achieved by the differential expression of the signaling lymphocyte activation molecule (SLAM) markers, CD150 and CD48 (Kiel et al., 2005). Combining these different strategies the most commonly used combination of markers to identify an extremely purified HSC population is LKS CD150+ CD48- CD34- (Wilson et al., 2007). Moreover, early progenitors in the hematopoietic hierarchy could be identified by up-regulation of CD34 and CD48, followed by down regulation of CD150 (Wilson et al., 2007).

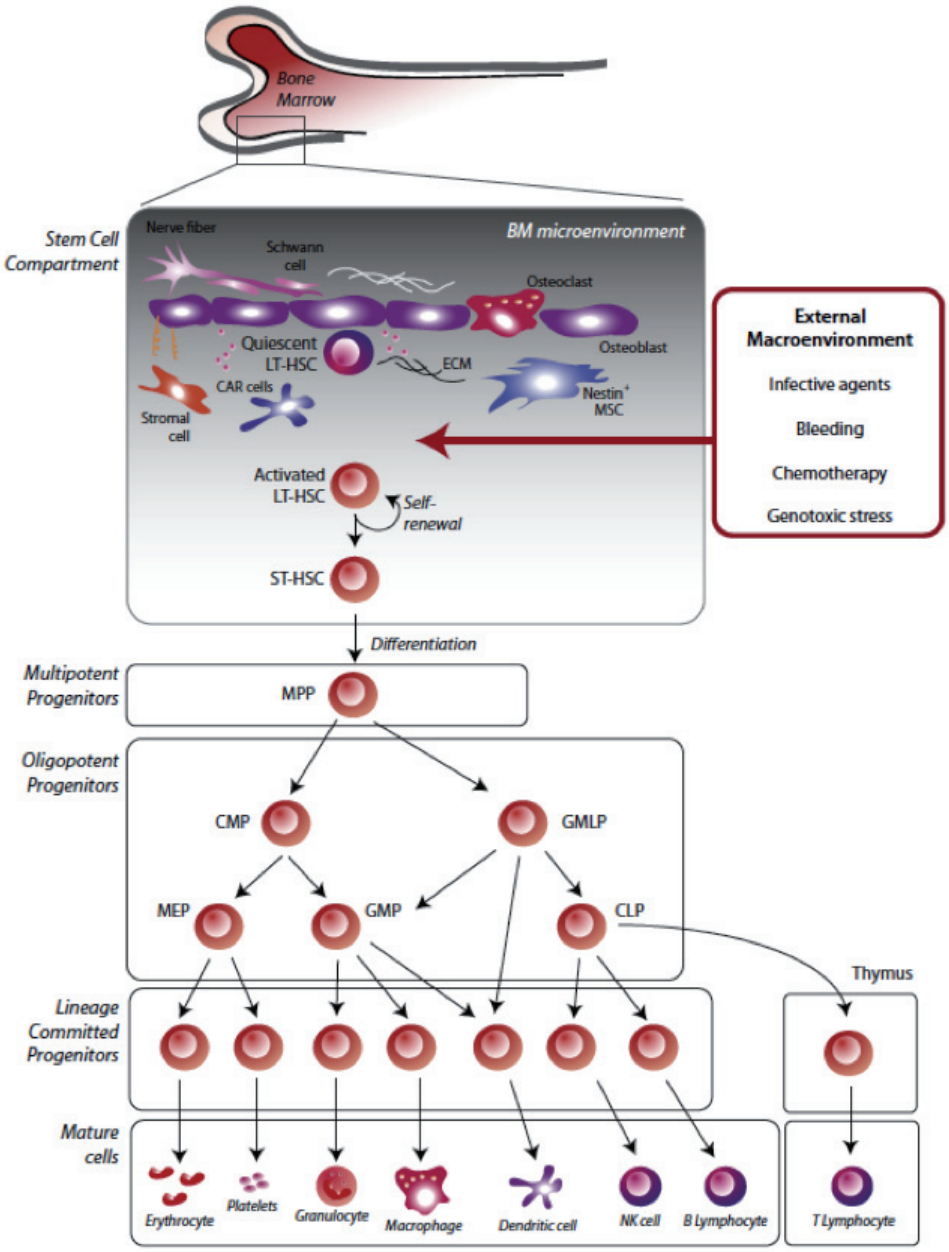

Figure 1.1: The hematopoietic hierarchy. HSCs at top of the hierarchy have a dual role of self-renewal and commitment, ensuring life long blood production. CAR:CXCL12-abundant reticular cells, ECM: Extracellular matrix, MSC: Mesenchymal stem cells, MPP: Multi potent progenitor, CMP: Common myeloid progenitor, GMLP: Granulocyte-macrophage-lymphoid progenitor, MEP: Megakaryocyte-erythrocyte progenitor, GMP: Granulocyte-macrophage progenitor, CLP: Common lymphoid progenitor. Image from (Rossi et al., 2012).

Hematopoietic stem cell niche

The concept of the HSC "Niche" was first proposed by Schofield, at that time it was believed that the HSCs associate with other cells in the bone marrow (BM) capable of determining their behavior (Schofield, 1978). Moreover, this association was hypothesized to be critical for the maintenance of HSC stemness. The first evidence of the existence of the niche came from investigation of two mutant mouse strains (W strain and Sl strain) that showed severe anemia and lack of mast cell production

(Bernstein et al., 1968; Geissler and Russell, 1983; Kitamura and Go, 1979; Kitamura et al., 1978). When normal BM cells were transplanted into W mutant mice, hematopoiesis was restored, whereas transplanting normal BM cells into Sl mutant mice did not result in normal hematopoiesis. When W mutant BM was transplanted in either WT or Sl mice it did not show normal hematopoiesis. However, when Sl mutant BM was transplanted in W mutant mice hematopoiesis was restored. The outcomes of these transplants could be explained by concluding that the W locus was essential for functional HSCs while the Sl locus was an essential environmental component for hematopoiesis but was not present on HSCs (Jarboe and Huff, 1989; Mayer and Green, 1968; Russell and Bernstein, 1968). The W gene was later found to be cKit (Nocka et al., 1989; Reith et al., 1990), an essential cell surface receptor present on all HSCs, while the Sl gene was identified as Stem cell factor (SCF), the ligand for cKit (Zsebo et al., 1990) expressed by the niche cells. The idea of the niche has evolved ever since; it now includes various cell types in distinct anatomical locations, secreted cytokines, soluble factors, oxygen concentration and many other physical parameters (Figure 1.2) (Lutolf and Blau, 2009).

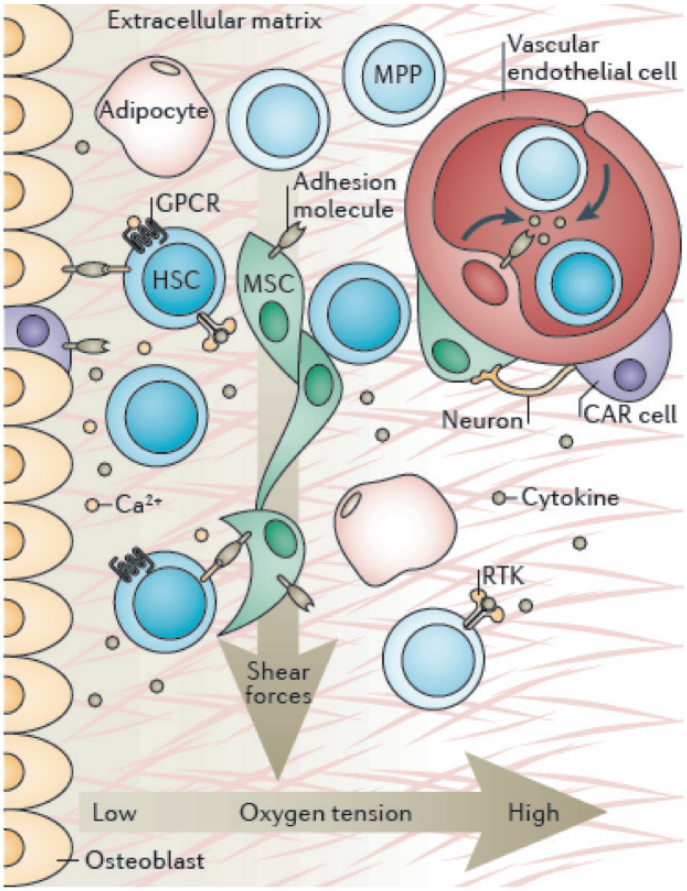

Figure 1.2: The hematopoietic stem cell niche. Various cellular as well as acellular components form the hematopoietic niche. Image from (Wang and Wagers, 2011).

The osteoblasts are one of the best-characterized niche cells. Interaction of the osteoblasts and HSCs at the interface of the bone and the marrow space forms the **endosteal niche** (Figure 1.3). Using transgenic mice it was shown that increase in osteoblast number resulted in an expanded HSC population (Calvi et al., 2003), whereas osteoblast ablation led to loss of the HSC pool (Visnjic et al., 2001; Visnjic et al., 2004). Moreover, *in vitro* studies demonstrated that long term reconstitution potential of HSCs could be maintained when co-cultured with osteoblasts (Chitteti et al., 2010; Nakamura et al., 2010; Taichman et al., 1996). Additionally, osteoblasts were shown to be the source of some key factors regulating HSC maintenance and survival; these include angiopoietin, N-cadherin, thrombopoietin, osteopontin, Wnt, and Notch (Arai et al., 2004; Fleming et al., 2008; Haug et al., 2008; Hosokawa et al., 2010a; Hosokawa et al., 2010b; Morrison and Spradling, 2008; Qian et al., 2007; Stier et al., 2005). Recent studies using high resolution *in vivo* imaging techniques have demonstrated close physical association of HSCs with osteoblasts (Kohler et al., 2009; Lo Celso et al., 2009; Takaku et al., 2010; Xie et al., 2009). Interestingly, progenitor cells were not seen in close proximity with the osteoblasts in these studies, suggesting that the interaction is specific to the HSC population.

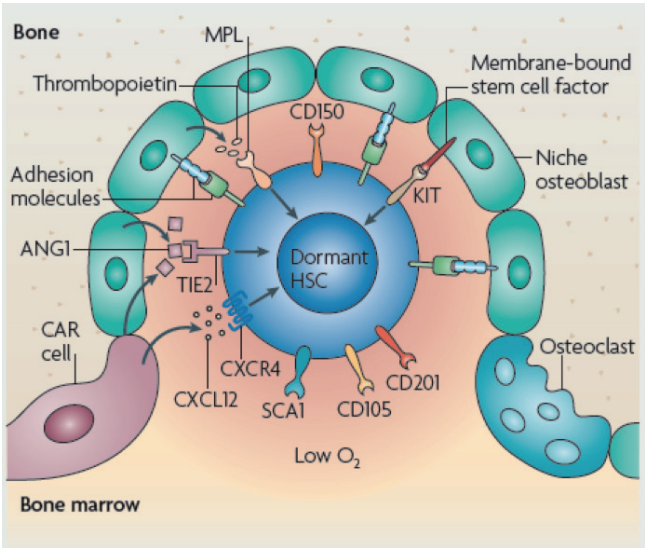

Figure 1.3: Interactions between HSCs and the niche cells. Image from (Trumpp et al., 2010).

In addition to the osteoblasts, HSCs were found in close association with the bone marrow sinusoids suggesting the existence of a **perivascular niche** (Kiel et al., 2005). Proximity to the vasculature is believed to act as a gateway for the HSCs transiting into or out of the bloodstream. Moreover, it might also be important in sensing systemic signals from the body and therefore regulate blood cell production. Various cell types in the perivascular space were shown to regulate HSC function; these include MSCs, CXCL12-adundant reticular (CAR) cells, endothelial cells and neural cells. Ablation of nestin positive MSCs resulted in a dramatic reduction of the HSC population (Mendez-Ferrer et al., 2010). Moreover, deletion of Cxcl12 expressing CAR cells resulted in 50% reduction in HSC numbers without any effect on the MPP

population (Omatsu et al., 2010). Interestingly, HSC population from CAR depleted mice was seen to be more quiescent (Omatsu et al., 2010). Additionally, conditional knock out of SCF in endothelial cells was shown to result in reduction in HSC numbers (Ding et al., 2012). However, all these cell types were identified using non-overlapping markers and it is possible that some of these marker systems identify overlapping populations. Intriguingly, components of the nervous system have also been implicated as an important part of the niche, non-myelinating Schwann cells were shown to sustain the HSC pool through TGF-β signaling (Yamazaki et al., 2011).

Apart from various cell types one of the most important characteristic of the niche is that it is extremely hypoxic. Staining with pimonidazole, a chemical marker for hypoxia revealed that HSCs are indeed hypoxic (Parmar et al., 2007). As a result, hypoxia inducible factor (HIF1α) was found to be highly upregulated in HSCs (Takubo et al., 2010). Recent work using live *in vivo* imaging of oxygen concentration using phosphorescence lifetime sensing nanoprobes revealed that the oxygen levels in the BM were significantly lower as compared to the cortical bone (Spencer et al., 2014). Together the cellular and acellular components of the niche are believed to regulate the HSC fate via a complex interplay of different signaling pathways (Lutolf and Blau, 2009; Morrison and Spradling, 2008; Nakamura-Ishizu and Suda, 2013; Suda et al., 2011; Wang and Wagers, 2011; Wilson and Trumpp, 2006).

Hematopoietic stem cell fate choices

Maintenance of the HSC pool and simultaneous production of committed progeny is essential for lifelong blood production. A fine balance of different HSC fate choices is crucial to successfully carry out both these objectives (Figure 1.4). Moreover, when the organism is under hematopoietic stress such as extreme blood loss or infection, this balance needs to be carefully adjusted to achieve the same outcomes. Therefore, the fate that an HSC chooses (or is instructed) to undertake must be tightly regulated.

Symmetric expansion divisions were shown to be most relevant during embryonic or fetal development in multiple systems. Since these developmental stages are associated with massive growth and increase in cell number, it is quite intuitive that the stem cell pool would also expand. The mouse hematopoietic stem cells were found to double every 24 hours during mid gestation (Morrison et al., 1995), suggesting that atleast some of the HSCs underwent symmetric expansion divisions. Moreover, imaging studies during embryonic development of mouse cerebral cortex and the epidermis, showed a rapid expansion of undifferentiated pool of cells before the differentiated progeny arises (Huttner et al., 2005; Lechler and Fuchs, 2005; Noctor et al., 2004). However, in adults, symmetric expansion divisions were observed to restore stem cell pools depleted after injury. In the hematopoietic system, granulocyte-colony stimulating factor (G-CSF) mediated mobilization of HSCs from

the bone marrow led to rapid proliferation and expansion of the HSC pool (Bodine et al., 1996; Morrison et al., 1997; Wright et al., 2001).

Asymmetric cell division results in the generation of two daughters with different stem potential (self-renewal). Under homeostasis, this fate choice presents an attractive strategy to maintain the HSC pool and produce differentiated progeny at the same time. Classical studies in *Drosophila* and *C. Elegans* have implicated both intrinsic (mediated by PAR family of proteins) (Doe and Bowerman, 2001; Strome and Wood, 1983) and extrinsic mechanisms (Yamashita et al., 2005) in mediating asymmetric cell division. In the hematopoietic system, pair daughter cell analysis revealed different colony forming potential (Takano et al., 2004) and *in vivo* reconstitution ability (Ema et al., 2000; Yamamoto et al., 2013) suggesting HSCs carry out asymmetric divisions. However, compelling evidence and mechanistic understanding of asymmetric division in adult HSCs is currently lacking.

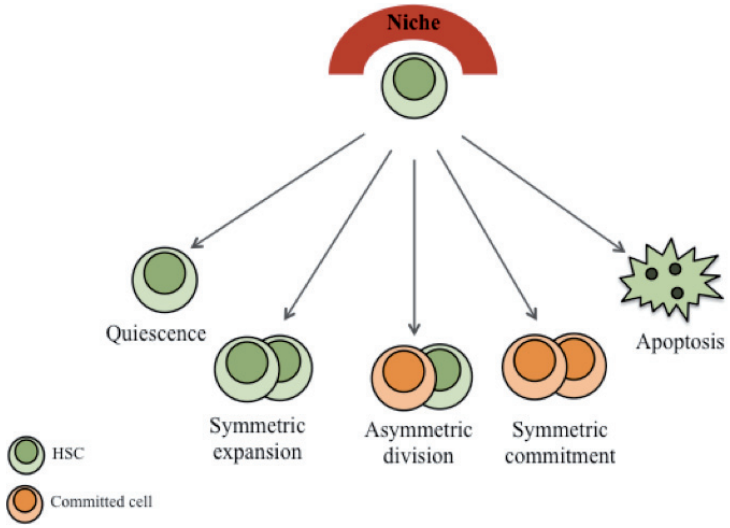

Figure 1.4: Hematopoietic stem cell fate choices. Different fate choices that an HSC can undertake. Fate choices are finely regulated by extrinsic signals from the niche and intracellular mechanisms in HSCs.

Despite immense self-renewing potential, most adult HSCs are found in the **quiescent** state (Cheshier et al., 1999; Wilson et al., 2008). The maintenance of quiescent fate is believed to be critical for stem cell maintenance and prevent proliferation-associated exhaustion (Orford and Scadden, 2008). Moreover, long-term BrdU incorporation experiments identified a highly quiescent sub-fraction of HSCs that divided only five times during the entire life of the mouse (Wilson et al., 2008) suggesting that these cells spend a majority of their time in a quiescent state. **Symmetric commitment divisions** if carried out indefinitely result in the exhaustion of the stem cell pool. Recent work has shown that chronic IFNα mediated activation of HSCs result in competitive disadvantage in long-term repopulation assays (Essers et al., 2009). Similar effects were reported upon IFNγ treatment (de Bruin et al., 2014). But this fate choice is believed to be less relevant in a normal homeostatic environment.

Regulation of hematopoietic stem cell fate

Tight regulation of HSC fate choice is important for life long maintenance of the blood system. Both, extrinsic cues from the niche and intrinsic HSC mechanisms are believed to regulate this process. Direct cell-cell adhesion and secreted cytokine mediated signals from the niche were shown to be critical for HSC maintenance (Figure 1.3). Stem cell factor (SCF), primarily expressed by osteoblasts (both as membrane bound form and secreted form), is one of the most important niche factors mediating HSC self-renewal (Miyazawa et al., 1995). Moreover, thrombopoietin (TPO) produced by niche cells, signals via the c-Mpl receptor on HSCs to promote self-renewal (Kimura et al., 1998; Solar et al., 1998). Exposure to various interleukins, such as IL-3, IL-6, and IL-11, results in rapid HSC proliferation, but leads to loss of long-term reconstitution potential (Peters et al., 1996). Moreover, osteopontin (OPN), secreted by osteoblasts has a negative effect on HSC number. OPN deficient mice were observed to have a two-fold increase in HSC number (Nilsson et al., 2005; Stier et al., 2005). This effect was shown to be primarily due to active maintenance of HSC quiescence by OPN (Nilsson et al., 2005).

As mentioned above, maintenance of HSC quiescence is believed to prevent proliferation based stem cell exhaustion (Orford and Scadden, 2008). Genetic studies on key cell cycle genes supported this notion. Deletion of Cdk inhibitors, such as p21, p27, and p57, lead to increased cell cycle entry and eventually HSC exhaustion (Matsumoto et al., 2011; Yu et al., 2006; Zou et al., 2011). Furthermore, conditional deletion of Pten, a tumour suppressor gene, led to increased proliferation and a concomitant loss of long-term engraftment potential (Zhang et al., 2006). Ang-1/Tie2 signaling was observed to be a key regulator of HSC quiescence. Ang-1 secreted by osteoblasts binds its receptor Tie2 on HSCs. Overexpression of Ang-1 resulted in increased HSC quiescence (Arai et al., 2004). Tie2 mediated signaling up regulated N-cadherin expression in HSCs, suggesting an increase in their anchoring in the endosteal niche by homotypic N-cadherin interaction with osteoblasts (Arai et al., 2004).

Additionally, many classical developmental signaling pathways have been shown to be relevant in HSC fate choice. Inhibition of notch signaling led to differentiation of HSCs *in vitro* and depletion of the HSC pool *in vivo* (Duncan et al., 2005). *In vitro* treatment with Wnt3a lead to increase in self-renewal capacity of HSCs, suggesting Wnt signaling as a promoter of HSC stemness (Fleming et al., 2008; Reya et al., 2003; Willert et al., 2003). Strikingly, lentiviral transduction of HoxB4 or *in vitro* treatment with HoxB4 protein was shown to result in the expansion of the HSC pool (Antonchuk et al., 2002; Krosl et al., 2003).

In addition to the cellular factors, the hypoxic environment of the niche is believed to play a key role in determining HSC fate choices by modulating various metabolic pathways (Ito and Suda, 2014; Suda et al., 2011) (Figure 1.5). Hypoxic culture

conditions induce quiescence (Hermitte et al., 2006; Shima et al., 2010) and result in increased reconstitution potential of HSCs (Cipolleschi et al., 1993; Danet et al., 2003). Hypoxia inducible factor-1α (HIF-1a) was shown to be the master regulator controlling various intracellular pathways in HSCs in response to the low oxygen microenvironment (Takubo et al., 2010). HIF-1a deletion led to increased cell cycle entry and loss of long-term engraftment potential (Takubo et al., 2010). Furthermore, HIF-1a increases the glycolytic flux in HSCs by activating glucose transporters and different glycolytic enzymes such as HK, PFK, LDHA (Iyer et al., 1998; Takubo et al., 2013). Moreover, it was shown to actively prevent oxidative phosphorylation (OXPHOS) through a pyruvate dehydrogenase kinase (PDK) mechanism that inhibits pyruvate dehydrogenase (PDH) responsible for the conversion of pyruvate to acetyl-CoA (Takubo et al., 2013)

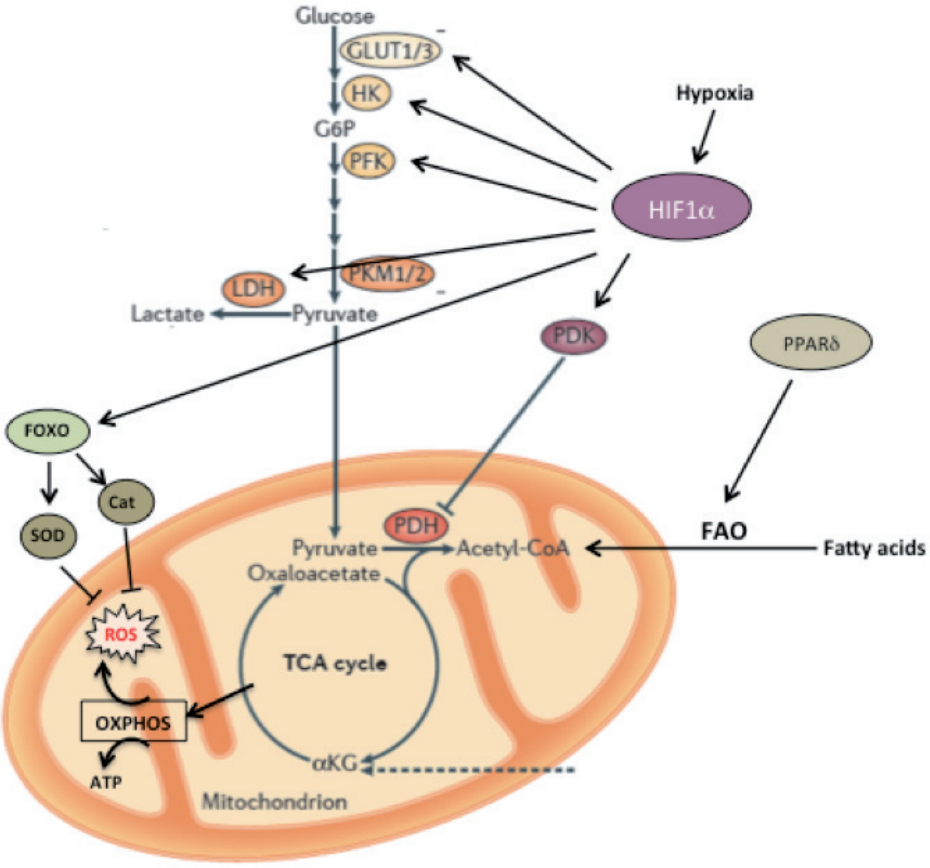

Figure 1.5: Metabolic regulation of Hematopoietic stem cell fate. Modified from (Ito and Suda, 2014)

Reactive oxygen species (ROS) primarily generated through mitochondrial oxidative phosphorylation were shown to have deleterious effects on HSC maintenance (Ito et al., 2004; Ito et al., 2006). To avoid ROS inflicted damage FOXO proteins activate expression of antioxidants such as super oxide dismutase and catalase, and therefore maintain HSC stemness (Jang and Sharkis, 2007; Miyamoto et al., 2007; Tothova et al., 2007). Several other reports propose a link between mitochondrial metabolism and

HSC fate. Inactivation of PTEN-like mitochondrial phosphatase (PTPMT1) was shown to block HSC differentiation divisions and subsequently lead to hematopoietic failure (Yu et al., 2013). Knocking down PGC-1a and PGC-1b, two key regulators of mitochondrial function and biogenesis, resulted in hematopoietic defects (Basu et al., 2013; Sahin et al., 2011). Moreover, the tumor suppressor and glucose sensor Lkb1 was shown to be crucial for HSC maintenance via a not yet fully understood mechanism involving mitochondrial biogenesis and function (Gan et al., 2010; Gurumurthy et al., 2010; Nakada et al., 2010). Additionally, recent work in the field has implicated the role of fatty acid oxidation in HSC fate decision. Inhibition of PPARδ or mitochondria fatty acid oxidation resulted in loss of the HSC pool by increased symmetric commitment divisions (Ito et al., 2012).

Collectively, these data show that a complex network of signals and constant crosstalk between the niche and the HSCs is necessary for the regulation of HSC fate decisions in the bone marrow. Further understanding of this intricate regulatory process will enable development of new culture systems for better maintenance of HSCs *in vitro*, and could potentially allow identification of markers to fish out functional HSCs from heterogeneous *in vitro* cultures.

References

Antonchuk, J., Sauvageau, G., and Humphries, R.K. (2002). HOXB4-induced expansion of adult hematopoietic stem cells ex vivo. Cell *109*, 39-45.

Arai, F., Hirao, A., Ohmura, M., Sato, H., Matsuoka, S., Takubo, K., Ito, K., Koh, G.Y., and Suda, T. (2004). Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche. Cell *118*, 149-161.

Ballen, K.K., Gluckman, E., and Broxmeyer, H.E. (2013). Umbilical cord blood transplantation: the first 25 years and beyond. Blood *122*, 491-498.

Basu, S., Broxmeyer, H.E., and Hangoc, G. (2013). PGC-1alpha Mediated Mitochondrial Biogenesis is Important for Hematopoietic Recovery in Response to Stress. Stem Cells Dev.

Bernstein, S.E., Russell, E.S., and Keighley, G. (1968). TWO HEREDITARY MOUSE ANEMIAS (Sl/Sld and W/Wv) DEFICIENT IN RESPONSE TO ERYTHROPOIETIN*. Annals of the New York Academy of Sciences *149*, 475-485.

Bodine, D.M., Seidel, N.E., and Orlic, D. (1996). Bone marrow collected 14 days after in vivo administration of granulocyte colony-stimulating factor and stem cell factor to mice has 10-fold more repopulating ability than untreated bone marrow. Blood *88*, 89-97.

Calvi, L.M., Adams, G.B., Weibrecht, K.W., Weber, J.M., Olson, D.P., Knight, M.C., Martin, R.P., Schipani, E., Divieti, P., Bringhurst, F.R.*, et al.* (2003). Osteoblastic cells regulate the haematopoietic stem cell niche. Nature *425*, 841-846.

Cheshier, S.H., Morrison, S.J., Liao, X., and Weissman, I.L. (1999). In vivo proliferation and cell cycle kinetics of long-term self-renewing hematopoietic stem cells. Proc Natl Acad Sci U S A *96*, 3120-3125.

Chitteti, B.R., Cheng, Y.H., Poteat, B., Rodriguez-Rodriguez, S., Goebel, W.S., Carlesso, N., Kacena, M.A., and Srour, E.F. (2010). Impact of interactions of cellular components of the bone marrow microenvironment on hematopoietic stem and progenitor cell function. Blood *115*, 3239-3248.

Christensen, J.L., and Weissman, I.L. (2001). Flk-2 is a marker in hematopoietic stem cell differentiation: a simple method to isolate long-term stem cells. Proc Natl Acad Sci U S A *98*, 14541-14546.

Cipolleschi, M.G., Dello Sbarba, P., and Olivotto, M. (1993). The role of hypoxia in the maintenance of hematopoietic stem cells. Blood *82*, 2031-2037.

Danet, G.H., Pan, Y., Luongo, J.L., Bonnet, D.A., and Simon, M.C. (2003). Expansion of human SCID-repopulating cells under hypoxic conditions. J Clin Invest *112*, 126-135.

de Bruin, A.M., Voermans, C., and Nolte, M.A. (2014). Impact of interferon-gamma on hematopoiesis. Blood *124*, 2479-2486.

Ding, L., Saunders, T.L., Enikolopov, G., and Morrison, S.J. (2012). Endothelial and perivascular cells maintain haematopoietic stem cells. Nature *481*, 457-462.

Doe, C.Q., and Bowerman, B. (2001). Asymmetric cell division: fly neuroblast meets worm zygote. Current opinion in cell biology *13*, 68-75.

Dorrell, C., Gan, O.I., Pereira, D.S., Hawley, R.G., and Dick, J.E. (2000). Expansion of human cord blood CD34(+)CD38(-) cells in ex vivo culture during retroviral transduction without a corresponding increase in SCID repopulating cell (SRC) frequency: dissociation of SRC phenotype and function. Blood *95*, 102-110.

Duncan, A.W., Rattis, F.M., DiMascio, L.N., Congdon, K.L., Pazianos, G., Zhao, C., Yoon, K., Cook, J.M., Willert, K., Gaiano, N*., et al.* (2005). Integration of Notch and Wnt signaling in hematopoietic stem cell maintenance. Nat Immunol *6*, 314-322.

Ema, H., Takano, H., Sudo, K., and Nakauchi, H. (2000). In vitro self-renewal division of hematopoietic stem cells. J Exp Med *192*, 1281-1288.

Essers, M.A., Offner, S., Blanco-Bose, W.E., Waibler, Z., Kalinke, U., Duchosal, M.A., and Trumpp, A. (2009). IFNalpha activates dormant haematopoietic stem cells in vivo. Nature *458*, 904-908.

Fleming, H.E., Janzen, V., Lo Celso, C., Guo, J., Leahy, K.M., Kronenberg, H.M., and Scadden, D.T. (2008). Wnt signaling in the niche enforces hematopoietic stem cell quiescence and is necessary to preserve self-renewal in vivo. Cell Stem Cell *2*, 274-283.

Gan, B., Hu, J., Jiang, S., Liu, Y., Sahin, E., Zhuang, L., Fletcher-Sananikone, E., Colla, S., Wang, Y.A., Chin, L.*, et al.* (2010). Lkb1 regulates quiescence and metabolic homeostasis of haematopoietic stem cells. Nature *468*, 701-704.

Geissler, E.N., and Russell, E.S. (1983). Analysis of the hematopoietic effects of new dominant spotting (W) mutations of the mouse. II. Effects on mast cell development. Exp Hematol *11*, 461-466.

Goodell, M.A., Brose, K., Paradis, G., Conner, A.S., and Mulligan, R.C. (1996). Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. J Exp Med *183*, 1797-1806.

Gurumurthy, S., Xie, S.Z., Alagesan, B., Kim, J., Yusuf, R.Z., Saez, B., Tzatsos, A., Ozsolak, F., Milos, P., Ferrari, F.*, et al.* (2010). The Lkb1 metabolic sensor maintains haematopoietic stem cell survival. Nature *468*, 659-663.

Haug, J.S., He, X.C., Grindley, J.C., Wunderlich, J.P., Gaudenz, K., Ross, J.T., Paulson, A., Wagner, K.P., Xie, Y., Zhu, R.*, et al.* (2008). N-cadherin expression level distinguishes reserved versus primed states of hematopoietic stem cells. Cell Stem Cell *2*, 367-379.

Hermitte, F., Brunet de la Grange, P., Belloc, F., Praloran, V., and Ivanovic, Z. (2006). Very low O2 concentration (0.1%) favors G0 return of dividing CD34+ cells. Stem Cells *24*, 65-73.

Hosokawa, K., Arai, F., Yoshihara, H., Iwasaki, H., Hembree, M., Yin, T., Nakamura, Y., Gomei, Y., Takubo, K., Shiama, H.*, et al.* (2010a). Cadherin-based adhesion is a potential target for niche manipulation to protect hematopoietic stem cells in adult bone marrow. Cell Stem Cell *6*, 194-198.

Hosokawa, K., Arai, F., Yoshihara, H., Iwasaki, H., Nakamura, Y., Gomei, Y., and Suda, T. (2010b). Knockdown of N-cadherin suppresses the long-term engraftment of hematopoietic stem cells. Blood *116*, 554-563.

Huttner, H.B., Lohmann, G., and von Cramon, D.Y. (2005). Magnetic resonance imaging of the human frontal cortex reveals differential anterior-posterior variability of sulcal basins. Neuroimage *25*, 646-651.

Ito, K., Carracedo, A., Weiss, D., Arai, F., Ala, U., Avigan, D.E., Schafer, Z.T., Evans, R.M., Suda, T., Lee, C.H.*, et al.* (2012). A PML-PPAR-delta pathway for fatty acid oxidation regulates hematopoietic stem cell maintenance. Nat Med.

Ito, K., Hirao, A., Arai, F., Matsuoka, S., Takubo, K., Hamaguchi, I., Nomiyama, K., Hosokawa, K., Sakurada, K., Nakagata, N.*, et al.* (2004). Regulation of oxidative stress by ATM is required for self-renewal of haematopoietic stem cells. Nature *431*, 997-1002.

Ito, K., Hirao, A., Arai, F., Takubo, K., Matsuoka, S., Miyamoto, K., Ohmura, M., Naka, K., Hosokawa, K., Ikeda, Y.*, et al.* (2006). Reactive oxygen species act through p38 MAPK to limit the lifespan of hematopoietic stem cells. Nat Med *12*, 446-451.

Ito, K., and Suda, T. (2014). Metabolic requirements for the maintenance of self-renewing stem cells. Nat Rev Mol Cell Biol *15*, 243-256.

Iyer, N.V., Kotch, L.E., Agani, F., Leung, S.W., Laughner, E., Wenger, R.H., Gassmann, M., Gearhart, J.D., Lawler, A.M., Yu, A.Y.*, et al.* (1998). Cellular and developmental control of O2 homeostasis by hypoxia-inducible factor 1 alpha. Genes Dev *12*, 149-162.

Jang, Y.Y., and Sharkis, S.J. (2007). A low level of reactive oxygen species selects for primitive hematopoietic stem cells that may reside in the low-oxygenic niche. Blood *110*, 3056-3063.

Jarboe, D.L., and Huff, T.F. (1989). The mast cell-committed progenitor. II. W/Wv mice do not make mast cell-committed progenitors and S1/S1d fibroblasts do not support development of normal mast cell-committed progenitors. J Immunol *142*, 2418-2423.

Kiel, M.J., Yilmaz, O.H., Iwashita, T., Yilmaz, O.H., Terhorst, C., and Morrison, S.J. (2005). SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. Cell *121*, 1109-1121.

Kimura, S., Roberts, A.W., Metcalf, D., and Alexander, W.S. (1998). Hematopoietic stem cell deficiencies in mice lacking c-Mpl, the receptor for thrombopoietin. Proc Natl Acad Sci U S A *95*, 1195-1200.

Kitamura, Y., and Go, S. (1979). Decreased production of mast cells in S1/S1d anemic mice. Blood *53*, 492-497.

Kitamura, Y., Go, S., and Hatanaka, K. (1978). Decrease of mast cells in W/Wv mice and their increase by bone marrow transplantation. Blood *52*, 447-452.

Kohler, A., Schmithorst, V., Filippi, M.D., Ryan, M.A., Daria, D., Gunzer, M., and Geiger, H. (2009). Altered cellular dynamics and endosteal location of aged early hematopoietic progenitor cells revealed by time-lapse intravital imaging in long bones. Blood *114*, 290-298.

Krosl, J., Austin, P., Beslu, N., Kroon, E., Humphries, R.K., and Sauvageau, G. (2003). In vitro expansion of hematopoietic stem cells by recombinant TAT-HOXB4 protein. Nat Med *9*, 1428-1432.

Lechler, T., and Fuchs, E. (2005). Asymmetric cell divisions promote stratification and differentiation of mammalian skin. Nature *437*, 275-280.

Lo Celso, C., Fleming, H.E., Wu, J.W., Zhao, C.X., Miake-Lye, S., Fujisaki, J., Cote, D., Rowe, D.W., Lin, C.P., and Scadden, D.T. (2009). Live-animal tracking of individual haematopoietic stem/progenitor cells in their niche. Nature *457*, 92-96.

Lutolf, M.P., and Blau, H.M. (2009). Artificial stem cell niches. Adv Mater *21*, 3255-3268.

Matsumoto, A., Takeishi, S., Kanie, T., Susaki, E., Onoyama, I., Tateishi, Y., Nakayama, K., and Nakayama, K.I. (2011). p57 is required for quiescence and maintenance of adult hematopoietic stem cells. Cell Stem Cell *9*, 262-271.

Mayer, T.C., and Green, M.C. (1968). An experimental analysis of the pigment defect caused by mutations at the W and S1 loci in mice. Dev Biol *18*, 62-75.

Mendez-Ferrer, S., Michurina, T.V., Ferraro, F., Mazloom, A.R., Macarthur, B.D., Lira, S.A., Scadden, D.T., Ma'ayan, A., Enikolopov, G.N., and Frenette, P.S. (2010). Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. Nature *466*, 829-834.

Miyamoto, K., Araki, K.Y., Naka, K., Arai, F., Takubo, K., Yamazaki, S., Matsuoka, S., Miyamoto, T., Ito, K., Ohmura, M.*, et al.* (2007). Foxo3a is essential for maintenance of the hematopoietic stem cell pool. Cell Stem Cell *1*, 101-112.

Miyazawa, K., Williams, D.A., Gotoh, A., Nishimaki, J., Broxmeyer, H.E., and Toyama, K. (1995). Membrane-bound Steel factor induces more persistent tyrosine kinase activation and longer life span of c-kit gene-encoded protein than its soluble form. Blood *85*, 641-649.

Morrison, S.J., Hemmati, H.D., Wandycz, A.M., and Weissman, I.L. (1995). The purification and characterization of fetal liver hematopoietic stem cells. Proc Natl Acad Sci U S A *92*, 10302-10306.

Morrison, S.J., and Spradling, A.C. (2008). Stem cells and niches: mechanisms that promote stem cell maintenance throughout life. Cell *132*, 598-611.

Morrison, S.J., and Weissman, I.L. (1994). The long-term repopulating subset of hematopoietic stem cells is deterministic and isolatable by phenotype. Immunity *1*, 661-673.

Morrison, S.J., Wright, D.E., and Weissman, I.L. (1997). Cyclophosphamide/granulocyte colony-stimulating factor induces hematopoietic stem cells to proliferate prior to mobilization. Proc Natl Acad Sci U S A *94*, 1908-1913.

Nakada, D., Saunders, T.L., and Morrison, S.J. (2010). Lkb1 regulates cell cycle and energy metabolism in haematopoietic stem cells. Nature *468*, 653-658.

Nakamura, Y., Arai, F., Iwasaki, H., Hosokawa, K., Kobayashi, I., Gomei, Y., Matsumoto, Y., Yoshihara, H., and Suda, T. (2010). Isolation and characterization of endosteal niche cell populations that regulate hematopoietic stem cells. Blood *116*, 1422-1432.

Nakamura-Ishizu, A., and Suda, T. (2013). Hematopoietic stem cell niche: an interplay among a repertoire of multiple functional niches. Biochim Biophys Acta *1830*, 2404-2409.

Nilsson, S.K., Johnston, H.M., Whitty, G.A., Williams, B., Webb, R.J., Denhardt, D.T., Bertoncello, I., Bendall, L.J., Simmons, P.J., and Haylock, D.N. (2005). Osteopontin, a key component of the hematopoietic stem cell niche and regulator of primitive hematopoietic progenitor cells. Blood *106*, 1232-1239.

Nocka, K., Majumder, S., Chabot, B., Ray, P., Cervone, M., Bernstein, A., and Besmer, P. (1989). Expression of c-kit gene products in known cellular targets of W mutations in normal and W mutant mice--evidence for an impaired c-kit kinase in mutant mice. Genes Dev *3*, 816-826.

Noctor, S.C., Martinez-Cerdeno, V., Ivic, L., and Kriegstein, A.R. (2004). Cortical neurons arise in symmetric and asymmetric division zones and migrate through specific phases. Nat Neurosci *7*, 136-144.

Okada, S., Nakauchi, H., Nagayoshi, K., Nishikawa, S., Miura, Y., and Suda, T. (1992). In vivo and in vitro stem cell function of c-kit- and Sca-1-positive murine hematopoietic cells. Blood *80*, 3044-3050.

Omatsu, Y., Sugiyama, T., Kohara, H., Kondoh, G., Fujii, N., Kohno, K., and Nagasawa, T. (2010). The essential functions of adipo-osteogenic progenitors as the hematopoietic stem and progenitor cell niche. Immunity *33*, 387-399.

Orford, K.W., and Scadden, D.T. (2008). Deconstructing stem cell self-renewal: genetic insights into cell-cycle regulation. Nat Rev Genet *9*, 115-128.

Osawa, M., Hanada, K., Hamada, H., and Nakauchi, H. (1996). Long-term lymphohematopoietic reconstitution by a single CD34-low/negative hematopoietic stem cell. Science *273*, 242-245.

Parmar, K., Mauch, P., Vergilio, J.A., Sackstein, R., and Down, J.D. (2007). Distribution of hematopoietic stem cells in the bone marrow according to regional hypoxia. Proc Natl Acad Sci U S A *104*, 5431-5436.

Passweg, J.R., Baldomero, H., Bregni, M., Cesaro, S., Dreger, P., Duarte, R.F., Falkenburg, J.H., Kroger, N., Farge-Bancel, D., Gaspar, H.B.*, et al.* (2013). Hematopoietic SCT in Europe: data and trends in 2011. Bone Marrow Transplant *48*, 1161-1167.

Passweg, J.R., Baldomero, H., Peters, C., Gaspar, H.B., Cesaro, S., Dreger, P., Duarte, R.F., Falkenburg, J.H., Farge-Bancel, D., Gennery, A.*, et al.* (2014). Hematopoietic SCT in Europe: data and trends in 2012 with special consideration of pediatric transplantation. Bone Marrow Transplant *49*, 744-750.

Peters, S.O., Kittler, E.L., Ramshaw, H.S., and Quesenberry, P.J. (1996). Ex vivo expansion of murine marrow cells with interleukin-3 (IL-3), IL-6, IL-11, and stem cell factor leads to impaired engraftment in irradiated hosts. Blood *87*, 30-37.

Qian, H., Buza-Vidas, N., Hyland, C.D., Jensen, C.T., Antonchuk, J., Mansson, R., Thoren, L.A., Ekblom, M., Alexander, W.S., and Jacobsen, S.E. (2007). Critical role of thrombopoietin in maintaining adult quiescent hematopoietic stem cells. Cell Stem Cell *1*, 671-684.

Reith, A.D., Rottapel, R., Giddens, E., Brady, C., Forrester, L., and Bernstein, A. (1990). W mutant mice with mild or severe developmental defects contain distinct point mutations in the kinase domain of the c-kit receptor. Genes Dev *4*, 390-400.

Reya, T., Duncan, A.W., Ailles, L., Domen, J., Scherer, D.C., Willert, K., Hintz, L., Nusse, R., and Weissman, I.L. (2003). A role for Wnt signalling in self-renewal of haematopoietic stem cells. Nature *423*, 409-414.

Rossi, L., Lin, K.K., Boles, N.C., Yang, L., King, K.Y., Jeong, M., Mayle, A., and Goodell, M.A. (2012). Less is more: unveiling the functional core of hematopoietic stem cells through knockout mice. Cell Stem Cell *11*, 302-317.

Russell, E.S., and Bernstein, S.E. (1968). Proof of whole-cell implant in therapy of W-series anemia. Arch Biochem Biophys *125*, 594-597.

Sahin, E., Colla, S., Liesa, M., Moslehi, J., Muller, F.L., Guo, M., Cooper, M., Kotton, D., Fabian, A.J., Walkey, C.*, et al.* (2011). Telomere dysfunction induces metabolic and mitochondrial compromise (vol 470, pg 359, 2011). Nature *475*.

Schofield, R. (1978). The relationship between the spleen colony-forming cell and the haemopoietic stem cell. Blood Cells *4*, 7-25.

Shima, H., Takubo, K., Tago, N., Iwasaki, H., Arai, F., Takahashi, T., and Suda, T. (2010). Acquisition of G(0) state by CD34-positive cord blood cells after bone marrow transplantation. Exp Hematol *38*, 1231-1240.

Solar, G.P., Kerr, W.G., Zeigler, F.C., Hess, D., Donahue, C., de Sauvage, F.J., and Eaton, D.L. (1998). Role of c-mpl in early hematopoiesis. Blood *92*, 4-10.

Spencer, J.A., Ferraro, F., Roussakis, E., Klein, A., Wu, J., Runnels, J.M., Zaher, W., Mortensen, L.J., Alt, C., Turcotte, R.*, et al.* (2014). Direct measurement of local oxygen concentration in the bone marrow of live animals. Nature *508*, 269-273.

Stier, S., Ko, Y., Forkert, R., Lutz, C., Neuhaus, T., Grunewald, E., Cheng, T., Dombkowski, D., Calvi, L.M., Rittling, S.R.*, et al.* (2005). Osteopontin is a hematopoietic stem cell niche component that negatively regulates stem cell pool size. J Exp Med *201*, 1781-1791.

Strome, S., and Wood, W.B. (1983). Generation of asymmetry and segregation of germ-line granules in early C. elegans embryos. Cell *35*, 15-25.

Suda, T., Takubo, K., and Semenza, G.L. (2011). Metabolic regulation of hematopoietic stem cells in the hypoxic niche. Cell Stem Cell *9*, 298-310.

Taichman, R.S., Reilly, M.J., and Emerson, S.G. (1996). Human osteoblasts support human hematopoietic progenitor cells in vitro bone marrow cultures. Blood *87*, 518-524.

Takaku, T., Malide, D., Chen, J., Calado, R.T., Kajigaya, S., and Young, N.S. (2010). Hematopoiesis in 3 dimensions: human and murine bone marrow architecture visualized by confocal microscopy. Blood *116*, e41-55.

Takano, H., Ema, H., Sudo, K., and Nakauchi, H. (2004). Asymmetric division and lineage commitment at the level of hematopoietic stem cells: inference from differentiation in daughter cell and granddaughter cell pairs. J Exp Med *199*, 295-302.

Takubo, K., Goda, N., Yamada, W., Iriuchishima, H., Ikeda, E., Kubota, Y., Shima, H., Johnson, R.S., Hirao, A., Suematsu, M.*, et al.* (2010). Regulation of the HIF-1alpha level is essential for hematopoietic stem cells. Cell Stem Cell *7*, 391-402.

Takubo, K., Nagamatsu, G., Kobayashi, C.I., Nakamura-Ishizu, A., Kobayashi, H., Ikeda, E., Goda, N., Rahimi, Y., Johnson, R.S., Soga, T.*, et al.* (2013). Regulation of glycolysis by Pdk functions as a metabolic checkpoint for cell cycle quiescence in hematopoietic stem cells. Cell Stem Cell *12*, 49-61.

Tan, S.S., Uyl-de Groot, C.A., Huijgens, P.C., and Fibbe, W.E. (2007). Stem cell transplantation in Europe: trends and prospects. Eur J Cancer *43*, 2359-2365.

Tothova, Z., Kollipara, R., Huntly, B.J., Lee, B.H., Castrillon, D.H., Cullen, D.E., McDowell, E.P., Lazo-Kallanian, S., Williams, I.R., Sears, C.*, et al.* (2007). FoxOs are critical mediators of hematopoietic stem cell resistance to physiologic oxidative stress. Cell *128*, 325-339.

Trumpp, A., Essers, M., and Wilson, A. (2010). Awakening dormant haematopoietic stem cells. Nat Rev Immunol *10*, 201-209.

Visnjic, D., Kalajzic, I., Gronowicz, G., Aguila, H.L., Clark, S.H., Lichtler, A.C., and Rowe, D.W. (2001). Conditional ablation of the osteoblast lineage in Col2.3deltatk transgenic mice. J Bone Miner Res *16*, 2222-2231.

Visnjic, D., Kalajzic, Z., Rowe, D.W., Katavic, V., Lorenzo, J., and Aguila, H.L. (2004). Hematopoiesis is severely altered in mice with an induced osteoblast deficiency. Blood *103*, 3258-3264.

Walasek, M.A., van Os, R., and de Haan, G. (2012). Hematopoietic stem cell expansion: challenges and opportunities. Ann N Y Acad Sci *1266*, 138-150.

Wang, L.D., and Wagers, A.J. (2011). Dynamic niches in the origination and differentiation of haematopoietic stem cells. Nat Rev Mol Cell Biol *12*, 643-655.

Willert, K., Brown, J.D., Danenberg, E., Duncan, A.W., Weissman, I.L., Reya, T., Yates, J.R., 3rd, and Nusse, R. (2003). Wnt proteins are lipid-modified and can act as stem cell growth factors. Nature *423*, 448-452.

Wilson, A., Laurenti, E., Oser, G., van der Wath, R.C., Blanco-Bose, W., Jaworski, M., Offner, S., Dunant, C.F., Eshkind, L., Bockamp, E.*, et al.* (2008). Hematopoietic stem cells reversibly switch from dormancy to self-renewal during homeostasis and repair. Cell *135*, 1118-1129.

Wilson, A., Oser, G.M., Jaworski, M., Blanco-Bose, W.E., Laurenti, E., Adolphe, C., Essers, M.A., Macdonald, H.R., and Trumpp, A. (2007). Dormant and self-renewing hematopoietic stem cells and their niches. Ann N Y Acad Sci *1106*, 64-75.

Wilson, A., and Trumpp, A. (2006). Bone-marrow haematopoietic-stem-cell niches. Nat Rev Immunol *6*, 93-106.

Wright, D.E., Cheshier, S.H., Wagers, A.J., Randall, T.D., Christensen, J.L., and Weissman, I.L. (2001). Cyclophosphamide/granulocyte colony-stimulating factor

causes selective mobilization of bone marrow hematopoietic stem cells into the blood after M phase of the cell cycle. Blood *97*, 2278-2285.

Xie, Y., Yin, T., Wiegraebe, W., He, X.C., Miller, D., Stark, D., Perko, K., Alexander, R., Schwartz, J., Grindley, J.C.*, et al.* (2009). Detection of functional haematopoietic stem cell niche using real-time imaging. Nature *457*, 97-101.

Yamamoto, R., Morita, Y., Ooehara, J., Hamanaka, S., Onodera, M., Rudolph, K.L., Ema, H., and Nakauchi, H. (2013). Clonal Analysis Unveils Self-Renewing Lineage-Restricted Progenitors Generated Directly from Hematopoietic Stem Cells. Cell *154*, 1112-1126.

Yamashita, Y.M., Fuller, M.T., and Jones, D.L. (2005). Signaling in stem cell niches: lessons from the Drosophila germline. J Cell Sci *118*, 665-672.

Yamazaki, S., Ema, H., Karlsson, G., Yamaguchi, T., Miyoshi, H., Shioda, S., Taketo, M.M., Karlsson, S., Iwama, A., and Nakauchi, H. (2011). Nonmyelinating Schwann cells maintain hematopoietic stem cell hibernation in the bone marrow niche. Cell *147*, 1146-1158.

Yu, H., Yuan, Y., Shen, H., and Cheng, T. (2006). Hematopoietic stem cell exhaustion impacted by p18 INK4C and p21 Cip1/Waf1 in opposite manners. Blood *107*, 1200-1206.

Yu, W.M., Liu, X., Shen, J., Jovanovic, O., Pohl, E.E., Gerson, S.L., Finkel, T., Broxmeyer, H.E., and Qu, C.K. (2013). Metabolic Regulation by the Mitochondrial Phosphatase PTPMT1 Is Required for Hematopoietic Stem Cell Differentiation. Cell Stem Cell *12*, 62-74.

Zhang, C.C., and Sadek, H.A. (2014). Hypoxia and metabolic properties of hematopoietic stem cells. Antioxid Redox Signal *20*, 1891-1901.

Zhang, J., Grindley, J.C., Yin, T., Jayasinghe, S., He, X.C., Ross, J.T., Haug, J.S., Rupp, D., Porter-Westpfahl, K.S., Wiedemann, L.M.*, et al.* (2006). PTEN maintains haematopoietic stem cells and acts in lineage choice and leukaemia prevention. Nature *441*, 518-522.

Zou, P., Yoshihara, H., Hosokawa, K., Tai, I., Shinmyozu, K., Tsukahara, F., Maru, Y., Nakayama, K., Nakayama, K.I., and Suda, T. (2011). p57(Kip2) and p27(Kip1) cooperate to maintain hematopoietic stem cell quiescence through interactions with Hsc70. Cell Stem Cell *9*, 247-261.

Zsebo, K.M., Williams, D.A., Geissler, E.N., Broudy, V.C., Martin, F.H., Atkins, H.L., Hsu, R.Y., Birkett, N.C., Okino, K.H., Murdock, D.C.*, et al.* (1990). Stem cell factor is encoded at the Sl locus of the mouse and is the ligand for the c-kit tyrosine kinase receptor. Cell *63*, 213-224.

Chapter 2

Divisional tracking of HSCs *in vitro*

Development of a cell-division tracking assay to follow hematopoietic stem cell fate

Manuscript under preparation

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Abstract

Although a majority of the adult hematopoietic stem cell (HSC) population is quiescent, their well-regulated cell divisions are necessary to maintain the stem cell pool and produce differentiated progeny to replenish the blood system. Divisional tracking using 5-bromo-2'-deoxyuridine (BrdU)-mediated DNA labeling, and carboxyfluorescein diacetate succinimidyl ester (CFSE)-based cytoplasmic labeling has shed light on steady state dynamics of HSC divisions *in vivo*. Cell cycle dynamics of HSCs cultured in predefined in *vitro* conditions have not been thoroughly characterized yet. Here, we used CFSE-based cell tracking combined with immunostaining to precisely map the phenotypic changes of HSCs that underwent up to four divisions, under culture conditions previously described to induce HSC self-renewal, commitment or maintenance (basal condition). We found significantly higher proliferation rates in self-renewing and commitment-inducing conditions as compared to the basal culture condition. Preliminary analyses of cell division patterns showed that the self-renewing condition maintained a higher proportion of cells with the HSC immunophenotype. Moreover, the proportion of these HSCs decreased with increasing number of cell divisions, supporting the notion that faster HSC cycling results in stem cell exhaustion.

Introduction

Adult HSCs predominantly reside in a quiescent cell cycle state (Cheshier et al., 1999; Wilson et al., 2008). The maintenance of this quiescent state is believed to be necessary to prevent stem cell exhaustion (Orford and Scadden, 2008). On the contrary, HSC divisions are indispensable for self-renewal and the generation of committed daughters for lifelong production of mature blood cells. Therefore, a fine balance between quiescence and cycling is critical for long-term blood production without exhausting the HSC pool. Various studies have used cell-tracking methods to study the division dynamics of HSCs in their native niche. For instance, *in vivo* incorporation of BrdU in HSCs was used to demonstrate that these rare cells enter cell cycle within three weeks and divided homogenously, with every HSC undergoing a division once in 57 days (Cheshier et al., 1999). Other studies used transient expression of histone 2B protein fused with green fluorescent protein (H2B-GFP), or a short BrdU pulse, followed by a long chase period, to identify two distinct populations within the phenotypically defined HSCs; one that divided faster (~36 days) and the other that divided much slower (~145 days) (Foudi et al., 2009; Wilson et al., 2008). Moreover, they demonstrated enhanced stem cell potential associated with the slower cycling fraction (Foudi et al., 2009; Wilson et al., 2008). Recent studies have tracked HSC divisions *in vivo* (Takizawa et al., 2011) by using carboxyfluorescein diacetate succinimidyl ester (CFSE), a cell permeable dye that is equally distributed in daughter cells upon division (Lyons and Parish, 1994; Weston and Parish, 1990). Interestingly, they found that both non-dividing (also termed "dormant") and fast cycling cells possess long-term blood reconstitution potential.

Strikingly, fast cycling cells were capable of reverting back to the dormant state, suggesting that these cell cycle states are reversible (Takizawa et al., 2011).
Similar studies to understand HSC cell cycle dynamics in predefined *in vitro* culture conditions are lacking. Earlier efforts could only identify a fast and a slow cycling population of cells using PKH-26, a lipophilic dye that non-covalently stains the cell membrane and distributes equally in daughter cells (Lee et al., 2002; Yan et al., 2003). Staining of Lin- or a human progenitor cell line by PKH-26 allowed only a crude identification of fast ($PKH^{low/null}$) and slow (PKH^{high}) cycling cells with no further resolution on the precise number of divisions and importantly, no relationship with immunophenotype (Yan et al., 2003; Lee et al., 2002).

Here we employed CFSE labeling to systematically analyze HSC progeny *in vitro.* Building on the methods developed by Takizawa and colleagues (Takizawa et al., 2011), we labeled freshly isolated HSCs (identified by Lin-cKit+Sca+CD150+CD48-CD34-) with CFSE, and performed flow cytometry analysis at various time points to assess changes in CFSE intensity correlating with HSC divisional history. We thus successfully tracked up to five HSC divisions in culture. We found cells cultured in self-renewing and commitment conditions to have faster proliferation rates compared to the basal condition. Furthermore, preliminary experiments showed that self-renewing condition maintained a higher proportion of cells retaining the HSC immunophenotype. The retention of HSCs negatively correlated with number of divisions, across all conditions. This finding is in congruence with the current view in the field that associates faster cycling with loss of stem cell activity (Orford and Scadden, 2008). Interestingly, even though the cycling rates of self-renewing and commitment condition were similar, changes in phenotype were found to be noticeably different, suggesting that external cues induce different stem cell fates. However, these experiments need to be repeated in order to confirm these preliminary findings. Altogether, this method allows high resolution tracking of HSC divisions *in vitro*, and can potentially be applied to isolate functional HSCs from different divisional states.

Experimental methods

Mice

Mice were purchased from Charles River Laboratories International and maintained at the Center for Studying Living System (CAV) at the EPFL in micro-isolator cages. Mice were provided continuously with sterile food, water and bedding. All experiments were carried out in accordance with the Swiss law.

Antibodies

The following antibodies were used: cKit-PeCy7 (2B8, Biolegend), Sca1-APC (D7, Biolegend) or -PerCPCy5.5 (E13-E161.7, Biolegend), CD150-PeCy5 (TC-15-12F12.2, Biolegend), CD48-PB (GM48-1, Biolegend), CD34-FITC or –efluor660 (RAM34, eBiosciences), SAV-PO (life technologies). A mixture of biotinylated

mAbs against CD3, CD11b, CD45R/B220, Ly-6G, Ly-6C and TER-119 was used as lineage depletion cocktail (BD Biosciences).

Flow cytometry and cell sorting

Flow cytometry analysis of hematopoietic stem and progenitor cells was performed on freshly isolated bone marrow (BM) from 8-12 weeks old C57Bl/6J mice. BM was extracted from crushed femora, tibia and hip bone. Cell suspension was then filtered through a 70μm cell strainer and erythroid cells were eliminated by incubation with red blood cells lysis buffer (eBioscences). Lineage-positive cells were removed with a magnetic lineage depletion kit (Miltenyi Biotech). Cell suspensions were stained with a panel of specific antibodies for stem and progenitor cells and FACS-sorted on BD FACS Aria II. The long term Hematopoietic stem cell (HSC) compartment was identified and sorted with the following cell surface phenotype Lin- cKit+ Sca1+ (LKS) CD150+ CD48- CD34-.

CFSE staining

Freshly sorted HSCs were incubated for 20 min at 37°C with 1:400 CFSE stock solution (Cayman chemicals; CFSE cell division assay kit). Cells were then pelleted and re-suspended in Stemline II (Sigma) containing 10% FBS for 20 min at 37°C. Thereafter, the cells were washed twice with Stemline II and put in culture.

HSC culture

Cells were cultured in U-bottom 96-well plates for three days at 5% CO_2 at 37^{o} C.
Cultures were maintained in serum free media (Stemline II, Sigma) supplemented with a different set of cytokines for different conditions. The "Basal" media included addition of 100ng/ml SCF (R&D Systems), 2ng/ml Flt3 ligand (R&D) and 0.5% P/S. The "Self-renewing" media included 10μg/ml Heparin (Sigma), 100ng/ml SCF (R&D Systems), 2ng/ml Flt3 ligand (R&D), 20ng/ml TPO (R&D Systems), 10ng/ml FGF-1 (Invitrogen), 500ng/ml IGFBP2 (R&D Systems), 100ng/ml AngL-3 (R&D Systems) and 0.5% P/S. The "Commitment" media included the addition of 20ng/ml IL-3 (R&D Systems) and 100ng/ml IL-6 (R&D Systems) to the basal media.

Post culture staining

At the end of the culture period cells were recovered from U-bottom 96 well plates. Cells were then stained with a panel of specific antibodies and analyzed on BD LSRII.

Results and Discussion

CFSE-based *in vitro* divisional tracking of HSCs

To analyze the progeny of HSCs in their distinct divisional states we decided to employ CFSE-based *in vitro* divisional tracking. CFSE, a cell permeable dye is retained in the cells via covalent attachment to intracellular amines. Upon cell division the dye is equally distributed in daughter cells, hence diluting-out by a factor of two with every division (Lyons and Parish, 1994; Takizawa et al., 2011; Weston and Parish, 1990). Therefore, by uniformly labeling cells at the beginning of the culture and assessing the dilution of the dye (*i.e* intensity) at later time points, it is possible to precisely track the number of divisions a cell has undergone (Figure 2.1B).

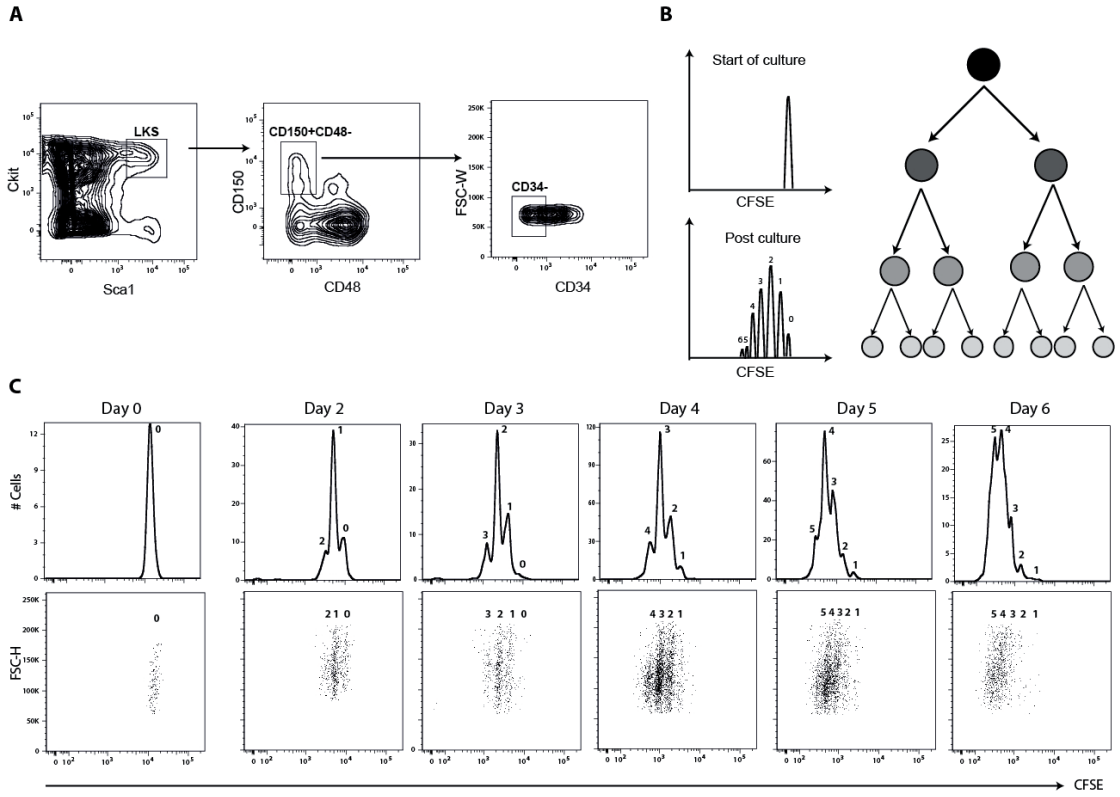

Figure 2.1: CFSE-based *in vitro* divisional tracking of HSCs. (A) Isolation of HSCs using flow cytometry. **(B)** Schematic of CFSE based divisional tracking of cells. CFSE dilutes out in every subsequent division. (**C**) CFSE based divisional tracking of HSCs, represented using histograms (above) and dot plots (below). Sharp peak corresponding to undivided cells was observed at the beginning of culture. Upto five HSC divisions could be precisely followed over a period of six days. Determination beyond 5 divisions with high resolution was not possible due to dilution of the dye. A representative example is shown here from the self-renewing condition.

To ascertain if this method could be used to track HSC divisions *in vitro*, we FACS sorted HSCs based on a commonly used combination of cell surface markers, Lin-cKit+Sca1+ (LKS) CD150+CD48-CD34- (Kiel et al., 2005; Wilson et al., 2008) (Figure 2.1A) and uniformly labeled them with CFSE. A small fraction of the labeled cells were analyzed by flow cytometry to determine the CFSE intensity corresponding to undivided cells (Figure 2.1C, left panel Day 0). The remaining cells were put in culture and analyzed by flow cytometry at various time points until six days (Figure

2.1C). We observed the emergence of precise peaks of CFSE intensities corresponding to a step-wise increase in the number of cell divisions (Figure 2.1C). A predominant peak corresponding to "1 division" after two days and "2 division" after three days in culture was observed (Figure 2.1C). These observed division kinetics corresponded well with our previous analysis using live cell imaging at single cell level (Vannini et al., 2012) (Roch et al., submitted), and we therefore rule out changes in cell cycle characteristics upon CFSE staining. Importantly, previous studies have demonstrated that CFSE labeled LKS cells show long-term blood reconstitution, ruling out labeling related impairment of stem cell function (Takizawa et al., 2011).

Linking HSC divisional history to phenotype in different culture conditions

Having established *in vitro* divisional tracking of HSCs, we wanted to test their division kinetics in different *in vitro* culture conditions and correlate it to changes in phenotype. We therefore cultured CFSE-labeled HSCs in well defined *in vitro* conditions, previously identified as promoting either HSC *self renewal* (Huynh et al., 2008; Huynh et al., 2011; Zheng et al., 2011), or *commitment,* i.e rapid loss of stem cell potential (Peters et al., 1996). In addition we used a minimal *basal* culture condition promoting HSC survival but also eventual loss of stem cell activity (Lutolf et al., 2009; Vannini et al., 2012). After three days in culture, HSC progeny generated in different culture conditions were stained with cell surface markers to assess their phenotype.

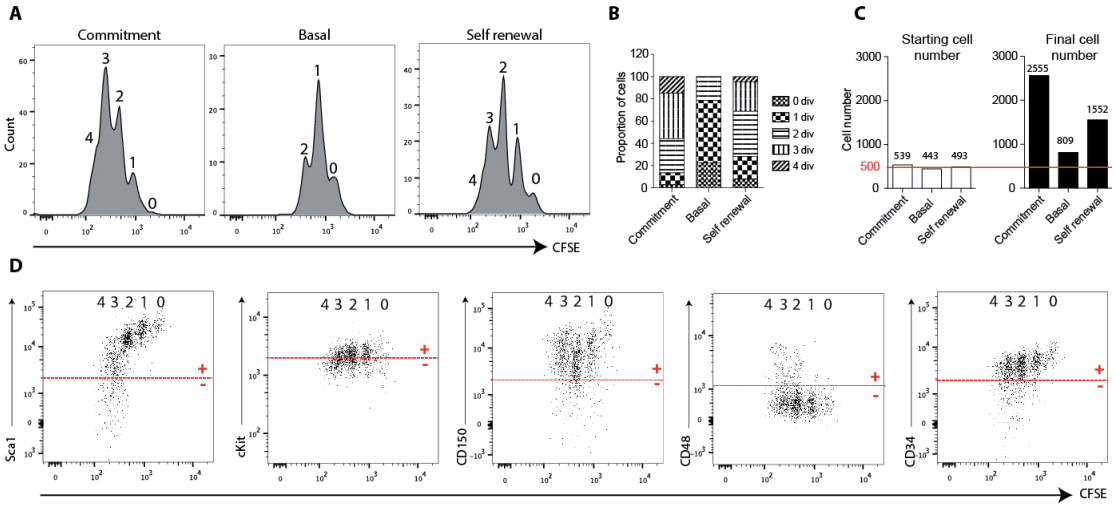

Figure 2.2: Divisional kinetics in different culture. (**A**) HSCs cultured in different *in vitro* conditions ("commitment", "basal", and "self-renewal") for 3 days. Commitment and self-renewing condition show faster proliferation with many cells having divided three or four times. Basal condition demonstrate slower proliferation with most cells having divided only once (**B**) Estimation of proportion of cells in each division state in the three culture conditions. (**C**) Starting cell number was back calculated using the total final cell number and number of cells in each division (from B). Commitment and self-renewing condition showed around 5 and 3 fold expansion in cell number, respectively. Basal condition had less than 2 fold expansion in the same culture duration (3 days) n=1. (**D**) HSC marker staining in combination with CFSE based divisional tracking. Representative example shown here from the self-renewing condition. Different markers showed distinct expression patterns. Sca1 expression dropped with increasing divisions, while cKit expression remained consistent across divisions. CD48 expression starts coming up only after two cell divisions. Moreover, most cells showed high CD34 expression, in line with previous studies that showed CD34 expression coming up in cycling cells. Gates for each channel were drawn using the unstained control (Figure 2.6, check appendix).

First we assessed the division kinetics of HSCs cultured in different conditions for three days (Figure 2.2A-C). The basal condition demonstrated slowest division kinetics, with most cells having undergone only one division, and no cells with more than two divisions (Figure 2.2A,B). HSCs grown under commitment and self-renewal conditions demonstrated faster division rates (Figure 2.2A), with a higher proportion of cells undergoing three divisions (in commitment condition) and two divisions (in self-renewal condition) (Figure 2.2A,B). With the final cell number (Figure 2.2C, right panel) and divisional history (Figure 2.2A,B) known, we estimated the approximate starting cell number in each condition (Figure 2.2C, left panel), allowing us to calculate the fold-expansion in each culture conditions. We found the starting cell number in each condition to be around 500 cells. Expectedly, commitment (~4.8 fold) and self-renewal (~3.2 fold) demonstrated higher expansion rates compared to the basal condition (~1.8 fold) in this experiment (n=1) (Figure 2.2C). Of note, since it is impossible to account for cells that could have died at different divisional states during the culture period, the initial cell number estimation is only approximate.

Next, we determined the expression levels of different HSC markers in these cells. To this end, we re-stained the cells at the end of the culture period with all markers used to isolate the starting HSC population (cKit, Sca1, CD150, CD48, and CD34). Gates for each channel were drawn based on the unstained control (Figure 2.6, check appendix). The changes in stem cell marker expression as a function of the number of cell divisions was found to be very marker specific. For example, under self-renewal conditions, while Sca1 expression dropped gradually with every division, cKit appeared to remain relatively constant across the entire divisional history, whereas CD48 positive cells started appearing only after cells had undergone at least two division (Figure 2.2D). Interestingly, majority of the cells showed high CD34 expression (Figure 2.2D), consistent with previous reports that demonstrated CD34 expression being up regulated in activated HSCs (Ogawa et al., 2001). Moreover CD34 expression was shown to be reversible, with *in vitro* activated CD34+ cells capable of reverting back to a CD34 negative stem cell (Ogawa et al., 2001; Sato et al., 1999).

HSC phenotype in different culture conditions

As identification of HSCs is based on a unique combination of surface markers, we next looked at the entire repertoire of HSC and multipotent progenitor markers in different culture conditions (Figure 2.3). We did not stain cells with lineage markers, as it was difficult to incorporate additional colors in our immunostaining scheme for FACS analysis. Moreover, these markers are expressed only in more mature cells. First we identified the cKit+Sca1+ cells, a heterogeneous population known to contain HSCs and mostly progenitors (Okada et al., 1992). We found a higher proportion of cells (>50%) cultured in the self-renewing condition falling in this gate (Figure 2.3A, B{right graph}, whereas commitment and basal conditions retained only ~20% cells with these two markers (Figure 2.3A, B{right graph}).

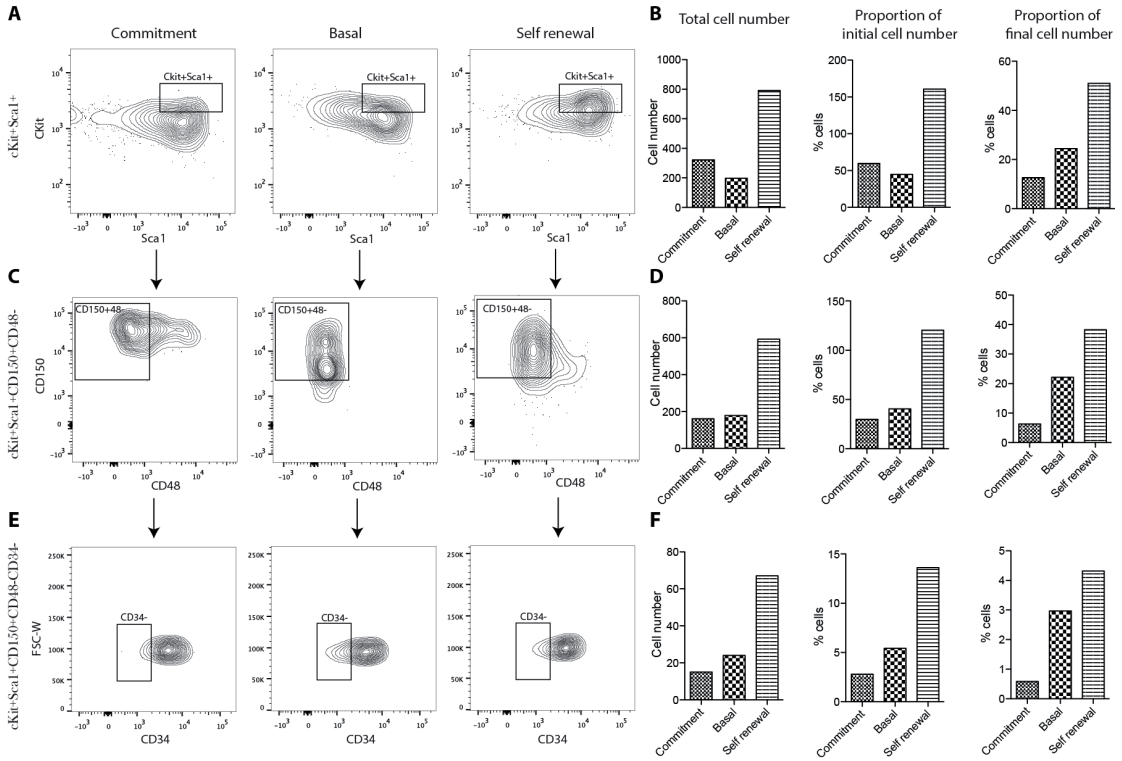

Figure 2.3: HSC phenotype in different culture conditions. (**A**) Identification of cKit+Sca1+ population in the three culture conditions. (**B**) Actual cell number maintaining the cKit+Sca1+ phenotype. Self-renewing condition shows marked increase in the number of cells expressing these two markers as compared to the other two conditions (left graph). In comparison to initial cell number, self-renewing condition shows an increase (~1.4 fold) in the number of cells with this phenotype, suggesting that some HSCs underwent symmetric expansion division in this condition (middle graph). The other two conditions show only about 50% of the initial cell number retaining this phenotype (middle graph). More than 50% of the final cell number in the self-renewing condition maintained the cKit+Sca1+ phenotype, while the other two conditions had less than 25% (right graph). (**C-D**) Identification of cKit+Sca1+ in combination with the SLAM code (CD150+ CD48-). Similar trends were observed as seen with only cKit+Sca1+ population. Self-renewing condition was observed to have an increase (~1.2 fold) as compared to the initial cell number, indicating HSC expansion divisions (middle graph). Moreover, self-renewing condition had ~40% of the final cell number retaining this phenotype, in contrast to the other two conditions, which had very low fraction in this gate; basal (~20%) and commitment (<5%) (right graph). (**E-F**) Identification of the CD34 expression in addition to the population gated in C-D. Most cells were observed to be positive for CD34, in line with previous studies that linked CD34 expression to cycling. Expectedly, a very small fraction of cells (<5%) retained CD34 negativity at the end of the culture (right graph). Since, CD34 negativity becomes unreliable for the identification of HSCs when they are cycling, we decided to not include CD34 expression for further interpretations. (n=1)

Furthermore, addition of the SLAM code (*i.e* differential expression of CD150 and CD48) to the cKit+Sca1+ population showed a similar trend, with self-renewing condition retaining close to 40% cells, while the commitment condition having less than 5% cells in this gate, primarily due to a marked increase of CD48 positivity (Figure 2.3C, D{right graph}). Moreover, most cells showed high levels of CD34, irrespective of the culture condition as they were actively cycling (Figure 2.5E), confirming earlier reports how CD34 negativity becomes unreliable to identify stem cells once they are cycling (Ogawa et al., 2001). However, this experiment needs to be repeated to confirm these observations.

As expected, absolute cell number in the cKit+Sca1+ gate (Figure 2.3B, left graph) and cKit+Sca1+CD150+CD48- gate (Figure 2.3D, left graph), revealed a marked difference between the self-renewing and the other two conditions. Moreover, the

self-renewing condition showed an increase from the initial cell number (Figure 2.3B,D, middle graph), indicating that this condition might facilitate symmetric expansion divisions in some cells (Huynh et al., 2008; Huynh et al., 2011; Zheng et al., 2011). Expectedly, under commitment and basal conditions, very few cells maintained these phenotypes, with the proportion going down to less than 40% of the initial cell number (Figure 2.3D, middle graph). Together, these data indicate that the self-renewing condition maintains a higher proportion of cells with the HSC phenotype compared to the other two conditions. Repetition of this analysis will be carried out in future to validate these initial findings.

HSC phenotype across cell divisional history

Previous studies have implicated a negative correlation between HSC cycling and stem cell potential (Orford and Scadden, 2008). Additionally, we observed that some stem cell markers such as Sca1 and CD48 change dramatically from slow- to fast-cycling cells (Figure 2.2D). Therefore, we next checked phenotypic changes across entire divisional history under various culture conditions, in this experiment (n=1).

To this end, we determined the proportion of cells expressing these markers as a function of the number of cell divisions (Figure 2.4). Interestingly, we found that in both, cKit+Sca1+ and cKit+Sca1+CD150+CD48- populations, the proportion of cells decreased systematically with increasing divisions (Figure 2.4A, commitment condition shown as a representative example). This preliminary observation revealed a similar trend in all three culture conditions (Figure 2.4B,C), suggesting that the stem cell potential might be inversely related to cycling kinetics.

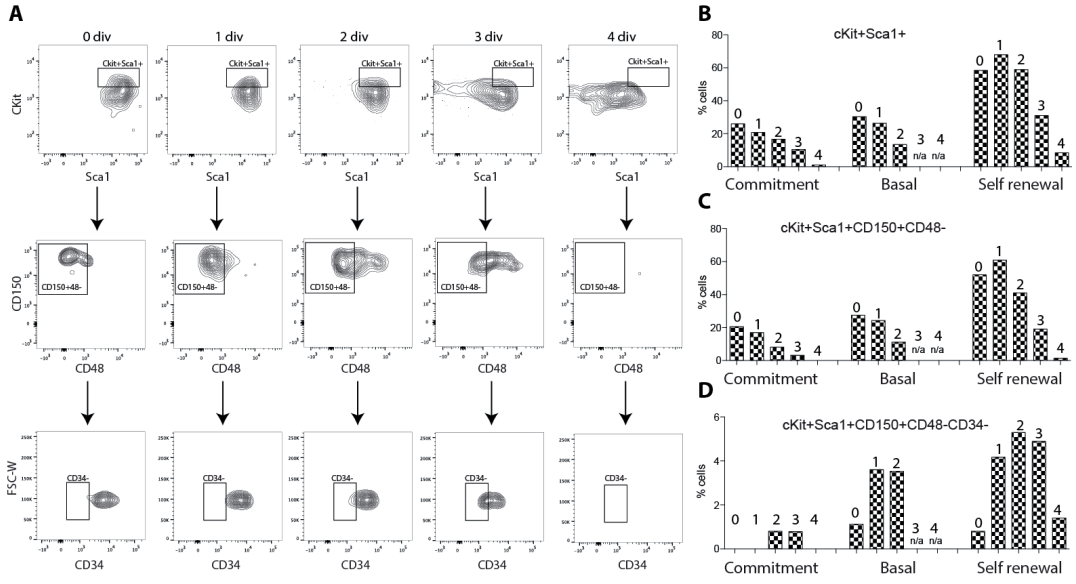

Figure 2.4: HSC phenotype across cell division history. (**A**) Expression pattern of different phenotypes (cKit+Sca1+ (top panel), cKit+Sca1+CD150+CD48- (middle panel), and cKit+Sca1+CD150+CD48-CD34- (bottom panel), across different divisions (commitment condition shown as a representative example). (**B-C**) The proportion of cells maintaining the cKit+Sca1+ (B) and Ckit+Sca1+CD150+CD48- (C) phenotype goes down with increasing divisions across all three conditions. The self-renewing condition contained a high proportion of cells maintaining these phenotypes upto two divisions. Interestingly by the fourth division most cells had lost the basic cKit+Sca1+ phenotype. (**D**) As mentioned before most cells were observed to be positive for CD34 and therefore a very low fraction was seen to be CD34- across different divisions in all three conditions. (n=1)

Of note, the proportion of cKit+Sca1+ cells decreased with increasing divisions primarily due to a dramatic loss of Sca1 expression (Figure 2.4A{upper panel},B). Furthermore, we detected an increase of CD48 levels in many cells after the second division, contributing to the rapid loss of the stem cell phenotype (Figure 2.4A {middle panel}, C). Intriguingly, by the fourth division, majority of the progeny had lost the basic cKit+Sca1+ phenotype under commitment and self-renewing conditions (Figure 2.4B), suggesting that these rapidly dividing cells have lost their stem cell potential.

Next we compared across culture conditions the marker expression for cells in a particular division state (Figure 2.5). We found that within the first two divisions the self-renewal condition promoted relatively higher proportion of phenotypic HSCs compared to the other two conditions (Figure 2.5A,B). As most cells lost the HSC phenotype after third division, the differences across conditions became much smaller (Figure 2.5A,B). This suggests that the first two divisions could be critical in maintaining stem cell phenotypes. These preliminary data reveal an interesting pattern in the dynamics evolution of HSC phenotypes, as a function of cell division history under different *in vitro* culture conditions, that can be further explored in future studies.

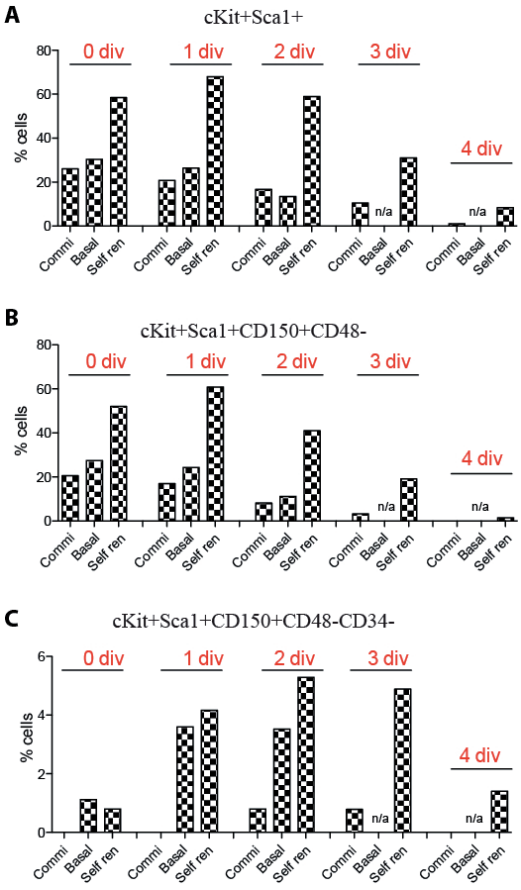

Figure 2.5: Comparison of the HSC phenotype in particular divisional state in different conditions. (**A-B**) Estimation of the proportion of cells maintaining cKit+Sca1+ (A) and cKit+Sca1+CD150+CD48- (B) phenotype, in different division states. Upto two divisions the self-renewing conditions shows a high proportion of cells in these phenotypes as compared to "commitment" and "basal" conditions. After the third divisions the difference becomes less pronounced, and majority of the cells were seen to have lost the HSC phenotype by the fourth division. (**C**) Most cells had lost CD34 negativity, as they were actively cycling. (n=1)

Conclusions

Here we established a novel framework for systematically assessing HSC behavior in culture, with a focus on cell division kinetics and phenotypic changes. By using CFSE based cell tracking we could reliably follow the precise number of HSC divisions *in vitro*. Moreover, combining divisional tracking with immunostaining allowed systematic analysis of the HSC progeny in different divisional states, under defined culture conditions imposing specific fates. Our preliminary findings indicate that compared to other conditions, the self-renewing cultures had the highest proportion of cells maintaining the HSC phenotype. However, this difference between different culture conditions became much smaller from third division onwards, suggesting the importance of the first two divisions in maintaining stem cell phenotypes *in vitro*. Importantly, we saw that the proportion of cells retaining the HSC phenotype systematically decreased with increasing number of cell divisions, supporting the current view in the field that correlates faster cycling with loss of stem cell activity via exhaustion. Collectively, these results shed light on distinct patterns in HSC phenotype expression in different divisional states *in vitro*. A thorough analysis with multiple repeats is being carried out at the moment to confirm these initial findings. Additionally, functional assays will be performed to confirm if this phenotype indeed correlates with long-term reconstitution potential in cultured cells. Finally, a comparative analysis between freshly isolated HSCs and cultured cells will be necessary to assess changes in marker expression due to *in vitro* culture.

References

Cheshier, S.H., Morrison, S.J., Liao, X., and Weissman, I.L. (1999). In vivo proliferation and cell cycle kinetics of long-term self-renewing hematopoietic stem cells. Proc Natl Acad Sci U S A *96*, 3120-3125.

Foudi, A., Hochedlinger, K., Van Buren, D., Schindler, J.W., Jaenisch, R., Carey, V., and Hock, H. (2009). Analysis of histone 2B-GFP retention reveals slowly cycling hematopoietic stem cells. Nat Biotechnol *27*, 84-90.

Huynh, H., Iizuka, S., Kaba, M., Kirak, O., Zheng, J., Lodish, H.F., and Zhang, C.C. (2008). Insulin-like growth factor-binding protein 2 secreted by a tumorigenic cell line supports ex vivo expansion of mouse hematopoietic stem cells. Stem Cells *26*, 1628-1635.

Huynh, H., Zheng, J., Umikawa, M., Zhang, C., Silvany, R., Iizuka, S., Holzenberger, M., Zhang, W., and Zhang, C.C. (2011). IGF binding protein 2 supports the survival and cycling of hematopoietic stem cells. Blood *118*, 3236-3243.

Kiel, M.J., Yilmaz, O.H., Iwashita, T., Terhorst, C., and Morrison, S.J. (2005). SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. Cell *121*, 1109-1121.

Lee, G.M., Fong, S.S., Oh, D.J., Francis, K., and Palsson, B.O. (2002). Characterization and efficacy of PKH26 as a probe to study the replication history of

the human hematopoietic KG1a progenitor cell line. In vitro cellular & developmental biology Animal *38*, 90-96.

Lutolf, M.P., Doyonnas, R., Havenstrite, K., Koleckar, K., and Blau, H.M. (2009). Perturbation of single hematopoietic stem cell fates in artificial niches. Integr Biol (Camb) *1*, 59-69.

Lyons, A.B., and Parish, C.R. (1994). Determination of lymphocyte division by flow cytometry. Journal of immunological methods *171*, 131-137.

Ogawa, M., Tajima, F., Ito, T., Sato, T., Laver, J.H., and Deguchi, T. (2001). CD34 expression by murine hematopoietic stem cells. Developmental changes and kinetic alterations. Ann N Y Acad Sci *938*, 139-145.

Okada, S., Nakauchi, H., Nagayoshi, K., Nishikawa, S., Miura, Y., and Suda, T. (1992). In vivo and in vitro stem cell function of c-kit- and Sca-1-positive murine hematopoietic cells. Blood *80*, 3044-3050.

Orford, K.W., and Scadden, D.T. (2008). Deconstructing stem cell self-renewal: genetic insights into cell-cycle regulation. Nat Rev Genet *9*, 115-128.

Peters, S.O., Kittler, E.L., Ramshaw, H.S., and Quesenberry, P.J. (1996). Ex vivo expansion of murine marrow cells with interleukin-3 (IL-3), IL-6, IL-11, and stem cell factor leads to impaired engraftment in irradiated hosts. Blood *87*, 30-37.

Sato, T., Laver, J.H., and Ogawa, M. (1999). Reversible expression of CD34 by murine hematopoietic stem cells. Blood *94*, 2548-2554.

Takizawa, H., Regoes, R.R., Boddupalli, C.S., Bonhoeffer, S., and Manz, M.G. (2011). Dynamic variation in cycling of hematopoietic stem cells in steady state and inflammation. J Exp Med *208*, 273-284.

Vannini, N., Roch, A., Naveiras, O., Griffa, A., Kobel, S., and Lutolf, M.P. (2012). Identification of in vitro HSC fate regulators by differential lipid raft clustering. Cell Cycle *11*, 1535-1543.

Weston, S.A., and Parish, C.R. (1990). New fluorescent dyes for lymphocyte migration studies. Analysis by flow cytometry and fluorescence microscopy. Journal of immunological methods *133*, 87-97.

Wilson, A., Laurenti, E., Oser, G., van der Wath, R.C., Blanco-Bose, W., Jaworski, M., Offner, S., Dunant, C.F., Eshkind, L., Bockamp, E.*, et al.* (2008). Hematopoietic stem cells reversibly switch from dormancy to self-renewal during homeostasis and repair. Cell *135*, 1118-1129.

Yan, F., Collector, M.I., Tyszko, S., and Sharkis, S.J. (2003). Using divisional history to measure hematopoietic stem cell self-renewal and differentiation. Exp Hematol *31*, 56-64.

Zheng, J., Huynh, H., Umikawa, M., Silvany, R., and Zhang, C.C. (2011). Angiopoietin-like protein 3 supports the activity of hematopoietic stem cells in the bone marrow niche. Blood *117*, 470-479.

Chapter 3

Mitochondrial activity and HSC fate

Mitochondrial activity determines hematopoietic stem cell fate decisions

Manuscript in review

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Abstract

A tight control of hematopoietic stem cell (HSC) fate is crucial for lifelong blood production. A fine balance of quiescence, self-renewal and differentiation are therefore key to maintain the HSC pool and at the same time produce progenitors to replenish various blood cell lineages. The mechanisms behind this regulation are still poorly understood. Here we show that mitochondrial activity is a key determinant of HSC fate. A low mitochondrial membrane potential (ΔΨm) predicts long-term multi-lineage blood reconstitution capability for both, freshly isolated and *in vitro*-cultured, HSCs. However, as *in vivo* both quiescent and cycling HSCs have comparable ΔΨm distributions, a low ΔΨm is not *per se* related to quiescence but is also found in cycling cells. Indeed, using *in vitro* divisional tracking, we demonstrate that daughter HSCs with a low ΔΨm maintain long-term reconstitution potential, whereas daughter cells with high ΔΨm undergo differentiation. Strikingly, lowering the ΔΨm by chemical uncoupling of the electron transport chain leads to HSC self-renewal under culture conditions that normally induce rapid differentiation. Taken together, these data show that mitochondrial activity and fate choice are causally related in HSCs.

Introduction

The maintenance of the blood system is ensured by a pool of hematopoietic stem cells (HSCs) residing in poorly defined hypoxic niches in the bone marrow. These rare cells are capable of lifelong self-renewal and commitment to multipotent progenitors (MPP). For many decades, HSCs have been successfully used for the treatment of hematological and immune diseases, but their limited number prevents more reliable and broader applications of HSC-based therapies. Several attempts to propagate HSCs *'ex vivo'* have failed, because long-term self-renewal capacity is rapidly lost in culture. The lack of reliable phenotypic or genetic markers to distinguish self-renewing HSCs from their earliest, functionally compromised progeny has made it difficult to elucidate the molecular mechanisms of self-renewal and fate decision-making. Gaining knowledge on these aspects could enable the design of better strategies for clinical HSC manipulation.

Classically, HSCs were identified by their ability to efflux the Hoechst dye through membrane transport pumps, and were shown to be enriched in the "side population" of Hoechst staining of LKS cells (Goodell et al., 1996). Recent advances in the field allow use of a combination of several surface markers to distinguish long-term HSCs (LT-HSC; Lin- cKit+ Sca-1+ (LKS) CD150+ CD34-) from short-term HSCs (ST-HSC; LKS CD150+CD34+) and MPPs (LKS CD150- CD34+) in the mouse. Although the progression of LT-HSCs to ST-HSCs and MMPs is mirrored by only small changes in the repertoire of cell surface markers, an increasing body of work has shown that the change in cell identity and potency during early commitment involves a profound alteration in metabolic programs of the cells (Broxmeyer and Mantel, 2012; Ito et al., 2012; Ito and Suda, 2014; Suda et al., 2011; Takubo et al.,

2013; Yu et al., 2013). LT-HSCs are mostly quiescent and fulfill their energy requirements predominantly through anaerobic glycolysis, which has been linked to their residence in low oxygen niches (Parmar et al., 2007; Simsek et al., 2010). In contrast, blood-producing stem and progenitor cell types with reduced self-renewal ability (*i.e.* ST-HSCs and rapidly proliferating MPPs) generate ATP primarily through mitochondrial oxidative phosphorylation (OXPHOS) (Takubo et al., 2013; Yu et al., 2013). The distinct metabolic program of LT-HSCs appears to play a critical role in maintaining their long-term *in vivo* function. Accordingly, limiting mitochondrial respiration protects the cells from cellular damage inflicted by reactive oxygen species (ROS) in active mitochondria (Chen et al., 2008; Ito et al., 2004; Ito et al., 2006; Tothova et al., 2007). Recent work in the field has shed light on some specific metabolic pathways and mechanisms by which HSC functionality is maintained. It was found that HSCs actively prevent OXPHOS through a pyruvate dehydrogenase kinase (PDK)-dependent mechanism that inhibits pyruvate dehydrogenase (PDH) responsible for the transformation of pyruvate to Acetyl-CoA (Takubo et al., 2013). In the same study HIF1α was shown to up regulate key glycolytic enzymes and therefore promote anaerobic glycolysis in HSCs (Takubo et al., 2013). Furthermore, knocking down PTEN-like mitochondrial phosphatase (PTPMT1) was shown to result in an induction of the uncoupling protein 2 (UCP2), diminishing the efficacy of OXPHOS and HSC differentiation (Yu et al., 2013). In addition, knocking out transcription factors PGC-1α and PGC-1β, two key regulators of mitochondria function and biogenesis, lead to similar detrimental defects in hematopoiesis (Basu et al., 2013; Sahin et al., 2011). Finally, the tumor suppressor and glucose sensor Lkb1 was shown to be crucial for HSC maintenance via a not yet fully understood mechanism involving mitochondrial biogenesis and function (Gan et al., 2010; Gurumurthy et al., 2010; Nakada et al., 2010).

Collectively, these data suggest that in HSCs, metabolism and function are intricately linked to each other. However, whether anaerobic glycolysis in HSCs is a cause or a consequence of this (hypoxic) niche induced quiescent state remains unclear. Furthermore, it is unknown how quiescent HSCs change their metabolism when they are activated and exit quiescence to undergo a self-renewal or differentiation division.
To address these questions, here we used the mitochondrial membrane potential (ΔΨm), indicated by tetramethylrhodamine, methyl ester (TMRM) fluorescence, as a surrogate for the metabolic state of HSCs and MPPs. We show that phenotypically defined HSCs and progenitor cells vary substantially in their ΔΨm. Using *in vivo* multi-lineage blood reconstitution assays, we show that functional short-term and long-term self-renewal activity within each population is restricted to subpopulations having lower ΔΨm. Intriguingly, by comparing ΔΨm distributions of HSCs separated by their cell cycle phase, we found that during homeostasis as well as under acute stress, quiescent and cycling HSCs have relatively similar mitochondrial activity profiles. This shows that the distinct metabolic programs of HSCs are rather indicative of fate choice (i.e. self-renewal versus commitment) and not *per se* a

hallmark of the quiescent (versus activated) state. Indeed, *in vitro*, in heterogeneous HSC expansion cultures, divisional tracking experiments show that actively self-renewing HSCs retain a low ΔΨm, in marked contrast to differentiating cells that have activated mitochondria (high ΔΨm). Strikingly, low-dose chemical uncoupling of the electron transport chain forces HSCs to self-renew under culture conditions that normally induce rapid differentiation. Collectively, our data identify low mitochondrial activity as a potent functional marker of LT-HSCs and reveal an intriguing causal relationship between metabolism and fate. Therefore, HSC metabolism is more than an adaptation to a specific microenvironment in the bone marrow niche, but appears to be a requirement for cells to be able to execute a particular cell fate choice.

Experimental methods

Mice

Mice were purchased from Charles River Laboratories International and maintained at the Center for Studying Living System (CAV) at the EPFL in micro-isolator cages. Mice were provided continuously with sterile food, water and bedding. All experiments were carried out in accordance with the Swiss law.

Antibodies

The following antibodies were used: cKit-PeCy7 (2B8, Biolegend), Sca1-APC (D7, Biolegend), CD150-PeCy5 (TC-15-12F12.2, Biolegend), CD48-PB (GM48-1, Biolegend), CD34-FITC (RAM34, eBiosciences), SAV-PO (life technologies), CD45.2-PB (104, Biolegend), CD45.1-FITC (A20, Biolegend), Gr1-APC (RB6-8C5, Biolegend), F4/80-APC (BM8, Biolegend), CD19-PE (6D5, Biolegend), CD3-PE (17A2, Biolegend). A mixture of biotinylated mAbs against CD3, CD11b, CD45R/B220, Ly-6G, Ly-6C and TER-119 was used as lineage depletion cocktail (BD Biosciences).

Flow cytometry and cell sorting

Flow cytometry analysis of hematopoietic stem and progenitor cells was performed on freshly isolated bone marrow (BM) from 8-12 weeks old C57Bl/6J mice. BM was extracted from crushed femora, tibia and hip bone. Cell suspension was then filtered through a 70μm cell strainer and erythroid cells were eliminated by incubation with red blood cells lysis buffer (eBioscences). Lineage-positive cells were removed with a magnetic lineage depletion kit (Miltenyi Biotech). Cell suspensions were stained with a panel of specific antibodies for stem and progenitor cells and analyzed or FACS-sorted respectively on a BD LSRII and BD FACS Aria II.

Analysis of mitochondrial activity

Freshly isolated BM cells after RBC lysis were incubated at 37°C for 1 hour with 200nM TMRM (Invitrogen) and then stained with specific antibodies for different hematopoietic stem/progenitor cell compartments. Labeled cells were FACS-sorted or

analyzed by flow cytometry. For confocal imaging, cells were sorted and placed on adherent poly-L-lysine (PLL)-coated glass slides for 6 hours at 37°C. 20nM TMRM was then added in the media and live cell images were acquired on a Leica SP5 confocal microscope. For MitoTracker®Deep Red (Invitrogen) staining, cells were incubated at 200nM for one hour at 37°C.

Transplantations

Double congenic (CD45.1/45.2) marker system was used for all transplantations. CD45.2 mice were lethally irradiated (850RAD) and transplanted with donor cells isolated from CD45.1 mice and either competitor or helper cells from CD45.1/45.2 mice. For LKS transplants, 1000 LKS ($TMRM^{low}$ or $TMRM^{high}$) donor cells were transplanted together with $250*10^3$ total BM competitor cells in recipient mice. For LKS CD150+ CD34- (LT-HSC) and LKS CD150+ CD34+ (ST-HSC) transplants, 80 donor cells were transplanted together with $250*10^3$ total BM competitor cells in recipient mice. Recipient mice were bled at 4, 8 and 16 weeks post transplant and peripheral blood was stained with specific antibodies to determine donor-derived chimerism. One repopulating unit (RU) is equivalent to the repopulating ability of 100,000 competitor bone marrow cells.

For transplantation of *in vitro*-cultured LT-HSCs, the progeny of 200 LT-HSCs cultured for five days were FACS-sorted based on their TMRM signal ($TMRM^{low}$ or $TMRM^{high}$) and transplanted together with $2*10^6$ helper cells. Helper cells were obtained from BM of CD45.1/45.2 mice that were depleted for $Sca1^+$ and $CD150^+$ cells using Sca1-PE (E13-161.7, Biolegend) and CD150-PE (TC15-12F12.2) antibodies and anti-PE microbeads (Miltenyi Biotech). Recipient mice were bled at 4, 8 and 16 weeks post transplant and peripheral blood was stained with specific antibodies to determine donor-derived chimerism.

For the CFSE-TMRM transplants, LT-HSCs were sorted and stained for CFSE. At the end of the 2-day culture period, cells were stained with TMRM followed by resorting based on $TMRM^{low}$ and $TMRM^{high}$ signals in cells that have undergone one division. Each recipient mouse was injected with 100 cells of either population together with $2*10^6$ helper cells. Recipient mice were bled at 4, 8 and 16 weeks post transplant and peripheral blood was stained with specific antibodies to determine donor-derived chimerism. For transplants of HSCs exposed to the uncoupler FCCP, 100 LT-HSC:$TMRM^{low}$ cells were cultured for 5 days under differentiation-inducing conditions in the presence or absence of 5μM FCCP. The progeny of these 100 cells were transplanted in lethally irradiated recipient mice together with $2*10^6$ helper cells. Recipient mice were bled at 4, 8 and 16 weeks post transplant and peripheral blood was stained with specific antibodies to determine donor-derived chimerism.

Secondary Transplantation: Secondary recipients CD45.2 mice were lethally irradiated (850RAD) and transplanted with 3 million BM cells from the primary

recipients. Recipient mice were bled at 8 and 16 weeks post transplant and peripheral blood was stained with specific antibodies to determine donor-derived chimerism.

Cell cycle analysis

FACS sorted HSCs were fixed and permeabilized using Cytofix/Cytoperm plus kit (BD Biosciences), according to the manufacturer instruction. Cells were then stained overnight with Ki67 FITC (BD Biosciences) at 4°C, and 10 minutes with Hoechst 33342 (Invitrogen).

In vivo activation of HSC

HSC were activated to exit dormancy by interferon-alpha (IFN-α) treatment following published protocols (Essers et al., 2009). Briefly, subcutaneous injections in C57Bl/6J mice were carried out with 10,000U of IFN-α (R&D systems) 48 and 24 hours prior to bone marrow extraction. Control mice were injected with an equivalent volume of the vehicle (PBS + 0.1% BSA).

CFSE staining

Freshly sorted LT-HSCs were incubated for 20 min at 37°C with 1:400 CFSE stock solution (Cayman chemicals; CFSE cell division assay kit). Cells were then pelleted and re-suspended in Stemline II (Sigma) containing 10% FBS for 20 min at 37°C. Thereafter, the cells were washed twice with Stemline II and put in culture.

HSC culture

Cells were cultured in U-bottom 96-well plates at 5% CO_2 and 37^{o}C. Cultures were maintained in serum free media (Stemline II, Sigma) supplemented with 10μg/ml Heparin (Sigma), 100ng/ml SCF (R&D Systems), 2ng/ml Flt3 ligand (R&D), 20ng/ml TPO (R&D Systems), 10ng/ml FGF-1 ((Invitrogen), 500ng/ml IGFBP2 (R&D Systems), 100ng/ml AngL-3 (R&D Systems). At the end of the culture period cells were stained with TMRM and analyzed or sorted by flow cytometry. To induce differentiation, HSCs were cultured in basal medium (Stemline II containing 100ng/ml SCF and 2ng/ml Flt3 ligand) supplemented with 20ng/ml IL-3 (R&D Systems) and 100ng/ml IL-6 (R&D Systems). For uncoupler experiments, 5μM carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) (Sigma) was added at the medium. FCCP stock solution was prepared by dissolving the powder in ethanol at 10mM concentration.

Colony forming unit (CFU) assay

Colony forming unit (CFU) assays were performed in complete M3434 methylcellulose (Stem Cell Technologies) following the manufacturer's instructions. 15'000 whole bone marrow or 100 LT-HSC:TMRM^{low} cells were plated in methylcellulose after two days of culture in the presence or absence of FCCP. Colonies were scored after 8 days or 21 days for total bone marrow and LT-HSC:TMRM^{low} cells, respectively.

Statistics

Data were statistically analyzed by Student's t-test, one-way ANOVA followed by Bonferroni's multiple comparison test and Mann Whitney test.

Results and Discussion

Phenotypically defined HSC and progenitor cells show distinct ΔΨm

We identified HSCs and their closely related progeny based on a commonly used combination of surface markers (Kiel et al., 2005; Osawa et al., 1996; Wilson et al., 2008) and analyzed their ΔΨm levels by flow cytometry and confocal microscopy (Figure 3.1). We used TMRM, a cell permeable dye that is readily sequestered by active (polarized) mitochondria, as a read-out of mitochondrial activity. Different fractions in the hematopoietic hierarchy were separated based on Lin, Ckit, Sca1, CD150, and CD34 cell surface markers (Figure 3.1A). Flow cytometry analysis revealed distinct levels of TMRM intensity in different populations, with a stepwise increase from the most primitive to the most committed population (Figure 3.1B, data from Prof. Olaia Naveiras). Furthermore, confocal imaging of different populations confirmed that indeed more primitive fractions have significantly lower amount of active mitochondria (Figure 3.1C,D, data from Dr. Nicola Vannini), with signals becoming barely detectable in LT-HSCs.

These data supports the idea that mitochondrial activity is limited in the most primitive HSC population, most likely as a strategy to protect them from ROS inflicted cellular damage (Ito et al., 2006; Jang and Sharkis, 2007; Piccoli et al., 2005; Tothova et al., 2007). In contrast, hematopoietic progenitors that are highly proliferative meet their increasing energy requirement by increasing mitochondrial metabolism, presumably through OXPHOS (Suda et al., 2011; Yu et al., 2013).

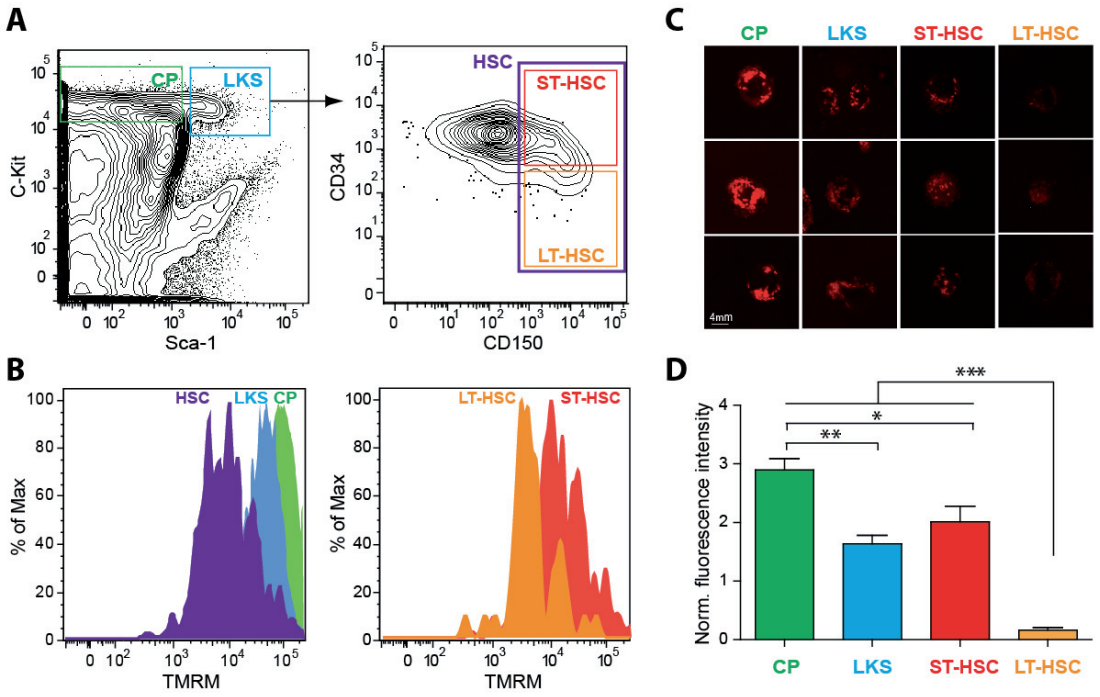

Figure 3.1: Phenotypically defined hematopoietic stem and progenitor cell populations show distinct ΔΨm. (**A**) Isolation by FACS of commonly used, phenotypically defined hematopoietic stem and progenitor populations. Committed progenitors, CPs: c-Kit+; LKS: Lin- c-Kit+ Sca-1+ (*i.e.* a population that comprises all multipotent stem and progenitor cells in the bone marrow); short-term HSCs: LKS CD150+ CD34+ (ST-HSC); long-term HSCs: LKS CD150+ CD34- (LT-HSC). (**B**) Flow cytometry analysis of CPs, LKS, ST-HSC and LT-HSC based

on ΔΨm labeled with TMRM. Each population is marked by a differential ΔΨm level with a stepwise increase from the most primitive to the most committed population. (**C,D**) Confocal imaging and image analysis of live TMRM-labeled stem/progenitor cells confirms an increase of ΔΨm with increasing commitment level (n=7). Three representative examples are shown for each cell population (**D**). ***P< 0.001, **P< 0.01 and *P< 0.05

Low ΔΨm marks HSC subpopulations with long-term blood reconstitution capacity

Having already established that HSCs have lower mitochondrial activity as compared to the progenitors, we wanted to test if ΔΨm could be used to identify functional HSCs from the LKS fraction (a heterogeneous population that contains HSCs and predominantly progenitor cells) in the bone marrow. We used *in vivo* multi-lineage blood reconstitution assays as a read-out of long-term stem cell function (Figure 3.2A). Engraftment was measured as repopulation unit (RU), one RU being equivalent to the repopulation ability found in 100,000 competitor bone marrow cells.

To this end, we first isolated by FACS LKS subpopulations separated by low (LKS:TMRM^{low}) and high (LKS:TMRM^{high}) ΔΨm levels. Transplantation of these two metabolically different phenotypes into lethally irradiated mice was performed using a double congenic allelic system (Figure 3.2A). Long-term multi-lineage blood reconstitution analysis showed that within the LKS population, only cells with low ΔΨm (*i.e.* LKS:TMRM^{low}) possess long-term multi-lineage blood reconstitution potential (Figure 3.2B,C). Therefore, the addition of a metabolic read-out to the existing surface marker repertoire allowed for purification of cells with long-term reconstitution capacity from a poorly defined population (LKS) containing mainly progenitors. We then extended the same sorting strategy to ST-HSCs (LKS CD150+CD34+), and compared ST-HSC:TMRM^{low} and ST-HSC:TMRM^{high} for their ability to reconstitute the blood system in irradiated recipients (Figure 3.2D,E). Strikingly, within the ST-HSC population, short-term multi-lineage reconstitution capacity was almost exclusively restricted to the TMRM^{low} fraction (Figure 3.2E) as demonstrated by a peak in engraftment at 4 weeks followed by a drop at later time points (8 and 16 weeks). Furthermore, phenotypically defined LT-HSCs (LKS CD150+CD34-) could be separated into two distinct populations with low (LT-HSC:TMRM^{low}) and high (LT-HSC:TMRM^{high}) mitochondrial activity (Figure 3.2D), where long term multi lineage reconstitution was restricted exclusively to the "low" fraction (Figure 3.2F).

We reanalyzed the four populations (based on CD34 and TMRM signal) by flow cytometry immediately after sorting (Figure 3.6, check appendix) and saw that the fractions remain almost exclusively in their respective gates, confirming that our metabolically defined populations are distinct and stable. More importantly propidium iodide (PI) staining did not show any measurable difference in viability between the four populations, even after 24 hours in culture (Figure 3.7, check appendix), ruling out that the lack of engraftment was a result of differential cell viability.

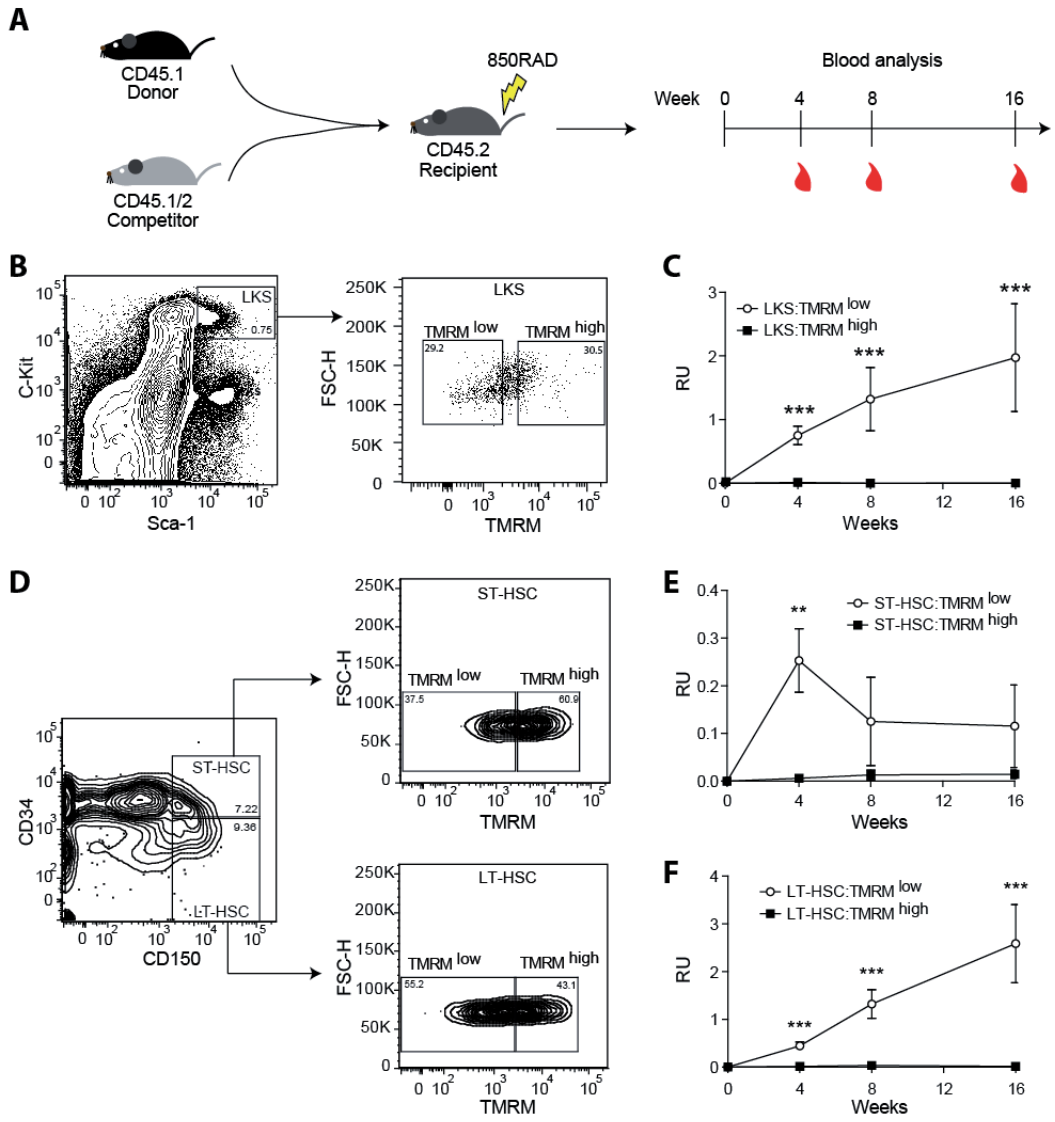

Figure 3.2: Low ΔΨm exclusively marks HSC subpopulations with multi-lineage reconstitution capacity. (**A**) Competitive transplantation strategy used to assess multi-lineage blood reconstitution levels from peripheral blood after 4,8 and 16 weeks. Engraftment is measured here as repopulating unit (RU), one RU being equivalent to the repopulating ability found in 100,000 competitor bone marrow cells. (**B,C**) Within LKS, containing all multipotent stem and progenitor cells in the bone marrow, long-term stemness is restricted to TMRM^{low}cells (LKS:TMRM^{low}) (n=8 for each condition). (**D,E**) In the phenotypically defined ST-HSC compartment, stemness is restricted to TMRM^{low} cells (ST-HSC:TMRM^{low}) (n=9 for each condition). (**D,F**) In the phenotypically defined LT-HSC compartment, stemness is restricted to TMRM^{low} cells (LT-HSC:TMRM^{low}) (n=9 for each condition).***P< 0.001, **P< 0.01 and *P< 0.05

Our *in vivo* data thus reveals a striking functional heterogeneity in phenotypically defined HSCs. The observation that LT-HSCs with activated mitochondria (*i.e.* LT-HSC:TMRM^{high}) do not show blood reconstitution (Figure 3.2F), suggests that these cells may not be hierarchically related to 'true' LT-HSCs. They may instead represent HSCs that give rise to long-term lineage-restricted progenitor cells, as shown by recent *in vivo* single-cell multi-lineage reconstitution assays performed on the same immunophenotypes (Yamamoto et al., 2013).

Quiescent and cycling HSCs in the bone marrow have similar ΔΨm distributions

Next, we wanted to test if quiescent and cycling LT-HSCs have similar levels of mitochondrial activity, *i.e.* that low ΔΨm is a hallmark of stemness independent of the cell cycle state. To this end, we performed cell cycle phase analyses using Ki67 and Hoechst staining on freshly isolated LT-HSCs, and looked at the mitochondrial activity of the quiescent and cycling fraction. Since cell cycle staining involves cell fixation that is not compatible with TMRM-based assays, we used MitoTracker Deep Red as a marker for ΔΨm (Simsek et al., 2010) for these experiments. This dye labels ΔΨm in stem and progenitor cells comparable to TMRM, with a step wise increase in intensity moving from the most primitive to the most committed fraction (Figure 3.8, check appendix).

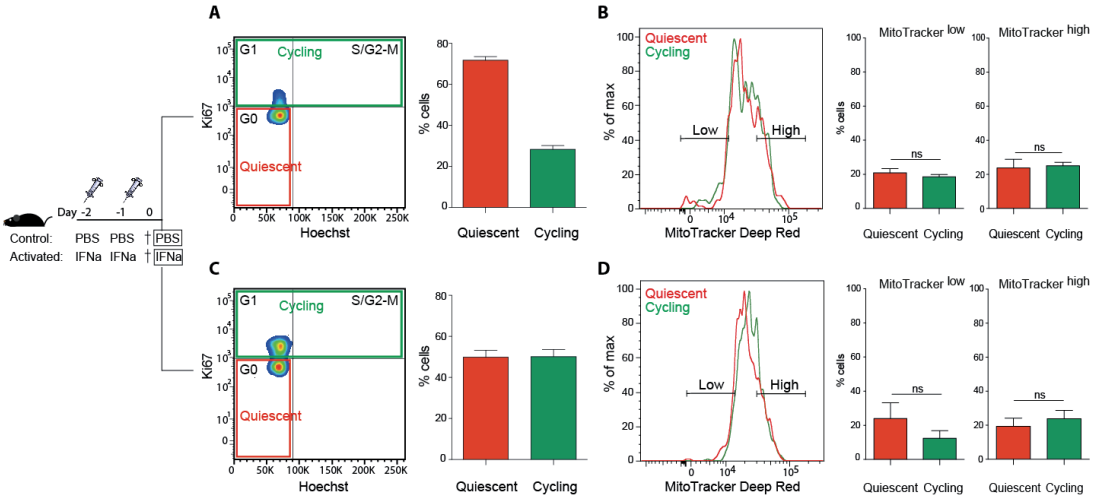

Figure 3.3: Quiescent and cycling HSC populations in the native niche have comparable mitochondrial activity levels. (**A**) Cell cycle analysis using Ki67 and Hoechst staining on freshly isolated HSCs indicate that more than 70% of the cells are in a quiescent state (G_0, red), with the remaining cells cycling (G_1 + S/G_2-M, green). (**B**) Flow cytometry analysis of quiescent and cycling HSCs based on ΔΨm labeled with MitoTracker® Deep Red. Both, quiescent and cycling HSCs show overlapping ΔΨm profiles. The proportion of $\Delta\Psi m^{low}$ and $\Delta\Psi m^{high}$ cells within the quiescent and cycling HSC populations is similar ($\Delta\Psi m^{low}$: P=0.44, $\Delta\Psi m^{high}$ P=0.81). (**C**) IFN-α stimulation results in *in vivo* activation of HSCs as demonstrated by Hoechst/Ki67 staining. (**D**) Flow cytometry analysis shows overlapping ΔΨm profiles of quiescent and cycling HSCs in IFN-αcondition. Similarly, the proportion of $\Delta\Psi m^{low}$ and $\Delta\Psi m^{high}$ cells in quiescent and cycling HSCs remains comparable ($\Delta\Psi m^{low}$: P=0.32, $\Delta\Psi m^{high}$ P=0.54), suggesting that mitochondrial activity is independent of HSC cell cycle state even under acute stress conditions.

Under homeostatic conditions, quiescent HSCs (identified by 2n DNA content and low Ki67) correspond to ~70% of cells, while cycling HSCs (identified by high Ki67) correspond to ~30% of cells (Figure 3.3A). Intriguingly, both these subfractions had nearly indistinguishable ΔΨm distributions (Figure 3.3B). We next performed a similar analysis on HSCs that were activated to exit dormancy directly *in vivo* using interferon-alpha (IFN-α) treatment of mice (Essers et al., 2009). To this end, we performed subcutaneous injections of IFN-α 48 and 24 hours prior to isolation of HSCs. Compared to control mice, IFN-α treatment resulted in a significant increase in the number of cycling (Ki67+) HSCs from 30 to 50% (Figure 3.3C), in line with previous reports (Essers et al., 2009). However, just like under homeostatic

conditions, ΔΨm distributions of quiescent and cycling HSCs were not significantly different (Figure 3.3D). This data shows that *in vivo*, low ΔΨm is not *per se* a hallmark of quiescent HSCs, but rather a functional marker for their stemness independent of cell cycle status.

Low ΔΨm marks self-renewing HSCs in culture

Having established low ΔΨm as a functional discriminator of stemness and revealing comparable ΔΨm levels of quiescent and cycling LT-HSCs *in vivo*, we sought to test whether ΔΨm levels would discriminate self-renewing from differentiating HSCs in heterogeneous bulk *in vitro* cultures (Figure 3.4). Like previously, we used *in vivo* multi-lineage blood reconstitution assays as readout of long-term stem cell function. To this end, we isolated LT-HSCs from CD45.1 mice based on surface marker expression and expanded *in vitro* in serum-free medium containing a cocktail of self-renewing factors Angiopoietin-like protein, Insulin-like growth factor binding protein 2, stem cell factor, fibroblast growth factor 1 and thrombopoietin (Huynh et al., 2008; Huynh et al., 2011; Zhang et al., 2006; Zheng et al., 2011) (Figure 3.4A). After 5 days in culture, cells were resorted based on $TMRM^{low}$ and $TMRM^{high}$ phenotypes, and transplanted into lethally irradiated CD45.2 recipient mice. Consistent with our previous results, low TMRM signal was also predictive of the long-term blood reconstitution capacity of cultured HSCs (Figure 3.4B). Analysis of peripheral blood chimerism 16 weeks post transplantation showed significantly higher levels of reconstitution in mice injected with $TMRM^{low}$ cells (Figure 3.4B). This data suggests that self-renewing HSCs in culture can be detected based on the same metabolic read-out as freshly isolated HSCs from the bone marrow (Figure 3.2F).

However, it is known that a small fraction of cultured HSCs can maintain their stem cell potential by remaining quiescent even after prolonged time in culture (Lutolf et al., 2009; Yamazaki et al., 2006), therefore reconstitution from $TMRM^{low}$ cells seen in Figure 3.4B might have come from non-dividing HSCs. To exclude this possibility and unequivocally identify self-renewing cells in these bulk cultures, we devised a cell-labeling strategy that allowed us to track the precise number of divisions an HSC had undergone in culture (Figure 3.4C). Freshly isolated LT-HSCs were uniformly labeled with carboxyfluoresceinsuccinimidyl ester (CFSE), a live cell-permeable dye that gets diluted with every cell division such that CFSE intensity is decreased by ~50% upon each division (Takizawa et al., 2011). Post CFSE staining, cells were put in culture for 2 days to allow the majority of them to divide. At the end of the culture period cells were stained with TMRM to prepare them for sorting. By these means, we FACS-sorted cultured HSCs that underwent precisely one division after two days and further separated them into $TMRM^{low}$ and $TMRM^{high}$ sub-populations (Figure 3.4C, lower panels). 100 cells of each population were transplanted into lethally irradiated mice and long-term multi lineage reconstitution was analyzed up to 16 weeks. In line with our previous findings, long-term multi lineage blood reconstitution was restricted to the $TMRM^{low}$ subpopulation (Figure 3.4D).

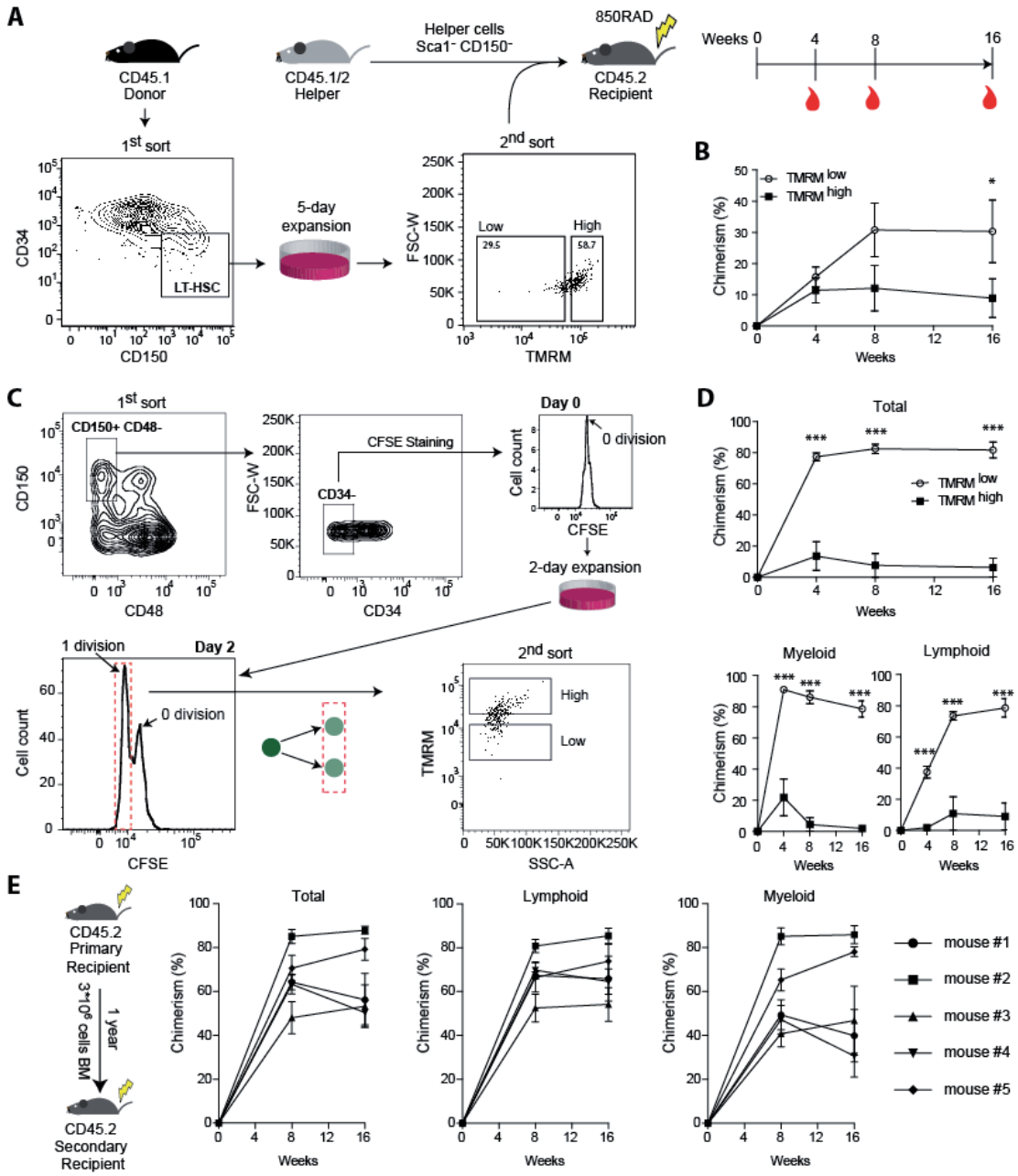

Figure 3.4: A low ΔΨm marks self-renewing HSCs in culture. (**A**) TMRM^{low}and TMRM^{high} cells of 5-day *in vitro*-expanded LT-HSCs were FACS-sorted and transplanted into lethally irradiated mice together with helper cells (Sca1-, CD150-). Multi-lineage blood reconstitution was measured at 4,8 and16 weeks (n=12 for each condition). (**B**) The TMRM^{low} cell fraction of culture-expanded HSC progeny show higher chimerism compared to TMRM^{high} cells. (**C**) CFSE-labeled LT-HSCs were cultured under expansion conditions for two days and progeny that underwent one division were sorted into TMRM^{low} and TMRM^{high} phenotypes and transplanted into lethally irradiated recipient mice together with helper cells. (**D**) The TMRM^{low} fraction of the first generation of daughter cells (*i.e.* dividing one time) exhibited strikingly higher long-term multi-lineage blood reconstitution efficiency compared to TMRM^{high} cells, providing evidence for self-renewing versus differentiating HSC divisions in culture (n=10 for each condition). Assessment of blood chimerism is shown for total blood (top panel) as well as the lymphoid and myeloid lineages (bottom panels). (**E**) BM derived from each of the TMRM^{low} primary recipients (from **D**) was injected into four secondary recipient mice after one year of the primary transplant. Blood chimerism (average of 4 secondary recipients corresponding to each primary recipient) show long-term multi-lineage reconstitution in secondary transplants. ***P< 0.001, **P< 0.01 and *P< 0.05

To further consolidate our findings that indeed TMRM^{low} cells were long term HSCs, we performed secondary transplantation after a period of one year (Figure 3.4E). Bone marrow derived from each of the TMRM^{low} primary recipients was injected into four secondary recipients (3 million cells each). Blood analysis revealed high level of long term multi lineage reconstitution in all secondary recipients (Figure 3.4E), confirming that low TMRM signal allows for successful isolation of functional self renewing long term HSCs from bulk cultures.

HSC fate conversion by electron transport chain uncoupling

Finally, we decided to test whether low mitochondrial activity is a determinant or a consequence of HSC function. More specifically we tested whether HSC fate could be altered by modulation of mitochondrial activity. We chose culture conditions that would push HSCs to differentiate, and asked whether blocking the establishment of a high $\Delta\Psi m$ would result in the maintenance of stemness. For that purpose, we used trifluoro carbonyl cyanide phenylhydrazone (FCCP) that permeabilizes the inner mitochondrial membrane and disrupts its potential (Figure 3.9, check appendix), uncoupling electron transport from ATP generation. We saw a concentration-dependent lowering of $\Delta\Psi m$ upon addition of FCCP (Figure 3.9, check appendix). We chose 5μM FCCP, as it was found to be the ideal concentration that resulted in reasonable lowering of $\Delta\Psi m$ without compromising on the viability of the cells (Figure 3.9, check appendix).

In a first series of experiments, we used quantitative clonal differentiation assays to test to which extent mitochondrial uncoupling could influence the colony forming behavior and efficiency of hematopoietic stem and progenitor cells (Figure 3.5A-D, data from Prof. Olaia Naveiras). 15'000 whole bone marrow cells cultured under differentiation conditions (SCF, Flt3, IL-3 and IL-6) for two days in the presence of FCCP showed a striking decrease in the total number of colonies, as well as an increase in the percentage of multi-lineage colonies (CFU-GEMMs: colony forming unit-granulocyte, erythrocyte, macrophage, megakaryocyte), which originate only from the most primitive progenitor cells (Figure 3.5B, right panel). In contrast, the percentage of lineage-restricted colonies derived from more committed progenitors (e.g. macrophage colonies, M, granulocyte/macrophage colonies, GM) were markedly decreased (Figure 3.5B, middle panel). Similarly, when 100 LT-HSC:TMRM^{low} cells were first cultured for two days under differentiation conditions in the presence or absence of FCCP (Figure 3.5C), we saw a striking decrease in the frequency of colonies derived from the more committed progenitors (Mk, G,M and GM) (Figure 3.6D, left and middle panel) and a concomitant increase of the highly proliferative colonies derived from the most primitive cells (CFU-GEMM) when cultured with FCCP (Figure 3.5D, right panel). These experiments suggested that mitochondrial uncoupling slows down rapid HSC differentiation, maintaining the cells in a more primitive, multipotent state.

Next, we performed long-term blood reconstitution assays wherein LT HSC:TMRM^{low} cells were cultured for five days under differentiation-inducing condition (SCF, Flt3, IL-3 and IL-6) (Peters et al., 1996) in the presence or absence of FCCP, and transplanted all progeny into lethally irradiated mice (Figure 3.5E). Strikingly, FCCP-exposed cells showed much higher levels of long-term multi-lineage blood reconstitution in recipient mice (Figure 3.5F). Importantly, by labeling HSCs with CFSE and tracking their divisional history, we could rule out that this effect was due to an induction of quiescence upon FCCP administration, as all cells in culture had divided multiple times (Figure 3.10, check appendix). This data clearly demonstrates that by disrupting the inner mitochondrial membrane potential, HSCs that would normally rapidly differentiate can be converted to undergo self-renewal divisions. Interestingly, self-renewing embryonic stem cells also have mitochondria with a low ΔΨm, indicative of high rates of glycolysis (Kondoh et al., 2007). Furthermore, during reprogramming of fibroblasts to the induced pluripotent stem cell state, a metabolic switch from OXPHOS to glycolysis is required (Folmes et al., 2011).

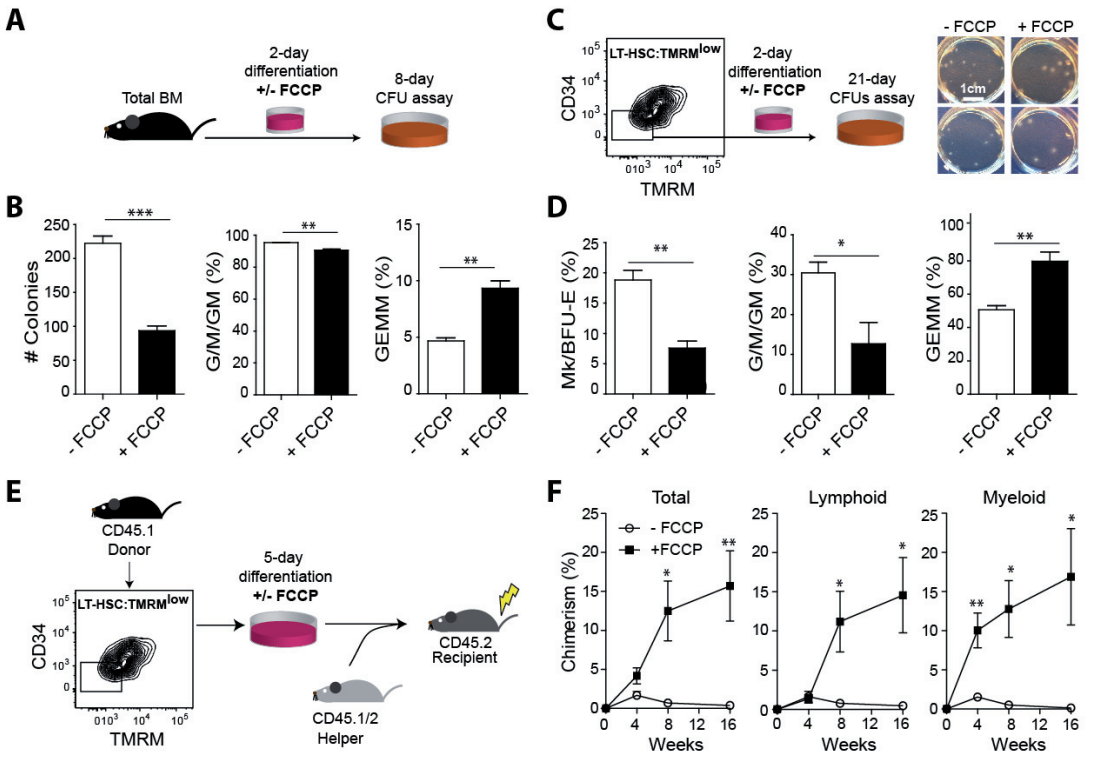

Figure 3.5: Modulation of mitochondrial metabolism alters HSC fate. (**A,C**) Short- and long-term colony forming unit (CFU) assays for measuring the influence of electron transport chain uncoupling on hematopoietic stem and progenitor cell differentiation. (**B,D**) Total bone marrow cells (**B**) as well as phenotypic LT-HSC:TMRM^{low} (**D**) showed a significant decrease in the formation of less primitive and a concomitant increase in the proportion of more primitive colony types when mitochondria were uncoupled. In short-term (8 day) assays, the total number of colonies from bone marrow cells is significantly lower in the presence of the uncoupler (**B**, panel left), suggesting that FCCP might slow down colony formation by enforcing self-renewal over differentiation. (CFU-)G: Colony forming unit-granulocyte; (CFU-)M: Colony forming unit-macrophage; (CFU-)GM: Colony forming unit-granulocyte, macrophage; (CFU-)GEMM: Colony forming unit-granulocyte, erythrocyte, macrophage, megakaryocyte); Mk/BFU-E: Megakaryocyte/Burst forming unit-erythroid. Photographs

of two representative examples of each experimental condition (+/- FCCP) reveal highly proliferative GEMM colonies (white) that are visible by naked eye when in presence of FCCP mitochondrial uncoupling (**C**, top panel on right). (**E**) LT-HSC:TMRM^{low} cultured for 5 days under differentiation conditions in the presence or absence of FCCP were transplanted in lethally irradiated recipient mice together with $2*10^6$ helper cells. (**F**) Cells cultured in the presence of FCCP show high levels of multi-lineage reconstitution, in contrast to controls lacking FCCP that result in rapid differentiation. ***$P< 0.001$, **$P< 0.01$ and *$P< 0.05$

Conclusions

We found a simple yet powerful approach of predicting and directing HSC fate based on mitochondrial activity. By adding a mitochondrial activity-based read-out to the existing surface marker repertoire, we are able to further purify functional hematopoietic stem cells by removing a cell population (LT-HSC:TMRM^{high}) that lacks long term multi-lineage blood reconstitution. Importantly, we found that this read-out marked functional HSCs independent of cell cycle status. Moreover, $\Delta\Psi m$ could be used to successfully isolate functional self-renewing HSCs from heterogeneous cultures. Importantly, we show that *in vitro* modulation of mitochondrial activity by treatment with an uncoupler can re-direct HSCs fate from rapid differentiation to self-renewal, suggesting that low mitochondrial activity is a cause of HSC stemness and not a consequence of a hypoxic bone marrow niche. These results provide evidence for the pivotal role of mitochondrial metabolism in HSC fate determination and suggest novel avenues for 'ex vivo' expansion and identification of HSCs for therapeutic manipulation.

References

Basu, S., Broxmeyer, H.E., and Hangoc, G. (2013). PGC-1alpha Mediated Mitochondrial Biogenesis is Important for Hematopoietic Recovery in Response to Stress. Stem Cells Dev.

Broxmeyer, H.E., and Mantel, C. (2012). A ROSy future for metabolic regulation of HSC division. Nat Med *18*, 1334-1336.

Chen, C., Liu, Y., Liu, R., Ikenoue, T., Guan, K.L., and Zheng, P. (2008). TSC-mTOR maintains quiescence and function of hematopoietic stem cells by repressing mitochondrial biogenesis and reactive oxygen species. J Exp Med *205*, 2397-2408.

Essers, M.A., Offner, S., Blanco-Bose, W.E., Waibler, Z., Kalinke, U., Duchosal, M.A., and Trumpp, A. (2009). IFNalpha activates dormant haematopoietic stem cells in vivo. Nature *458*, 904-908.

Folmes, C.D., Nelson, T.J., Martinez-Fernandez, A., Arrell, D.K., Lindor, J.Z., Dzeja, P.P., Ikeda, Y., Perez-Terzic, C., and Terzic, A. (2011). Somatic oxidative bioenergetics transitions into pluripotency-dependent glycolysis to facilitate nuclear reprogramming. Cell Metab *14*, 264-271.

Gan, B., Hu, J., Jiang, S., Liu, Y., Sahin, E., Zhuang, L., Fletcher-Sananikone, E., Colla, S., Wang, Y.A., Chin, L.*, et al.* (2010). Lkb1 regulates quiescence and metabolic homeostasis of haematopoietic stem cells. Nature *468*, 701-704.

Goodell, M.A., Brose, K., Paradis, G., Conner, A.S., and Mulligan, R.C. (1996). Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. J Exp Med *183*, 1797-1806.

Gurumurthy, S., Xie, S.Z., Alagesan, B., Kim, J., Yusuf, R.Z., Saez, B., Tzatsos, A., Ozsolak, F., Milos, P., Ferrari, F.*, et al.* (2010). The Lkb1 metabolic sensor maintains haematopoietic stem cell survival. Nature *468*, 659-663.

Huynh, H., Iizuka, S., Kaba, M., Kirak, O., Zheng, J., Lodish, H.F., and Zhang, C.C. (2008). Insulin-like growth factor-binding protein 2 secreted by a tumorigenic cell line supports ex vivo expansion of mouse hematopoietic stem cells. Stem Cells *26*, 1628-1635.

Huynh, H., Zheng, J., Umikawa, M., Zhang, C., Silvany, R., Iizuka, S., Holzenberger, M., Zhang, W., and Zhang, C.C. (2011). IGF binding protein 2 supports the survival and cycling of hematopoietic stem cells. Blood *118*, 3236-3243.

Ito, K., Carracedo, A., Weiss, D., Arai, F., Ala, U., Avigan, D.E., Schafer, Z.T., Evans, R.M., Suda, T., Lee, C.H.*, et al.* (2012). A PML-PPAR-delta pathway for fatty acid oxidation regulates hematopoietic stem cell maintenance. Nat Med.

Ito, K., Hirao, A., Arai, F., Matsuoka, S., Takubo, K., Hamaguchi, I., Nomiyama, K., Hosokawa, K., Sakurada, K., Nakagata, N.*, et al.* (2004). Regulation of oxidative stress by ATM is required for self-renewal of haematopoietic stem cells. Nature *431*, 997-1002.

Ito, K., Hirao, A., Arai, F., Takubo, K., Matsuoka, S., Miyamoto, K., Ohmura, M., Naka, K., Hosokawa, K., Ikeda, Y.*, et al.* (2006). Reactive oxygen species act through p38 MAPK to limit the lifespan of hematopoietic stem cells. Nat Med *12*, 446-451.

Ito, K., and Suda, T. (2014). Metabolic requirements for the maintenance of self-renewing stem cells. Nat Rev Mol Cell Bio *15*, 243-256.

Jang, Y.Y., and Sharkis, S.J. (2007). A low level of reactive oxygen species selects for primitive hematopoietic stem cells that may reside in the low-oxygenic niche. Blood *110*, 3056-3063.

Kiel, M.J., Yilmaz, O.H., Iwashita, T., Terhorst, C., and Morrison, S.J. (2005). SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. Cell *121*, 1109-1121.

Kondoh, H., Lleonart, M.E., Nakashima, Y., Yokode, M., Tanaka, M., Bernard, D., Gil, J., and Beach, D. (2007). A high glycolytic flux supports the proliferative potential of murine embryonic stem cells. Antioxid Redox Sign *9*, 293-299.

Lutolf, M.P., Doyonnas, R., Havenstrite, K., Koleckar, K., and Blau, H.M. (2009). Perturbation of single hematopoietic stem cell fates in artificial niches. Integr Biol (Camb) *1*, 59-69.

Nakada, D., Saunders, T.L., and Morrison, S.J. (2010). Lkb1 regulates cell cycle and energy metabolism in haematopoietic stem cells. Nature *468*, 653-658.

Osawa, M., Hanada, K., Hamada, H., and Nakauchi, H. (1996). Long-term lymphohematopoietic reconstitution by a single CD34-low/negative hematopoietic stem cell. Science *273*, 242-245.

Parmar, K., Mauch, P., Vergilio, J.A., Sackstein, R., and Down, J.D. (2007). Distribution of hematopoietic stem cells in the bone marrow according to regional hypoxia. Proc Natl Acad Sci U S A *104*, 5431-5436.

Peters, S.O., Kittler, E.L., Ramshaw, H.S., and Quesenberry, P.J. (1996). Ex vivo expansion of murine marrow cells with interleukin-3 (IL-3), IL-6, IL-11, and stem cell factor leads to impaired engraftment in irradiated hosts. Blood *87*, 30-37.

Piccoli, C., Ria, R., Scrima, R., Cela, O., D'Aprile, A., Boffoli, D., Falzetti, F., Tabilio, A., and Capitanio, N. (2005). Characterization of mitochondrial and extra-mitochondrial oxygen consuming reactions in human hematopoietic stem cells. Novel evidence of the occurrence of NAD(P)H oxidase activity. J Biol Chem *280*, 26467-26476.

Sahin, E., Colla, S., Liesa, M., Moslehi, J., Muller, F.L., Guo, M., Cooper, M., Kotton, D., Fabian, A.J., Walkey, C.*, et al.* (2011). Telomere dysfunction induces metabolic and mitochondrial compromise (vol 470, pg 359, 2011). Nature *475*.

Simsek, T., Kocabas, F., Zheng, J., Deberardinis, R.J., Mahmoud, A.I., Olson, E.N., Schneider, J.W., Zhang, C.C., and Sadek, H.A. (2010). The distinct metabolic profile of hematopoietic stem cells reflects their location in a hypoxic niche. Cell Stem Cell *7*, 380-390.

Suda, T., Takubo, K., and Semenza, G.L. (2011). Metabolic regulation of hematopoietic stem cells in the hypoxic niche. Cell Stem Cell *9*, 298-310.

Takizawa, H., Regoes, R.R., Boddupalli, C.S., Bonhoeffer, S., and Manz, M.G. (2011). Dynamic variation in cycling of hematopoietic stem cells in steady state and inflammation. J Exp Med *208*, 273-284.

Takubo, K., Nagamatsu, G., Kobayashi, C.I., Nakamura-Ishizu, A., Kobayashi, H., Ikeda, E., Goda, N., Rahimi, Y., Johnson, R.S., Soga, T.*, et al.* (2013). Regulation of glycolysis by Pdk functions as a metabolic checkpoint for cell cycle quiescence in hematopoietic stem cells. Cell Stem Cell *12*, 49-61.

Tothova, Z., Kollipara, R., Huntly, B.J., Lee, B.H., Castrillon, D.H., Cullen, D.E., McDowell, E.P., Lazo-Kallanian, S., Williams, I.R., Sears, C.*, et al.* (2007). FoxOs are critical mediators of hematopoietic stem cell resistance to physiologic oxidative stress. Cell *128*, 325-339.

Wilson, A., Laurenti, E., Oser, G., van der Wath, R.C., Blanco-Bose, W., Jaworski, M., Offner, S., Dunant, C.F., Eshkind, L., Bockamp, E.*, et al.* (2008). Hematopoietic stem cells reversibly switch from dormancy to self-renewal during homeostasis and repair. Cell *135*, 1118-1129.

Yamamoto, R., Morita, Y., Ooehara, J., Hamanaka, S., Onodera, M., Rudolph, K.L., Ema, H., and Nakauchi, H. (2013). Clonal Analysis Unveils Self-Renewing Lineage-

Restricted Progenitors Generated Directly from Hematopoietic Stem Cells. Cell *154*, 1112-1126.

Yamazaki, S., Iwama, A., Takayanagi, S., Morita, Y., Eto, K., Ema, H., and Nakauchi, H. (2006). Cytokine signals modulated via lipid rafts mimic niche signals and induce hibernation in hematopoietic stem cells. EMBO J *25*, 3515-3523.

Yu, W.M., Liu, X., Shen, J., Jovanovic, O., Pohl, E.E., Gerson, S.L., Finkel, T., Broxmeyer, H.E., and Qu, C.K. (2013). Metabolic Regulation by the Mitochondrial Phosphatase PTPMT1 Is Required for Hematopoietic Stem Cell Differentiation. Cell Stem Cell *12*, 62-74.

Zhang, C.C., Kaba, M., Ge, G., Xie, K., Tong, W., Hug, C., and Lodish, H.F. (2006). Angiopoietin-like proteins stimulate ex vivo expansion of hematopoietic stem cells. Nat Med *12*, 240-245.

Zheng, J., Huynh, H., Umikawa, M., Silvany, R., and Zhang, C.C. (2011). Angiopoietin-like protein 3 supports the activity of hematopoietic stem cells in the bone marrow niche. Blood *117*, 470-479.

Chapter 4

Asymmetric HSC divisions

Identification of asymmetrically expressed genes in paired daughter HSCs

Manuscript under preparation

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Abstract

The maintenance of hematopoietic stem cell (HSC) pool and concomitant production of committed daughter cells are critical for the life long production of blood. Under homeostasis, HSCs in the native niche are believed to achieve this dual role by undergoing a process called asymmetric cell division (ACD), whereby one daughter cell maintains stem cell potential while the other differentiates to give rise to various blood cell lineages. Since *in vitro* methods lack critical instructive signals of the *in vivo* niche, it is currently not possible to study self-renewal and ACD in a dish. Here we explored the possibility to 'pre-instruct' fate in activated HSCs directly in the *in vivo* niche, in order to assess fate choices of dividing HSCs *in vitro* in a cell-intrinsic manner. To this end, we activated HSCs by Interferon-α (IFNa) and analyzed paired daughter cells (PDCs) by *in vitro* tracking of cell division and multi gene expression analysis at the single cell level. IFNa treatment was found to significantly increase the proportion of asynchronously dividing HSC daughters, indicating a possible involvement of asymmetric divisions. Indeed, gene expression analysis of PDC revealed a set of 12 asymmetrically expressed genes, among them were, stem cell markers such as CD150 and CD34; enzymes such as hexokinase-2 and aconitase-1, that are part of key metabolic pathways; Glut-1, a glucose transporter; and, Tie2, a recently used asymmetry marker in the HSC field. Activated pairs showed significantly higher asymmetry index (A.I) values, a parameter we developed to quantitate asymmetry in expression.

Introduction

A fine balance of different HSC fate choices is indispensable for life long blood production. Uncontrolled stem cell expansion or differentiation can be detrimental for the survival of the organism. Therefore, asymmetric cell division (ACD) appears to be an attractive strategy that allows for both, the maintenance of stem cell number as well as the production of differentiated progeny, at the same time (Morrison and Kimble, 2006). In mammals, poor accessibility to stem cells and limited understanding of niche biology has made it difficult to elucidate ACD.

Classical studies in lower organisms such as *Drosophila Melanogaster* and *C. Elegans* have shed light on both extrinsic and cell intrinsic mechanisms mediating asymmetric cell division in stem cell systems. *C. Elegans* zygote, which divides asymmetrically to produce one large blastomere (taking on an ectoderm fate) and one small blastomere (taking on a mesoderm and endoderm fate), is an example of ACD where intrinsic mechanisms mediate asymmetry (Doe and Bowerman, 2001). Asymmetric localization of partitioning defective (PAR) family of proteins, such as PAR-3, PAR-6 and aPKC, govern mitotic spindle orientation and asymmetric segregation of key cell fate determinants resulting in ACD of the *C.Elegans* zygote (Mello et al., 1996; Strome and Wood, 1983; Suzuki and Ohno, 2006). Similar mechanisms regulate ACD in the *Drosophila* neuroblast, where *Numb*, a cell fate

determinant, localizes only to the daughter cell that is destined to differentiate (Spana et al., 1995). Interestingly, this mechanism was found to be conserved in the mammalian system such as in neural progenitors in the developing rodent brain where *Numb* is asymmetrically segregated to precursors destined for neurogenesis (Shen et al., 2002; Zhong et al., 1996). *Drosophila* germ line stem cells provide an interesting example where extrinsic mechanisms orchestrate ACD. Correct orientation of division ensures that one daughter remains in the stem-cell niche and retains stem cell identity, while the other daughter placed away from the niche begins to differentiate (Yamashita et al., 2005). Instructive cues from the niche ("cap cell" in the ovary and "hub cell" in the testis), lead to activation of BMP (in ovary) or JAK-STAT (in testis) signaling, which eventually represses differentiation in the daughter destined to maintain stem cell potential (Kiger et al., 2001; Song et al., 2004; Tulina and Matunis, 2001).

In the adult hematopoietic system, ACD is believed to be the primary mechanism by which HSC numbers could be maintained under homeostasis (Congdon and Reya, 2008). Since adult HSCs are primarily quiescent (Cheshier et al., 1999; Wilson et al., 2008) it becomes difficult to study the mechanisms controlling their division in the native bone marrow. However, PDC analyses have revealed different colony forming potential (Giebel et al., 2006; Takano et al., 2004) and *in vivo* reconstitution ability (Ema et al., 2000; Yamamoto et al., 2013) in sister cells, suggesting the occurrence of ACD in HSCs. Interestingly, unequal *Numb* segregation in dividing HSCs and therefore asymmetric Notch inhibition was demonstrated using a Notch reporter mouse (Wu et al., 2007). Since Notch signaling was earlier shown to be important in maintaining HSCs in an undifferentiated state (Duncan et al., 2005), this asymmetric inhibition by *Numb* was believed to mediate ACD (Wu et al., 2007). Moreover, when cultured on an osteoblastic cell line, HSCs underwent mostly ACD whereas a generic stromal cell line induced mostly symmetric divisions (Wu et al., 2007), pointing to the role of niche instructed cues, in addition to intrinsic mechanisms, in controlling HSC fate. Additionally, several recent reports have indicated asymmetric segregation of various proteins during HSC division but their link to asymmetric cell fate was difficult to establish (Beckmann et al., 2007; Nteliopoulos and Gordon, 2012; Ting et al., 2012).

To elucidate *in vivo* niche-mediated mechanisms regulating ACD, here we transiently activated HSCs out of their quiescence in the native niche using an acute dose of IFNa (Essers et al., 2009). Analysis of single cell proliferation kinetics via time-lapse microscopy revealed significant increase in asynchronous divisions upon IFNa activation. Using single cell gene expression analyses we identified genes that were asymmetrically expressed in PDCs. This asymmetric gene set included HSC markers such as CD150 (SLAM) and CD34, in addition to metabolic enzymes involved in glycolysis and the TCA cycle. Moreover, IFNa-activated pairs showed higher asymmetry in gene expression compared to control pairs. Strikingly, this asymmetry negatively correlated with time of 1st division, suggesting *in vivo* activated HSCs

undergo ACD. Flow cytometry analysis of some protein candidates showed that they were present at different levels in HSCs and progenitor cells. Collectively, *in vivo* programming of HSC fate by activation of quiescent cells combined with single cell gene expression analysis enabled us to shed light on niche-mediated processes dictating HSC fate choice.

Experimental methods

Mice

Mice were purchased from Charles River Laboratories International and maintained at the Center for Studying Living System (CAV) at the EPFL in micro-isolator cages. Mice were provided continuously with sterile food, water and bedding. All experiments were carried out in accordance with the Swiss law.

In vivo activation of HSCs

HSCs were activated to exit dormancy by interferon-alpha (IFN-α) treatment following published protocols (Essers et al., 2009). Briefly, subcutaneous injections in C57Bl/6J mice were carried out with 10,000U of IFN-α (R&D systems) 48 and 24 hours prior to bone marrow extraction. Control mice were injected with an equivalent volume of the vehicle (PBS + 0.1% BSA).

Antibodies

The following antibodies were used: cKit-PeCy7 (2B8, Biolegend), Sca1-APC (D7, Biolegend), CD150-PeCy5 (TC-15-12F12.2, Biolegend), CD48-PB (GM48-1, Biolegend), CD34-FITC (RAM34, eBiosciences), SAV-PO (life technologies), HK2-Alexa647 (bs-3993R, Bioss), Aco1-Alexa647 (bs-9848R, Bioss). A mixture of biotinylated mAbs against CD3, CD11b, CD45R/B220, Ly-6G, Ly-6C and TER-119 was used as lineage depletion cocktail (BD Biosciences).

Microwell array fabrication

Poly ethylene glycol (PEG) microwells were formed at the bottom of 96-well micro plate (BD) or 4-well plate (Nunc) by crosslinking 4arm-PEG-thiol (PEG-SH, 10kDa) with 8arm-PEG-vinylsulfones (PEGVS, 10kDa) at 5% w/v. Hydrogel films were micropatterned by soft embossing with PDMS (polydimethylsiloxane, VWR) stamps for one hour to create an array of microwells.

Flow cytometry and cell sorting

Flow cytometry analysis of hematopoietic stem and progenitor cells was performed on freshly isolated bone marrow (BM) from 8-12 weeks old C57Bl/6J mice. BM was extracted from crushed femora, tibia and hip bone. Cell suspension was then filtered through a 70μm cell strainer and erythroid cells were eliminated by incubation with red blood cells lysis buffer (eBioscences). Lineage-positive cells were removed with a magnetic lineage depletion kit (Miltenyi Biotech). Cell suspensions were stained with

a panel of specific antibodies for stem and progenitor cells and FACS-sorted on BD FACS Aria II. The hematopoietic stem cell (HSC) compartment was identified and sorted with the following cell surface phenotype Lin- Ckit+ Sca1+ (LKS) CD150+ CD48- CD34-.

HSC culture

HSC cultures were maintained at 5% CO_2 at 37^{o} C in serum free media (Stemline II, Sigma) supplemented with 100ng/ml SCF (R&D Systems), 2ng/ml Flt3 ligand (R&D) and 0.5% P/S.

Cell cycle analysis

FACS sorted HSCs were fixed and permeabilized using Cytofix/Cytoperm plus kit (BD Biosciences), according to the manufacturer instruction. Cells were then stained overnight with Ki67 FITC (BD Biosciences) at 4°C, and 10 minutes with Hoechst 33342 (Invitrogen).

Single cell proliferation analysis

Individual cells cultured in microwells were imaged on a Zeiss Axio Observer Z1 inverted microscope equipped with a motorized stage. An incubation chamber maintaining temperature and CO_2 levels allowed for live cell imaging. The stage was programmed to scan the microwell array surface and acquire images of multiple positions every 3 hours. Single cell proliferation kinetics were assessed based on time-lapse movies.

Micromanipulation of paired daughter cells for single cell analysis

Single cells in microwells having undergone one division to give rise to two daughter cells were identified after 40 hours in culture. Paired daughter cells were isolated by micromanipulation in 20um diameter micro capillaries (Eppendorf). Single cells were ejected from the micro capillaries into lysis solution for subsequent single cell PCR.

Selection of candidate genes

47 candidate genes listed in Table 4.1 were selected for expression analysis in PDCs. Some of those genes were identified in a microarray analysis where they were differentially expressed in HSCs compared to MPPs (Forsberg et al., 2005), or compared to mobilized or leukemic HSCs (Forsberg et al., 2010). These included, key cell surface molecules such as Jam3, Tie2, ProCR, and Esam1; intracellular adaptor proteins Grb10 and Fhl1; and, cycling dependent kinase inhibitor p57 (Umemoto et al., 2005). After a careful review of published work we included, cell cycle genes p21, p27, and p130, which were shown to be important in HSC quiescence (Cheng et al., 2000; Passegue et al., 2005; Zou et al., 2011); HSC maintenance genes b-cat (Perry et al., 2011), Pten (Zhang et al., 2006), and Gata3 (Ku et al., 2012); and, important self renewal mediators, HoxB4 (Krosl et al., 2003) and c-myc (Satoh et al., 2004; Wilson et al., 2004). With recent developments in the field, and our own work (chapter 3), strongly implying the role of metabolism in HSC fate choices, we included a variety

of metabolic genes for our analysis. These included, a glucose transporter (Glut1), key glycolytic enzymes (HK2, PFKFB3, Ldha) (Takubo et al., 2013), enzymes involved in TCA cycle (CS, Acyl, Aco1, Suclg1, Mdh2), and some key components of the oxidative phosphorylation machinery. Based on recent work that implicated fatty acid oxidation as a key to HSC maintenance (Ito et al., 2012), we included two acyl-coA dehydrogenases (MCad and Lcad) and an enzyme that facilitates the entry of fatty acids into the mitochondria (Cpt1a). With growing amount of information on the detrimental effects of ROS on HSC function, we included two key antioxidants (SOD2 and Cat) that were shown to be instrumental in keeping ROS levels low in HSCs (Takubo et al., 2010; Takubo et al., 2013).

Table 4.1: List of genes tested in single cell multi gene expression analysis

Classification	Gene name		Classification	Gene name
House keeping gene	HPRT		**Glycolysis**	HK2
ECM proteins	Tgm2			PFKFB3
	Bgn			Glut1
Membrane proteins	Esam1			Ldha
	Tie2		**TCA cycle**	CS
	Jam3			Acly
HSC markers	CD150			Aco1
	CD48			Suclg1
	CD34			Mdh2
	C-kit		**Oxidative Phosphorylation**	Cyt-C
Intracellular adaptors	Grb10			NDUFA2
	ProCR			COX2 (Sdhd)
	Fhl1			ATP5g1
	b-catenin			COX1
Cell cycle regulators	P57 (cdkn1c)			COX3
	P27 (cdkn1b)			COX4
	P21 (cdkn1a)		**Mitochondrial biogenesis**	Mfn2
	P130 (rab3gap)			Tfam
	Pten		**Antioxidants**	SOD2
Transcription factors	Pbx1			Catalase
	Gata3		**Fatty acid oxidation**	MCad
	c-myc			LCad
	Hoxb4			CPT1a
DNA repair	Gadd45			

Single cell qRT-PCR

Micromanipulated single cells were ejected in 0.2ml PCR tubes containing 10µl of Lysis solution (9µl single cell lysis solution+ 1µl single cell Dnase I). Cells were incubated in the lysis solution at RT for upto 30min followed by addition of 1µl of single cell stop solution and incubation at RT for upto 20min. The samples were then stored at -20°C. Reverse transcription and pre-amplification were performed sequentially on the lysed cell sample using Single Cell-to-Ct Kit (Life Technologies). Conditions for reverse transcription were 10min at 25°C, 60min at 42°C, and 5min at

42°C. Gene Expression TaqMan Assays (Life Technologies) corresponding to the gene set (Table 4.1) were pooled and diluted at 0.2X in 1X TE buffer pH8.0 for pre-amplification. Samples were incubated for 10min at 95°C and pre-amplified for 14 cycles of 15 sec at 95°C and 4 min at 60°C on a thermal cycler. The pre-amplified samples were diluted 1:15 in 1X TE buffer pH8.0 and stored at -20°C. Real-time quantitative PCR was performed with Gene Expression TaqMan Assays (Life Technologies) on a 7900HT system (Applied BioSystems). Conditions for amplification were 2 min at 50°C and 10 min at 94.5°C followed by 40 cycles of 5sec at 97°C and 1min at 59.7°C. Expression values over the threshold of the machine (ct=40) were set to 40.

Marker staining for FACS analysis

Cells were fixed and permeabilized using Cytofix/Cytoperm plus kit (BD Biosciences), according to the manufacturer instruction. Cells were stained with anti Aco1-Alexa647 or anti HK2-Alexa647 for 1 hour at 4°C followed by washing with PBS before being used for analysis. For Glut1 analysis, live cells were stained with anti Glut1-PE for 1 hour at 4°C followed by washing with PBS before being used for analysis.

CFSE staining

Freshly sorted HSCs were incubated for 20 min at 37°C with 1:400 CFSE stock solution (Cayman chemicals; CFSE cell division assay kit). Cells were then pelleted and re-suspended in Stemline II (Sigma) containing 10% FBS for 20 min at 37°C. Thereafter, the cells were washed twice with Stemline II and put in culture.

Transplantation

Double congenic (CD45.1/45.2) marker system was used for transplantations. CD45.2 mice were lethally irradiated (850RAD) and transplanted with donor cells isolated from CD45.1 mice and helper cells from CD45.1/45.2 mice. HSCs were isolated from IFNa activated and control mice. 100 cells were transplanted together with $2*10^6$ helper cells. Helper cells were obtained from BM of CD45.1/45.2 mice that were depleted for $Sca1^+$ and $CD150^+$ cells using Sca1-PE (E13-161.7, Biolegend) and CD150-PE (TC15-12F12.2) antibodies and anti-PE microbeads (Miltenyi Biotech). Recipient mice were bled at 4, 8 and 16 weeks post transplant and peripheral blood was stained with specific antibodies to determine donor-derived chimerism.

Statistics

Data was statistically analyzed by student t test, Fisher's exact test. Spearman's rank correlation coefficient was determined for correlation analysis.

Results and Discussion

In vivo activation of HSCs by IFNa increases asynchronous cell divisions

We used IFNa treatment to activate HSCs to exit their quiescent state *in vivo* (Essers et al., 2009). To this end, subcutaneous injections of IFNa were performed 48 and 24 hours prior to HSC isolation, control mice were injected with vehicle (Essers et al., 2009). HSCs were FACS sorted based on a combination of different cell surface markers, Lin-ckit+Sca1+ (LKS) CD150+CD48-CD34- (Kiel et al., 2005; Osawa et al., 1996; Wilson et al., 2008) (Figure 4.1A). Freshly isolated HSCs were immediately fixed and stained to determine their cell cycle status. IFNa treatment resulted in a significant increase in the proportion of cycling HSCs (Ki67+) as compared to untreated cells, from 25% to almost 60% (Figure 4.1B).

To assess the proliferation kinetics of freshly isolated IFNa-activated HSCs, we used our PEG microwell platform in combination with live cell imaging (Lutolf et al., 2009; Vannini et al., 2012). This allowed us to measure single cell behavior and division dynamics in high throughput over a period of up to five days (Figure 4.1C). IFNa-activated HSCs carried out the first division much faster as compared to control HSCs (Figure 4.1E). The time until half of the population had divided was around 45 hours for control and was shortened by approximately 9 hours in IFNa-activated cells (Figure 4.1E). Proliferation curves showed similar average expansion over a period of five days, with IFNa-activated cells demonstrating faster proliferation in the initial phases of the culture due to a shorter time to 1st division (Figure 4.1D,E). This data confirms that acute IFNa treatment leads to activation of HSCs.

One key property of HSCs is their slower cycling rates as compared to progenitors (Nygren et al., 2006; Orford and Scadden, 2008; Wilson et al., 2008). Therefore, we thought an analysis of the proliferation kinetics of the first generation of HSC daughters could reveal information on the maintenance of stem cell properties such as slower cycling rates. We assessed the difference in time of division of PDC to define the synchrony of cell division (ΔT). The two paired daughter cells were defined here as asynchronous if ΔT was longer than 6 hours (Figure 4.1F). We observed that the fraction of asynchronous divisions was significantly higher in IFNa-activated HSCs as compared to control cells (Figure 4.1G). This increase in asynchrony suggested a possible increase in asymmetric division where one daughter would retain stem cell potential (slowly cycling) whereas the other daughter would undergo commitment (fast cycling). Previous work has provided some evidence for the production of two non-identical daughter cells with different colony forming and blood reconstitution levels *in vitro* (Ema et al., 2000; Giebel et al., 2006; Takano et al., 2004; Yamamoto et al., 2013).

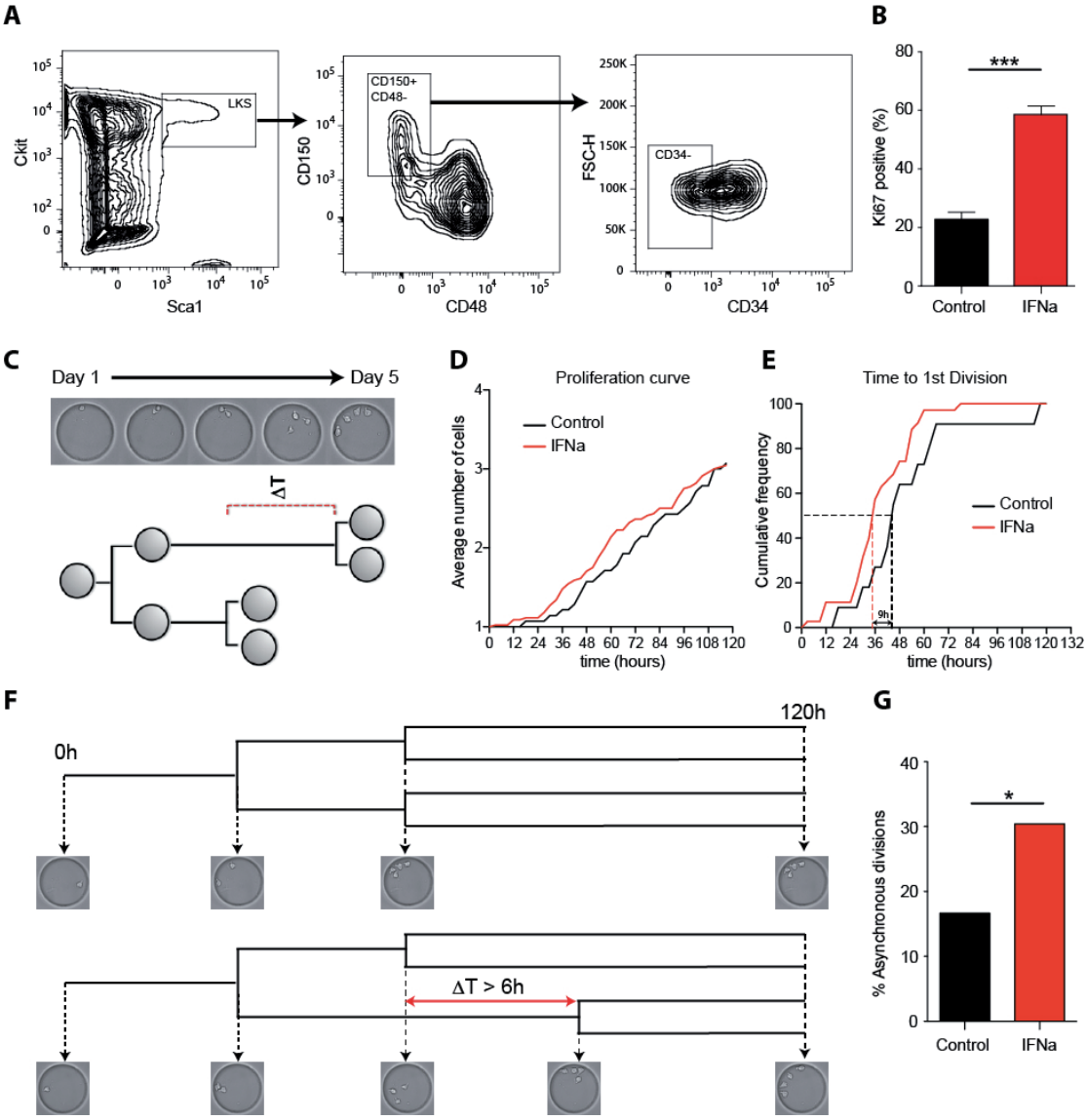

Figure 4.1: *In vivo* activation of HSCs by IFNa increases asynchronous cell divisions. (A) Isolation of long term HSCs based on commonly used cell surface markers (LKSCD150+CD48-CD34-). (**B**) Cell cycle analysis using Ki67 and Hoechst staining on freshly isolated HSCs from control and IFNa treated mice show a significant increase in the cycling fraction (Ki67+) upon IFNa administration. (**C**) Single cell proliferation kinetics analysis using live cell imaging of HSCs in PEG microwells (Lutolf et al., 2009). (**D-E**) Proliferation kinetics of HSCs isolated from control and IFNa administered mice. The average proliferation over 5 days remains similar in the two conditions, with IFNa activated cells carrying out the first division much faster (~9h) as compared to control HSCs. (**F**) Representative example of synchronous division (top) and an asynchronous division (below) with ΔT of greater than 6 hours being used as a threshold. (**G**) IFNa activated HSCs show a significant increase in the proportion of asynchronous divisions. ***$P< 0.001$, **$P< 0.01$ and *$P< 0.05$

Gene expression analysis in PDCs from IFNa-activated HSCs

Previous studies have shown that acute activation by IFNa results in HSC self renewal (Essers et al., 2009). As IFNa mediated activation occurs in the native bone marrow niche without loss of stem cell potential (Figure 4.7, check appendix, (Essers et al., 2009)) and *in vitro* leads to increase in asynchronous divisions (Figure 4.1G), we reasoned that this could be a suitable system to study HSC fate choices and specifically the process of ACD. To test this, we performed multi gene expression analysis in PDCs and compared their gene expression profiles. To this end, we

isolated IFNa-activated HSCs from mice and cultured them at single cell level in PEG microwells, in basal serum-free culture condition (described in chapter 2), to follow their proliferation kinetics (Figure 4.2A). Upon division, PDCs were isolated from the microwells by micromanipulation (Figure 4.2B, images by Dr. Aline Roch), and analyzed for their gene expression profile using single cell quantitative RT-PCR.

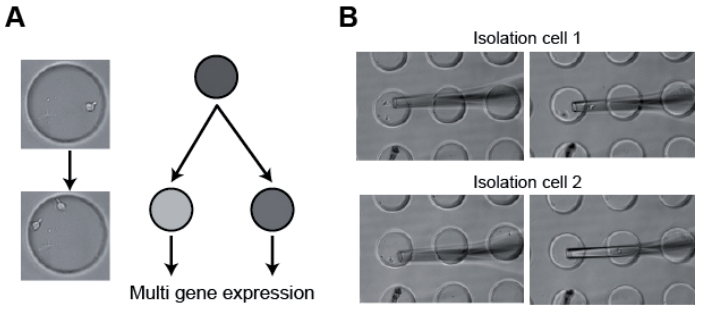

Figure 4.2: Gene expression analysis in PDCs from IFNa-activated HSCs. (**A**) Freshly isolated HSC were cultured in PEG microwells (Lutolf et al., 2009) and allowed to carry out a division. (**B)** Micromanipulation of paired daughter cells (PDCs) generated from a single HSC after one division, for multi gene expression analysis (Images by Dr. Aline Roch).

Identification of the asymmetric genes using PDC analysis

All candidate genes (described in experimental methods) were analyzed in 24 sets of PDCs. Strikingly, when Log_2(Ct) values of the two cells were plotted against each other, different genes showed distinct patterns of expression. Qualitative analysis revealed that on one hand, some genes showed extremely symmetric behavior, with almost all pairs found close to the diagonal (Figure 4.3A). On the other hand, various genes were asymmetrically expressed (Figure 4.3B), with one cell of the pair expressing the gene at higher levels (low Ct) and the other at lower levels (high Ct). However, for most of these potentially asymmetric genes there remained a sub population of pairs that appeared close to the diagonal (Figure 4.3B).

In order to identify asymmetrically expressed genes, we decided to give a quantitative value to every gene to determine where it ranked in comparison to the others. To this end we obtained the average ΔCt for every gene across all 24 pairs and designated it as "*Gene score"*.

$$Gene\ score = \frac{1}{n}\left(\sum_{i=1}^{n\ pairs} (\Delta Ct)\right) \qquad where\ \Delta Ct = |Ct\ cella - Ct\ cellb|$$

A gene score of 1 would indicate an average 2-fold difference in expression in the PDCs, and would increment to 4-, 8-, 16- and 32-fold, with gene scores of 2, 3 4 and 5. We found a huge range of this score with some genes as low as 0.195 (~1.15 fold difference), while others as high as 5 (~32 fold difference) (Figure 4.4A). Despite the

wide range it appeared to be a gradual curve with no disparate groups (Figure 4.4A). Therefore, we decided to put a stringent (although arbitrary) threshold to select out the most highly asymmetric genes for further analysis. A cutoff of 10-fold difference was applied to identify the asymmetric gene set. A group of 12 genes were identified as being above this threshold (Figure 4.4A,B).

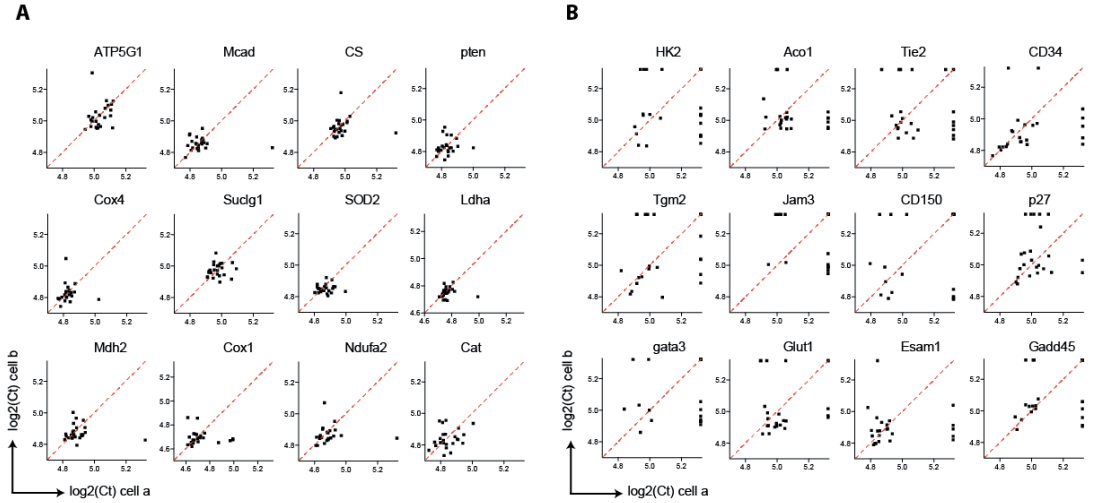

Figure 4.3: Qualitative analysis of distribution of gene expression in PDCs. Each gene is shown for the expression of 24 pairs of cells, with each dot representing a pair. (**A**) Representative examples of genes showing symmetric expression across most pairs. Most pairs appear close to the "symmetric diagonal" (in red). (**B**) Representative examples of genes showing asymmetric expression in pair daughter cells. Most pairs appearing far away from the "symmetric diagonal" (in red). Interestingly most of theses genes had a subpopulation of pairs that showed symmetric behavior.

Among the asymmetrically expressed genes, we found some metabolic genes such as Glut1, HK2 and Aco1 present in this category (Figure 4.4A-B). Glut-1, a glucose transporter across the cell membrane was recently implicated to be important for HSC self-renewal in the *in vivo* niche (Ito and Suda, 2014; Takubo et al., 2013). HK2, a glycolytic enzyme involved in the conversion of glucose to glucose-6-phosphate, was shown to be upregulated by HIF1a (Mathupala et al., 2001), a key component involved in HSC function (Takubo et al., 2013). Aco1, an important TCA cycle enzyme in the mitochondria converting citrate to iso-citrate, its asymmetric expression fitting well with our previous finding implicating mitochondrial activity as a determinant of HSC fate (chapter 3). Apart from these metabolic genes we also found Jam3, p27, and gadd45, genes implicated in HSC function ((Chen et al., 2014; Liebermann and Hoffman, 2007; Zou et al., 2011), Roch et al., submitted), to be asymmetrically expressed. Interestingly, Tie2 (receptor of Ang-1) was highly asymmetric (~22 fold), confirming previous reports of its importance in HSC maintenance (Arai et al., 2004) and use as an asymmetry marker (Ito et al., 2012). Unexpectedly, we found CD34 to be also asymmetrically expressed in PDCs unlike in previously (chapter 2), where almost all cells expressed it once put in culture. This difference could be due to the fact HSCs here were activated in their native niche *in vivo* while previous experiments were carried out with un-manipulated cells.

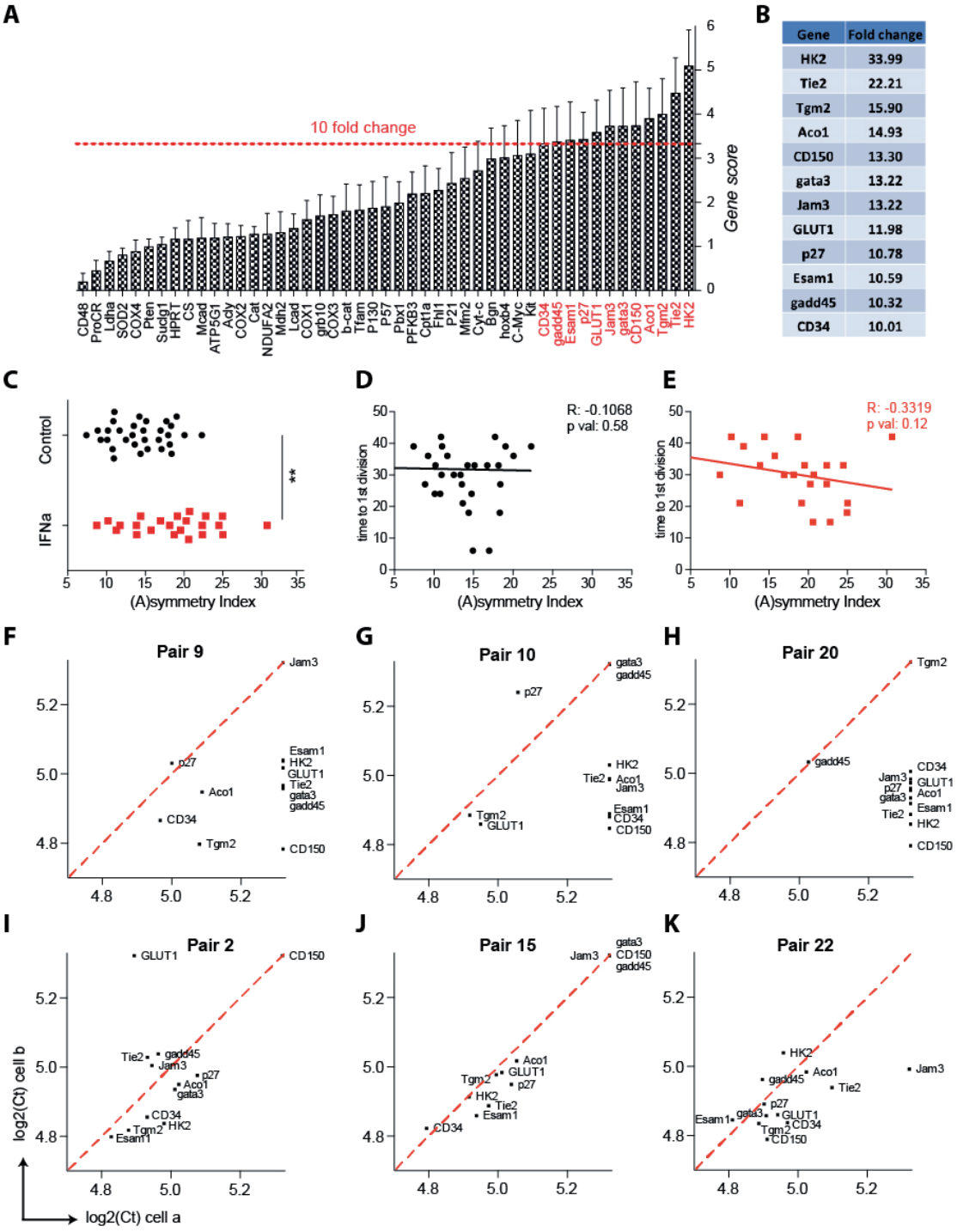

Figure 4.4: Identification of the asymmetric genes using PDC analysis. (**A**) Gene score values (of all 47 candidates) show a wide variation from gene to gene. A very stringent threshold (average 10-fold difference in gene expression between PDCs) was put to identify the "asymmetric gene set". (**B**) Table showing 12 candidates including some key metabolic genes that were seen to cross the 10-fold threshold (fold change values in table). (**C**) IFNa activated HSCs showed significantly higher values of (A)symmetry index suggesting increase in asymmetric divisions in this condition (N=32 for control, N-24 for IFNa). (**D-E**) Correlation analysis between time to 1st division and A.I value revealed that these two variables were negative correlated in both control (D) and in IFNa activated cells (E), with IFNa conditions showing higher value of negative correlation (r=-0.3319 for IFNa and -0.1068 for control). (**F-K**) Expression of asymmetric genes in various pairs from the IFNa condition. (F-H) Representative examples of pairs with high A.I values. Most genes were seen away from the symmetry diagonal (in red). (I-K) Representative examples of pairs with low A.I values. Most genes seen very close to the symmetry diagonal (in red). ***P< 0.001, **P< 0.01 and *P< 0.05

IFNa-activated HSCs show higher asymmetry than control PDCs

Having identified a set of asymmetrically expressed genes we next checked the asymmetry associated in individual pairs linked to this gene set. To obtain a single quantitative readout of symmetry for a pair we calculated the Euclidian distance between two cells of a pair by summing up the square ΔCt value for all genes in the "asymmetric" gene set and calculating its square root. We called this value as the (A)symmetry index (A.I) for a pair, with a higher value of A.I reflecting higher asymmetric gene expression.

$$A.I = \sqrt{\left(\sum_{i=1}^{N\ genes} (Ct\ \{cell\ a\} - Ct\ \{cell\ b\})^2 \right)}$$

Pairs with high A.I value (representative examples shown in Figure 4.4F-H) showed asymmetric expression of these genes and very often the expression was restricted to only one cell of the pair (Figure 4.4F-H). On the contrary, pairs with low A.I value (representative examples shown in Figure 4.4I-K) showed most genes being expressed symmetrically and were found very close to the diagonal (Figure 4.4I-K). A comparative analysis revealed that IFNa-activated pairs had significantly higher value of A.I than their control counterparts (Figure 4.4C).

From our previous cell cycle analysis (Figure 4.1B) we saw that IFNa treatment did not activate all HSCs, consistent with published data (Essers et al., 2009). Approximately 60% of the cells were cycling (Ki67+) while the remaining 40% were still in the quiescent state at the time of isolation (Figure 4.1B). We thus hypothesized that the activated fraction of cells would divide faster in our cultures and would have a higher possibility to carry out the niche-instructed program of asymmetric division, whereas cells that were still in the quiescent state at the time of isolation would be lacking the *in vivo* cues and therefore, execute an *in vitro* program that is niche-independent, resulting most likely in symmetric commitment divisions. To test this hypothesis we checked the correlation between A.I and time to 1st division. Intriguingly, we found that A.I and time to 1st division were negatively correlated (Figure 4.4D-E), and the extent of this negative correlation was higher in the IFNa activated cells (Figure 4.4E). This data supported the idea that HSCs have a higher propensity to divide asymmetrically when being instructed in the intact niche.

HSCs and progenitor populations demonstrate differential expression of metabolic genes

Having identified that metabolic genes Glut1, Aco1 and HK2 are asymmetrically expressed in PDCs (Figure 4.3), we next assessed the protein expression levels of these markers by flow cytometry. To this end, we stained different cell populations in the hematopoietic hierarchy with antibodies against these proteins. Strikingly, we

found that the most primitive population LT-HSCs (LKS CD150+CD48-CD34-) had a higher fraction of Aco1-low cells as compared to the more committed populations (ST-HSC: LKS CD150+CD48-CD34+ and MPP: LKS CD150-) (Figure 4.5B). These data supports our findings that low mitochondrial activity is a key determinant of HSC state (chapter 3). HK2 expression showed an opposite trend, with the fraction of HK2-low cells increasing in the MPPs compared to LT-HSCs (Figure 4.5A). This supports the notion that HSCs fulfill their energy needs through glycolysis while keeping their mitochondrial activity low (Ito and Suda, 2014; Simsek et al., 2010; Takubo et al., 2013). Interestingly, Glut1 expression showed a stepwise increase from the most primitive population (LT-HSCs) to the most committed population (MPPs) (Figure 4.5C). Repetition of this analysis will be carried out to confirm these findings.

Of note, previous flow cytometry analysis performed in the lab had identified three of these asymmetric gene candidates (Esam1, Tie2 and Jam3) to be highly expressed in LT-HSCs as compared to committed progenitor cells (Figure 4.8, check appendix, figure from Dr. Aline Roch's thesis). The differential expression pattern of these candidates in LT-HSCs and progenitors suggested that these genes could be used as potential markers to identify asymmetric cell division.

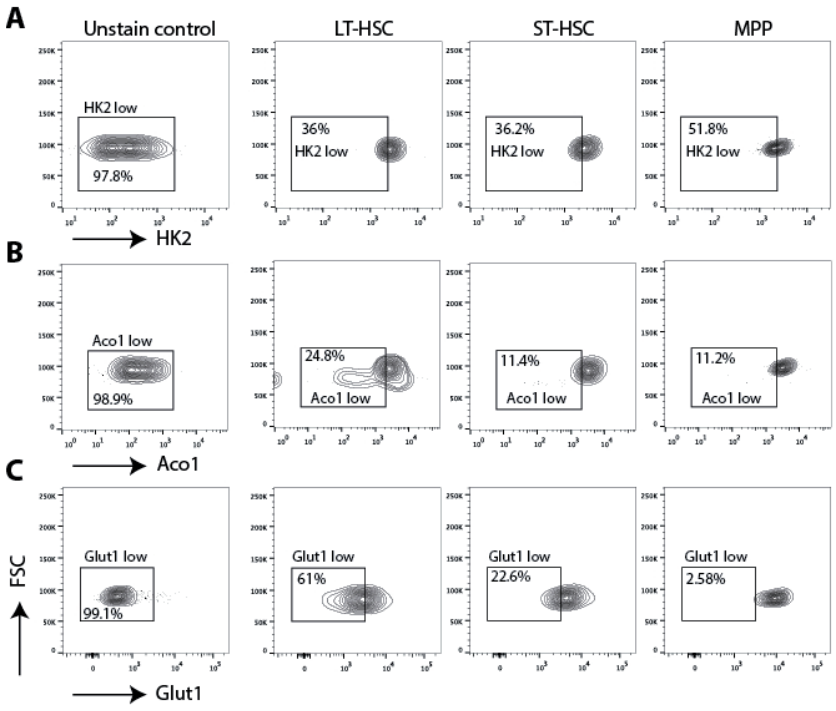

Figure 4.5: HSCs and progenitor populations demonstrate differential expression of metabolic genes. Expression of key metabolic markers identified in figure 4.4 was tested in various populations of the hematopoietic hierarchy using flow cytometry. Long term HSCs (LT-HSCs: LKSCD150+CD48-CD34-), short term HSCs (ST-HSCs: LKSCD150+CD48-CD34+) and multipotent progenitors (MPPs: LKSCD150-) were identified using a combination of cell surface markers. Cells were then fixed and stained (for HK2 and Aco1) or directly stained (for Glut1) to be analyzed by flow cytometry. (**A**) HK2 levels in HSCs were observed to be higher in HSCs than in MPP (HK2 low population increasing from ~36% to ~52% from LT-HSCs to MPPs). (**B**) Aco1 levels were observed to be low in LT-HSCs (Aco1 low ~25%) and increased in short term HSCs and MPPs (Aco1 low ~11%). (**C**) Glut1 levels changed dramatically from the LT-HSCs (Glut1 low ~61%) to ST-HSCs (Glut1 low ~23%) to MPPs (Glut1 low ~2.5%), with concomitant increase as one moves down in the hematopoietic hierarchy. (n=1)

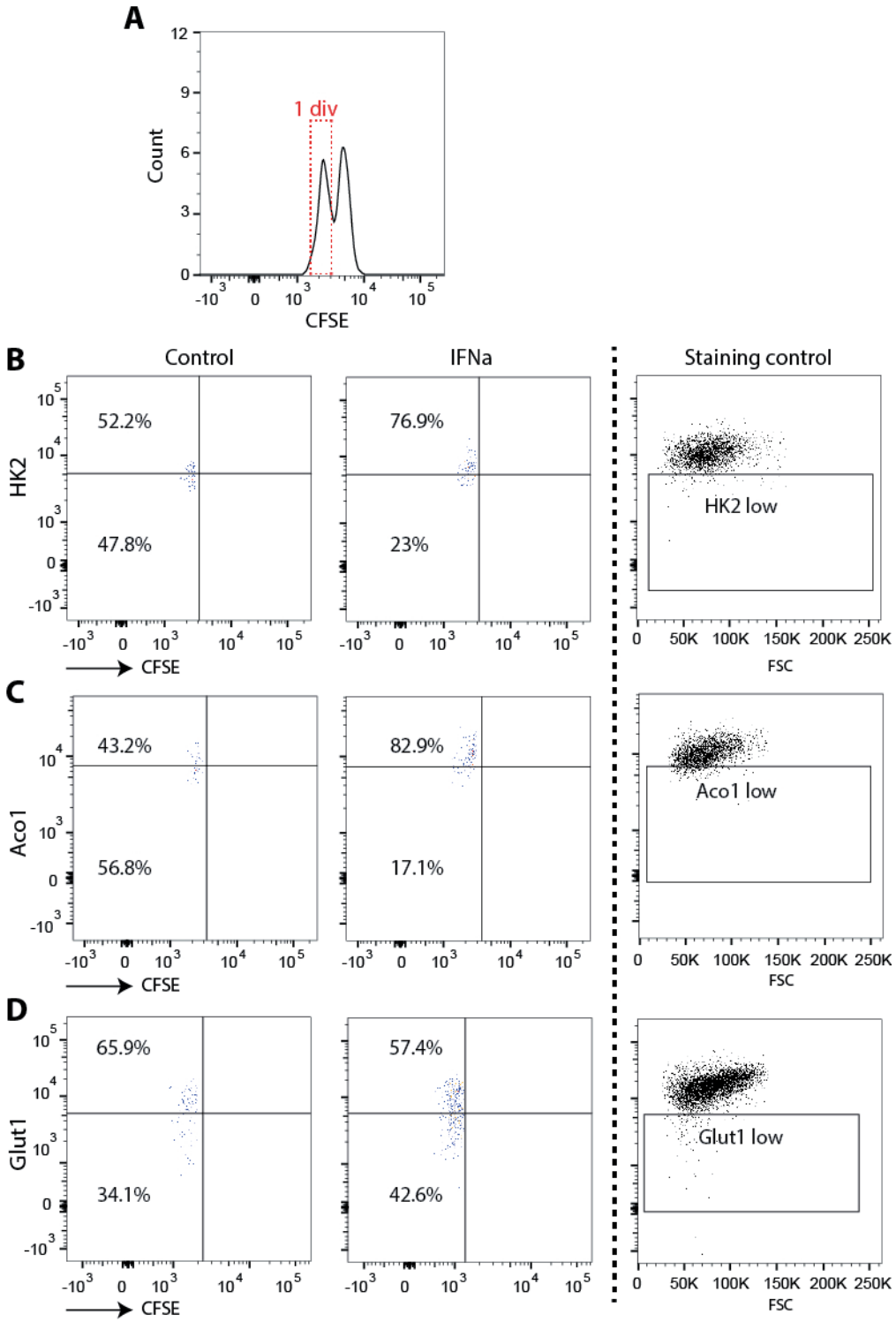

Figure 4.6: HSCs upon division show distinct expression levels of metabolic asymmetry markers. Divisional tracking (by CFSE) was combined with markers analysis to check the expression profile of the metabolic markers upon HSC division. (**A**) CFSE staining was used to identify HSC divisions and cells that underwent precisely one division were analyzed for their expression profiles in control and IFNa condition. (**B**) A large proportion of cells with high HK2 levels were seen in both conditions. (**C**) Aco1 low fraction in the control condition was found to be much higher than IFNa. This could be due to overall higher expression of Aco1 in IFNa treated cells (**D**) Glut1 staining also showed two distinct populations with different expression levels. (**B-D**) Since HSC cell number is often limiting to see distinct populations we used staining controls (LKS cells) to define low and high gates for our markers. (n=1)

We next tested whether a proportion of first generation of daughter cells maintains these newly identified metabolic stem cell characteristics, a high-HK2, low-Aco1 and low-Glut1 (Figure 4.5). Retention of these characteristics in cells post division would indicate an occurrence of ACD. To this end, we combined divisional tracking using our CFSE assay (described in chapter 2) with staining for the metabolic markers; based on the CFSE signal we identified cells that had divided precisely one time (Figure 4.6A). In both control and IFNa we found a large proportion of cells showing high HK2 levels (Figure 4.6B). Also, a substantial fraction retained the Glut1-low characteristic in both control and IFNa derived daughters (Figure 4.6D). Interestingly, Aco1-low fraction was found to be reduced in IFNa (17%) as compared to control (~57%) (Figure 4.6C). This could result from an overall higher expression of Aco1 in HSCs due to IFNa treatment. Since only a fraction of the first generation daughter cells retain HSC characteristics, it suggests that some HSCs carried out ACD. However, more repeats will be performed to confirm these initial findings

Conclusions

Here we describe a unique experimental strategy to assess niche-instructed HSC fate choices in an *in vitro* set up. By using acute IFNa treatment we could transiently activate HSCs in their native *in vivo* microenvironment. Strikingly, live single cell imaging revealed a significant increase in asynchronous divisions in activated HSCs, suggesting the execution of a niche-instructed asymmetric cell division (ACD) program. Using single-cell gene expression analysis in paired daughter cells (PDCs) we identified a group a 12 asymmetrically expressed genes, including enzymes from glycolytic and mitochondrial TCA cycle pathways. Moreover, PDCs arising from niche-instructed HSCs demonstrated a significant increase in asymmetry index value, a quantitative read out for asymmetric gene expression. Furthermore, preliminary flow cytometry analysis indicated that the protein expression of these metabolic candidates was different in HSCs compared to the progenitors, suggesting maintenance of a unique metabolic state in HSCs.

References

Arai, F., Hirao, A., Ohmura, M., Sato, H., Matsuoka, S., Takubo, K., Ito, K., Koh, G.Y., and Suda, T. (2004). Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche. Cell *118*, 149-161.

Beckmann, J., Scheitza, S., Wernet, P., Fischer, J.C., and Giebel, B. (2007). Asymmetric cell division within the human hematopoietic stem and progenitor cell compartment: identification of asymmetrically segregating proteins. Blood *109*, 5494-5501.

Chen, Y., Ma, X., Zhang, M., Wang, X., Wang, C., Wang, H., Guo, P., Yuan, W., Rudolph, K.L., Zhan, Q., *et al.* (2014). Gadd45a regulates hematopoietic stem cell stress responses in mice. Blood *123*, 851-862.

Cheng, T., Rodrigues, N., Shen, H., Yang, Y., Dombkowski, D., Sykes, M., and Scadden, D.T. (2000). Hematopoietic stem cell quiescence maintained by p21cip1/waf1. Science *287*, 1804-1808.

Cheshier, S.H., Morrison, S.J., Liao, X., and Weissman, I.L. (1999). In vivo proliferation and cell cycle kinetics of long-term self-renewing hematopoietic stem cells. Proc Natl Acad Sci U S A *96*, 3120-3125.

Congdon, K.L., and Reya, T. (2008). Divide and conquer: how asymmetric division shapes cell fate in the hematopoietic system. Current opinion in immunology *20*, 302-307.

Doe, C.Q., and Bowerman, B. (2001). Asymmetric cell division: fly neuroblast meets worm zygote. Current opinion in cell biology *13*, 68-75.

Duncan, A.W., Rattis, F.M., DiMascio, L.N., Congdon, K.L., Pazianos, G., Zhao, C., Yoon, K., Cook, J.M., Willert, K., Gaiano, N.*, et al.* (2005). Integration of Notch and Wnt signaling in hematopoietic stem cell maintenance. Nat Immunol *6*, 314-322.

Ema, H., Takano, H., Sudo, K., and Nakauchi, H. (2000). In vitro self-renewal division of hematopoietic stem cells. J Exp Med *192*, 1281-1288.

Essers, M.A., Offner, S., Blanco-Bose, W.E., Waibler, Z., Kalinke, U., Duchosal, M.A., and Trumpp, A. (2009). IFNalpha activates dormant haematopoietic stem cells in vivo. Nature *458*, 904-908.

Forsberg, E.C., Passegue, E., Prohaska, S.S., Wagers, A.J., Koeva, M., Stuart, J.M., and Weissman, I.L. (2010). Molecular signatures of quiescent, mobilized and leukemia-initiating hematopoietic stem cells. PLoS One *5*, e8785.

Forsberg, E.C., Prohaska, S.S., Katzman, S., Heffner, G.C., Stuart, J.M., and Weissman, I.L. (2005). Differential expression of novel potential regulators in hematopoietic stem cells. PLoS genetics *1*, e28.

Giebel, B., Zhang, T., Beckmann, J., Spanholtz, J., Wernet, P., Ho, A.D., and Punzel, M. (2006). Primitive human hematopoietic cells give rise to differentially specified daughter cells upon their initial cell division. Blood *107*, 2146-2152.

Ito, K., Carracedo, A., Weiss, D., Arai, F., Ala, U., Avigan, D.E., Schafer, Z.T., Evans, R.M., Suda, T., Lee, C.H.*, et al.* (2012). A PML-PPAR-delta pathway for fatty acid oxidation regulates hematopoietic stem cell maintenance. Nat Med.

Ito, K., and Suda, T. (2014). Metabolic requirements for the maintenance of self-renewing stem cells. Nat Rev Mol Cell Biol *15*, 243-256.

Kiel, M.J., Yilmaz, O.H., Iwashita, T., Terhorst, C., and Morrison, S.J. (2005). SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. Cell *121*, 1109-1121.

Kiger, A.A., Jones, D.L., Schulz, C., Rogers, M.B., and Fuller, M.T. (2001). Stem cell self-renewal specified by JAK-STAT activation in response to a support cell cue. Science *294*, 2542-2545.

Krosl, J., Austin, P., Beslu, N., Kroon, E., Humphries, R.K., and Sauvageau, G. (2003). In vitro expansion of hematopoietic stem cells by recombinant TAT-HOXB4 protein. Nat Med *9*, 1428-1432.

Ku, C.J., Hosoya, T., Maillard, I., and Engel, J.D. (2012). GATA-3 regulates hematopoietic stem cell maintenance and cell-cycle entry. Blood *119*, 2242-2251.

Liebermann, D.A., and Hoffman, B. (2007). Gadd45 in the response of hematopoietic cells to genotoxic stress. Blood cells, molecules & diseases *39*, 329-335.

Lutolf, M.P., Doyonnas, R., Havenstrite, K., Koleckar, K., and Blau, H.M. (2009). Perturbation of single hematopoietic stem cell fates in artificial niches. Integr Biol (Camb) *1*, 59-69.

Mathupala, S.P., Rempel, A., and Pedersen, P.L. (2001). Glucose catabolism in cancer cells: identification and characterization of a marked activation response of the type II hexokinase gene to hypoxic conditions. J Biol Chem *276*, 43407-43412.

Mello, C.C., Schubert, C., Draper, B., Zhang, W., Lobel, R., and Priess, J.R. (1996). The PIE-1 protein and germline specification in C. elegans embryos. Nature *382*, 710-712.

Morrison, S.J., and Kimble, J. (2006). Asymmetric and symmetric stem-cell divisions in development and cancer. Nature *441*, 1068-1074.

Nteliopoulos, G., and Gordon, M.Y. (2012). Protein segregation between dividing hematopoietic progenitor cells in the determination of the symmetry/asymmetry of cell division. Stem Cells Dev *21*, 2565-2580.

Nygren, J.M., Bryder, D., and Jacobsen, S.E. (2006). Prolonged cell cycle transit is a defining and developmentally conserved hemopoietic stem cell property. J Immunol *177*, 201-208.

Orford, K.W., and Scadden, D.T. (2008). Deconstructing stem cell self-renewal: genetic insights into cell-cycle regulation. Nat Rev Genet *9*, 115-128.

Osawa, M., Hanada, K., Hamada, H., and Nakauchi, H. (1996). Long-term lymphohematopoietic reconstitution by a single CD34-low/negative hematopoietic stem cell. Science *273*, 242-245.

Passegue, E., Wagers, A.J., Giuriato, S., Anderson, W.C., and Weissman, I.L. (2005). Global analysis of proliferation and cell cycle gene expression in the regulation of hematopoietic stem and progenitor cell fates. J Exp Med *202*, 1599-1611.

Perry, J.M., He, X.C., Sugimura, R., Grindley, J.C., Haug, J.S., Ding, S., and Li, L. (2011). Cooperation between both Wnt/{beta}-catenin and PTEN/PI3K/Akt signaling promotes primitive hematopoietic stem cell self-renewal and expansion. Genes Dev *25*, 1928-1942.

Satoh, Y., Matsumura, I., Tanaka, H., Ezoe, S., Sugahara, H., Mizuki, M., Shibayama, H., Ishiko, E., Ishiko, J., Nakajima, K.*, et al.* (2004). Roles for c-Myc in self-renewal of hematopoietic stem cells. J Biol Chem *279*, 24986-24993.

Shen, Q., Zhong, W., Jan, Y.N., and Temple, S. (2002). Asymmetric Numb distribution is critical for asymmetric cell division of mouse cerebral cortical stem cells and neuroblasts. Development *129*, 4843-4853.

Simsek, T., Kocabas, F., Zheng, J., Deberardinis, R.J., Mahmoud, A.I., Olson, E.N., Schneider, J.W., Zhang, C.C., and Sadek, H.A. (2010). The distinct metabolic profile of hematopoietic stem cells reflects their location in a hypoxic niche. Cell Stem Cell *7*, 380-390.

Song, X., Wong, M.D., Kawase, E., Xi, R., Ding, B.C., McCarthy, J.J., and Xie, T. (2004). Bmp signals from niche cells directly repress transcription of a differentiation-promoting gene, bag of marbles, in germline stem cells in the Drosophila ovary. Development *131*, 1353-1364.

Spana, E.P., Kopczynski, C., Goodman, C.S., and Doe, C.Q. (1995). Asymmetric localization of numb autonomously determines sibling neuron identity in the Drosophila CNS. Development *121*, 3489-3494.

Strome, S., and Wood, W.B. (1983). Generation of asymmetry and segregation of germ-line granules in early C. elegans embryos. Cell *35*, 15-25.

Suzuki, A., and Ohno, S. (2006). The PAR-aPKC system: lessons in polarity. J Cell Sci *119*, 979-987.

Takano, H., Ema, H., Sudo, K., and Nakauchi, H. (2004). Asymmetric division and lineage commitment at the level of hematopoietic stem cells: inference from differentiation in daughter cell and granddaughter cell pairs. J Exp Med *199*, 295-302.

Takubo, K., Goda, N., Yamada, W., Iriuchishima, H., Ikeda, E., Kubota, Y., Shima, H., Johnson, R.S., Hirao, A., Suematsu, M.*, et al.* (2010). Regulation of the HIF-1alpha level is essential for hematopoietic stem cells. Cell Stem Cell *7*, 391-402.

Takubo, K., Nagamatsu, G., Kobayashi, C.I., Nakamura-Ishizu, A., Kobayashi, H., Ikeda, E., Goda, N., Rahimi, Y., Johnson, R.S., Soga, T.*, et al.* (2013). Regulation of glycolysis by Pdk functions as a metabolic checkpoint for cell cycle quiescence in hematopoietic stem cells. Cell Stem Cell *12*, 49-61.

Ting, S.B., Deneault, E., Hope, K., Cellot, S., Chagraoui, J., Mayotte, N., Dorn, J.F., Laverdure, J.P., Harvey, M., Hawkins, E.D.*, et al.* (2012). Asymmetric segregation and self-renewal of hematopoietic stem and progenitor cells with endocytic Ap2a2. Blood *119*, 2510-2522.

Tulina, N., and Matunis, E. (2001). Control of stem cell self-renewal in Drosophila spermatogenesis by JAK-STAT signaling. Science *294*, 2546-2549.

Umemoto, T., Yamato, M., Nishida, K., Yang, J., Tano, Y., and Okano, T. (2005). p57Kip2 is expressed in quiescent mouse bone marrow side population cells. Biochem Biophys Res Commun *337*, 14-21.

Vannini, N., Roch, A., Naveiras, O., Griffa, A., Kobel, S., and Lutolf, M.P. (2012). Identification of in vitro HSC fate regulators by differential lipid raft clustering. Cell Cycle *11*, 1535-1543.

Wilson, A., Laurenti, E., Oser, G., van der Wath, R.C., Blanco-Bose, W., Jaworski, M., Offner, S., Dunant, C.F., Eshkind, L., Bockamp, E.*, et al.* (2008). Hematopoietic stem cells reversibly switch from dormancy to self-renewal during homeostasis and repair. Cell *135*, 1118-1129.

Wilson, A., Murphy, M.J., Oskarsson, T., Kaloulis, K., Bettess, M.D., Oser, G.M., Pasche, A.C., Knabenhans, C., Macdonald, H.R., and Trumpp, A. (2004). c-Myc controls the balance between hematopoietic stem cell self-renewal and differentiation. Genes Dev *18*, 2747-2763.

Wu, M., Kwon, H.Y., Rattis, F., Blum, J., Zhao, C., Ashkenazi, R., Jackson, T.L., Gaiano, N., Oliver, T., and Reya, T. (2007). Imaging hematopoietic precursor division in real time. Cell Stem Cell *1*, 541-554.

Yamamoto, R., Morita, Y., Ooehara, J., Hamanaka, S., Onodera, M., Rudolph, K.L., Ema, H., and Nakauchi, H. (2013). Clonal Analysis Unveils Self-Renewing Lineage-Restricted Progenitors Generated Directly from Hematopoietic Stem Cells. Cell *154*, 1112-1126.

Yamashita, Y.M., Fuller, M.T., and Jones, D.L. (2005). Signaling in stem cell niches: lessons from the Drosophila germline. J Cell Sci *118*, 665-672.

Zhang, J., Grindley, J.C., Yin, T., Jayasinghe, S., He, X.C., Ross, J.T., Haug, J.S., Rupp, D., Porter-Westpfahl, K.S., Wiedemann, L.M.*, et al.* (2006). PTEN maintains haematopoietic stem cells and acts in lineage choice and leukaemia prevention. Nature *441*, 518-522.

Zhong, W., Feder, J.N., Jiang, M.M., Jan, L.Y., and Jan, Y.N. (1996). Asymmetric localization of a mammalian numb homolog during mouse cortical neurogenesis. Neuron *17*, 43-53.

Zou, P., Yoshihara, H., Hosokawa, K., Tai, I., Shinmyozu, K., Tsukahara, F., Maru, Y., Nakayama, K., Nakayama, K.I., and Suda, T. (2011). p57(Kip2) and p27(Kip1) cooperate to maintain hematopoietic stem cell quiescence through interactions with Hsc70. Cell Stem Cell *9*, 247-261.

Chapter 5

Discussion and future perspectives

Several attempts to expand HSCs *in vitro* have failed, primarily due to the lack of instructive niche signals in the dish. A complex interplay of niche based signals, and intracellular mechanisms, are believed to guide HSC fate choices *in vivo* (Nakamura-Ishizu and Suda, 2013; Wang and Wagers, 2011; Wilson and Trumpp, 2006). Moreover, the hypoxic nature of the niche (Parmar et al., 2007; Spencer et al., 2014) presents an extreme metabolic environment, imposing HSCs to attain a unique metabolic identity in comparison to its committed progeny (Ito and Suda, 2014; Suda et al., 2011). This thesis has employed innovative experimental strategies to investigate a link between metabolism and HSC fate choices. A deeper understanding of the metabolic regulation of HSC fate will be crucial in developing novel assays for the expansion and identification of HSCs for their use in the clinic.

Divisional tracking of HSCs *in vitro*

In the first part of this thesis a cell-tracking strategy was developed to systematically follow HSC fates *in vitro.* Current methods involving PKH dyes only identify, very crudely, a fast and a slow cycling population *in vitro*, without any resolution on the precise number of divisions and more importantly, without any relationship to immunophenotypes (Lee et al., 2002; Yan et al., 2003).

Using CFSE, a cell permeable dye, we could reliably follow up to five HSC divisions *in vitro*. Moreover, CFSE-based tracking combined with immunostaining allowed mapping of the HSC phenotype across different divisional states, in defined culture conditions promoting specific HSC fates. Preliminary findings indicate that the self-renewing condition maintained the highest proportion of cells expressing the HSC phenotype, compared to the other conditions. Nevertheless, this difference became minimal after the second divisional state, suggesting the importance of the first two divisions in maintaining HSC phenotypes in culture. Intriguingly, we found a systematic loss of the HSC phenotype with increasing divisions in all conditions, supporting the current view in the field of cycling induced stem cell exhaustion (Orford and Scadden, 2008). However, functional assays still need to be performed to ascertain if this phenotype indeed reports *in vivo* long-term blood reconstitution potential.

This to our knowledge is a first attempt to rigorously characterize HSC phenotypes as a function of divisional history *in vitro*. Moreover, it opens up avenues to test the evolution of other stem cell markers in different divisional states. Some examples of the widespread use of this assay are reported in chapter 3 and 4 of this thesis, where it was successfully combined with different metabolic or intracellular markers.

Mitochondrial metabolism and HSC fate

There is growing evidence in the field supporting the role of metabolic cues from the niche in determining HSC fate *in vivo* (Ito and Suda, 2014; Suda et al., 2011). As

highlighted before, the hypoxic nature of the niche is believed to maintain HSCs in a distinct metabolic state (Takubo et al., 2010; Takubo et al., 2013).

In the second part of this thesis, we report a powerful approach of predicting, and directing, HSC fate based on mitochondrial metabolism. We found that phenotypic HSCs and the progenitor cells vary substantially in their mitochondrial activity profiles, suggesting that HSCs are indeed in a distinct metabolic state. Using long-term *in vivo* blood reconstitution assays, we showed that functional stem cells were restricted only to the low mitochondrial activity subpopulation of phenotypic HSCs, indicating a striking functional heterogeneity in phenotypically defined HSCs currently used in the field. This finding calls for the inclusion of metabolic readouts, such as mitochondrial activity, to the existing cell surface marker repertoire, to isolate functional HSCs from the bone marrow. Recent work in the field has implicated some mechanisms that facilitate maintenance of low mitochondrial activity in HSCs. For example, HIF1a was shown to up regulate key glycolytic enzymes in order to increase the glycolytic flux in HSCs. Moreover, low mitochondrial activity was maintained via up regulation of PDKs that inhibited PDH mediated conversion of pyruvate to acetyl-coA, and therefore reducing flux into the TCA cycle (Takubo et al., 2013).

A comparison of the mitochondrial activity of quiescent and cycling HSCs revealed overlapping profiles, indicating that distinct metabolic programs are rather indicative of fate choice and not per se a hallmark of the quiescent (versus activated) state. This particular finding goes against the current view in the field that links low metabolic activity to a quiescent cell cycle status (Ito and Suda, 2014; Suda et al., 2011). However, recent findings have demonstrated that rapidly dividing embryonic stem cells self-renew by maintaining low mitochondrial activity (Kondoh et al., 2007). Moreover, reprogramming of fibroblasts to induced pluripotent stem cells state, requires a metabolic switch from mitochondrial oxidative phosphorylation to glycolysis (Folmes et al., 2011). On similar lines, we believe, that maintaining this metabolic identity is a prerequisite for HSCs undergoing self-renewing divisions. Indeed, *in vitro* divisional tracking experiments (developed in chapter 2) in combination with long term *in vivo* assays, revealed that self-renewing HSCs retain low mitochondrial activity, in marked contrast to differentiated cells that show high mitochondrial activity.

Moreover, as the current set of cell surface markers have limited reliability for the identification of functional HSCs *in vitro* (Bunting et al., 1999; Dorrell et al., 2000; Zhang and Lodish, 2005), this single metabolic read out provides a robust marker for prospective isolation of self-renewing HSCs from heterogeneous *in vitro* cultures. However, a thorough functional analysis comparing surface marker expression and this metabolic read-out, in cultured HSCs still needs to be undertaken.

Strikingly, we found artificial lowering of mitochondrial activity could induce self-renewal fate under conditions that normally would induce rapid commitment.

Therefore, we believe HSC mitochondrial metabolism is not a mere adaptation to the *in vivo* hypoxic microenvironment but is a determinant of HSC fate. However, whether such modulation can be induced *in vivo*, and its effect on HSC function still needs to be ascertained.

Asymmetric HSC divisions

Amongst the different fate choices that an HSC can undertake *in vivo*, asymmetric cell division (ACD) appears to be the most relevant one under homeostasis (Congdon and Reya, 2008; Morrison and Kimble, 2006). The production of one stem and one committed daughter cell, allows the organism to maintain stem cell number and give rise to various blood cell lineages at the same time. However, absence of niche-based instructive signals *in vitro*, result in rapid commitment of HSCs. Due to this limitation it is currently impossible to study self-renewal and ACD in a dish. In the last part of this thesis we report a unique experimental strategy to analyze niche-instructed fate choices in an *in vitro* set up. Using acute IFNa treatment (Essers et al., 2009), we could transiently activate HSCs from their quiescent state in the native *in vivo* environment. Analysis on cell-cycle properties and gene expression profiles of paired daughter cells (PDCs), arising from these activated HSCs provided key insights on niche-instructed programs guiding HSC fate.

Live cell imaging revealed a significant increase in asynchronous divisions in activated HSCs, suggesting the execution of a niche-instructed ACD program. Previous studies in invertebrates have elegantly demonstrated the role of the niche in orchestrating ACD. For example, in *Drosophila*, close association with the niche (cap cell in the ovary and hub cell in the testis) is instrumental in the execution of ACD of germline stem cells. Instructive cues from the niche lead to the activation of BMP (in ovary) or JAK-STAT (in testis) signaling, which eventually represses differentiation in the daughter cell destined to maintain stemness (Kiger et al., 2001; Song et al., 2004; Tulina and Matunis, 2001; Yamashita et al., 2005). PDC analyses in the hematopoietic system have revealed, different colony forming potential (Giebel et al., 2006; Takano et al., 2004) and *in vivo* reconstitution ability (Ema et al., 2000; Yamamoto et al., 2013), in sister cells, suggesting the occurrence of ACD in HSCs. However, the precise mechanisms that regulate this fate choice are currently unknown.

Gene expression analysis led to the identification of a group of 12 asymmetrically expressed genes in on PDCs. Importantly, the three metabolic genes in this group belonged to either the glycolytic (HK2, Glut1) or mitochondrial (Aco1) pathways, suggesting the role of these metabolic pathways in mediating niche-instructed HSC fate choice. Recent studies have implicated hypoxia-inducible factor (HIF1a) in promoting glycolytic- and inhibiting mitochondrial-flux in HSCs *in vivo* (Takubo et al., 2013). However, our cultures were carried out under normoxia, where HIF1a is actively degraded (Suda et al., 2011). It will be interesting to see the expression of

these metabolic genes in HSCs cultured under hypoxic conditions. Additionally, it needs to be tested if the expression of these metabolic genes can be regulated by other mechanisms in HSCs. Moreover, long-term blood reconstitution assays will be necessary to ascertain whether asymmetry in gene expression corresponds to asymmetric fate of daughter cells.

Perspective on human HSCs

The characterization of the human hematopoietic system began fairly recently. With the development of xenotransplantation models and robust functional assays, it is now possible to study the human hematopoietic hierarchy (Doulatov et al., 2012). Engraftment of human cells in severe combined immune deficient (Scid) mice allowed for their marker characterization. Surprisingly, a lack of consistency between the mouse and human HSC markers was observed; for example, human HSCs express FLT3 receptor (Sitnicka et al., 2003) while mouse cells do not, and mouse HSCs express CD150 while human cells do not (Larochelle et al., 2011). Although, many groups now believe HSCs are enriched in the CD34+ CD38- Thy1+ CD45RA- compartment (Baum et al., 1992; Bhatia and McGlave, 1997; Conneally et al., 1997; Lansdorp et al., 1990), there are contrasting reports of the existence of a rare CD34- population, that is extremely quiescent and harbors stem cell potential (Anjos-Afonso et al., 2013). With this discrepancy in mind it will be useful to check if low mitochondrial activity (independent or in addition to these markers) can be used to identify functional HSCs from human samples. Moreover, prospective isolation of human HSCs based on the mitochondrial activity read-out from heterogeneous *in vitro* cultures could be a boon for their use in the clinic. Furthermore, clinically approved mitochondrial modulators could be tested for their capacity to expand human HSCs in culture. Additionally, *in vivo* modulation of HSC mitochondrial metabolism, in principle can induce faster blood production and therefore, could expedite the recovery of patients post-transplantation. An interesting avenue that still remains unexplored is the effect of mitochondrial activity modulation on bone marrow niche cells.

References

Anjos-Afonso, F., Currie, E., Palmer, H.G., Foster, K.E., Taussig, D.C., and Bonnet, D. (2013). CD34(-) cells at the apex of the human hematopoietic stem cell hierarchy have distinctive cellular and molecular signatures. Cell Stem Cell *13*, 161-174.

Baum, C.M., Weissman, I.L., Tsukamoto, A.S., Buckle, A.M., and Peault, B. (1992). Isolation of a candidate human hematopoietic stem-cell population. Proc Natl Acad Sci U S A *89*, 2804-2808.

Bhatia, R., and McGlave, P.B. (1997). Autologous stem cell transplantation for the treatment of chronic myelogenous leukemia. Cancer treatment and research *77*, 357-374.

Bunting, K.D., Galipeau, J., Topham, D., Benaim, E., and Sorrentino, B.P. (1999). Effects of retroviral-mediated MDR1 expression on hematopoietic stem cell self-renewal and differentiation in culture. Ann N Y Acad Sci *872*, 125-140; discussion 140-121.

Congdon, K.L., and Reya, T. (2008). Divide and conquer: how asymmetric division shapes cell fate in the hematopoietic system. Current opinion in immunology *20*, 302-307.

Conneally, E., Cashman, J., Petzer, A., and Eaves, C. (1997). Expansion in vitro of transplantable human cord blood stem cells demonstrated using a quantitative assay of their lympho-myeloid repopulating activity in nonobese diabetic-scid/scid mice. Proc Natl Acad Sci U S A *94*, 9836-9841.

Dorrell, C., Gan, O.I., Pereira, D.S., Hawley, R.G., and Dick, J.E. (2000). Expansion of human cord blood CD34(+)CD38(-) cells in ex vivo culture during retroviral transduction without a corresponding increase in SCID repopulating cell (SRC) frequency: dissociation of SRC phenotype and function. Blood *95*, 102-110.

Doulatov, S., Notta, F., Laurenti, E., and Dick, J.E. (2012). Hematopoiesis: a human perspective. Cell Stem Cell *10*, 120-136.

Ema, H., Takano, H., Sudo, K., and Nakauchi, H. (2000). In vitro self-renewal division of hematopoietic stem cells. J Exp Med *192*, 1281-1288.

Essers, M.A., Offner, S., Blanco-Bose, W.E., Waibler, Z., Kalinke, U., Duchosal, M.A., and Trumpp, A. (2009). IFNalpha activates dormant haematopoietic stem cells in vivo. Nature *458*, 904-908.

Folmes, C.D., Nelson, T.J., Martinez-Fernandez, A., Arrell, D.K., Lindor, J.Z., Dzeja, P.P., Ikeda, Y., Perez-Terzic, C., and Terzic, A. (2011). Somatic oxidative bioenergetics transitions into pluripotency-dependent glycolysis to facilitate nuclear reprogramming. Cell Metab *14*, 264-271.

Giebel, B., Zhang, T., Beckmann, J., Spanholtz, J., Wernet, P., Ho, A.D., and Punzel, M. (2006). Primitive human hematopoietic cells give rise to differentially specified daughter cells upon their initial cell division. Blood *107*, 2146-2152.

Ito, K., and Suda, T. (2014). Metabolic requirements for the maintenance of self-renewing stem cells. Nat Rev Mol Cell Biol *15*, 243-256.

Kiger, A.A., Jones, D.L., Schulz, C., Rogers, M.B., and Fuller, M.T. (2001). Stem cell self-renewal specified by JAK-STAT activation in response to a support cell cue. Science *294*, 2542-2545.

Kondoh, H., Lleonart, M.E., Nakashima, Y., Yokode, M., Tanaka, M., Bernard, D., Gil, J., and Beach, D. (2007). A high glycolytic flux supports the proliferative potential of murine embryonic stem cells. Antioxid Redox Sign *9*, 293-299.

Lansdorp, P.M., Sutherland, H.J., and Eaves, C.J. (1990). Selective expression of CD45 isoforms on functional subpopulations of CD34+ hemopoietic cells from human bone marrow. J Exp Med *172*, 363-366.

Larochelle, A., Savona, M., Wiggins, M., Anderson, S., Ichwan, B., Keyvanfar, K., Morrison, S.J., and Dunbar, C.E. (2011). Human and rhesus macaque hematopoietic stem cells cannot be purified based only on SLAM family markers. Blood *117*, 1550-1554.

Lee, G.M., Fong, S.S., Oh, D.J., Francis, K., and Palsson, B.O. (2002). Characterization and efficacy of PKH26 as a probe to study the replication history of the human hematopoietic KG1a progenitor cell line. In vitro cellular & developmental biology Animal *38*, 90-96.

Morrison, S.J., and Kimble, J. (2006). Asymmetric and symmetric stem-cell divisions in development and cancer. Nature *441*, 1068-1074.

Nakamura-Ishizu, A., and Suda, T. (2013). Hematopoietic stem cell niche: an interplay among a repertoire of multiple functional niches. Biochim Biophys Acta *1830*, 2404-2409.

Orford, K.W., and Scadden, D.T. (2008). Deconstructing stem cell self-renewal: genetic insights into cell-cycle regulation. Nat Rev Genet *9*, 115-128.

Parmar, K., Mauch, P., Vergilio, J.A., Sackstein, R., and Down, J.D. (2007). Distribution of hematopoietic stem cells in the bone marrow according to regional hypoxia. Proc Natl Acad Sci U S A *104*, 5431-5436.

Sitnicka, E., Buza-Vidas, N., Larsson, S., Nygren, J.M., Liuba, K., and Jacobsen, S.E. (2003). Human CD34+ hematopoietic stem cells capable of multilineage engrafting NOD/SCID mice express flt3: distinct flt3 and c-kit expression and response patterns on mouse and candidate human hematopoietic stem cells. Blood *102*, 881-886.

Song, X., Wong, M.D., Kawase, E., Xi, R., Ding, B.C., McCarthy, J.J., and Xie, T. (2004). Bmp signals from niche cells directly repress transcription of a differentiation-promoting gene, bag of marbles, in germline stem cells in the Drosophila ovary. Development *131*, 1353-1364.

Spencer, J.A., Ferraro, F., Roussakis, E., Klein, A., Wu, J., Runnels, J.M., Zaher, W., Mortensen, L.J., Alt, C., Turcotte, R.*, et al.* (2014). Direct measurement of local oxygen concentration in the bone marrow of live animals. Nature *508*, 269-273.

Suda, T., Takubo, K., and Semenza, G.L. (2011). Metabolic regulation of hematopoietic stem cells in the hypoxic niche. Cell Stem Cell *9*, 298-310.

Takano, H., Ema, H., Sudo, K., and Nakauchi, H. (2004). Asymmetric division and lineage commitment at the level of hematopoietic stem cells: inference from differentiation in daughter cell and granddaughter cell pairs. J Exp Med *199*, 295-302.

Takubo, K., Goda, N., Yamada, W., Iriuchishima, H., Ikeda, E., Kubota, Y., Shima, H., Johnson, R.S., Hirao, A., Suematsu, M.*, et al.* (2010). Regulation of the HIF-1alpha level is essential for hematopoietic stem cells. Cell Stem Cell *7*, 391-402.

Takubo, K., Nagamatsu, G., Kobayashi, C.I., Nakamura-Ishizu, A., Kobayashi, H., Ikeda, E., Goda, N., Rahimi, Y., Johnson, R.S., Soga, T.*, et al.* (2013). Regulation of

glycolysis by Pdk functions as a metabolic checkpoint for cell cycle quiescence in hematopoietic stem cells. Cell Stem Cell *12*, 49-61.

Tulina, N., and Matunis, E. (2001). Control of stem cell self-renewal in Drosophila spermatogenesis by JAK-STAT signaling. Science *294*, 2546-2549.

Wang, L.D., and Wagers, A.J. (2011). Dynamic niches in the origination and differentiation of haematopoietic stem cells. Nat Rev Mol Cell Biol *12*, 643-655.

Wilson, A., and Trumpp, A. (2006). Bone-marrow haematopoietic-stem-cell niches. Nat Rev Immunol *6*, 93-106.

Yamamoto, R., Morita, Y., Ooehara, J., Hamanaka, S., Onodera, M., Rudolph, K.L., Ema, H., and Nakauchi, H. (2013). Clonal Analysis Unveils Self-Renewing Lineage-Restricted Progenitors Generated Directly from Hematopoietic Stem Cells. Cell *154*, 1112-1126.

Yamashita, Y.M., Fuller, M.T., and Jones, D.L. (2005). Signaling in stem cell niches: lessons from the Drosophila germline. J Cell Sci *118*, 665-672.

Yan, F., Collector, M.I., Tyszko, S., and Sharkis, S.J. (2003). Using divisional history to measure hematopoietic stem cell self-renewal and differentiation. Exp Hematol *31*, 56-64.

Zhang, C.C., and Lodish, H.F. (2005). Murine hematopoietic stem cells change their surface phenotype during ex vivo expansion. Blood *105*, 4314-4320.

Appendix

Figures related to chapter 2

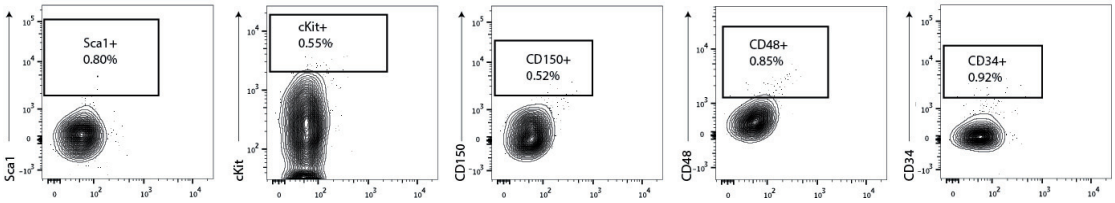

Figure 2.6: Unstained control used for gate setting in different channels

Figures related to chapter 3

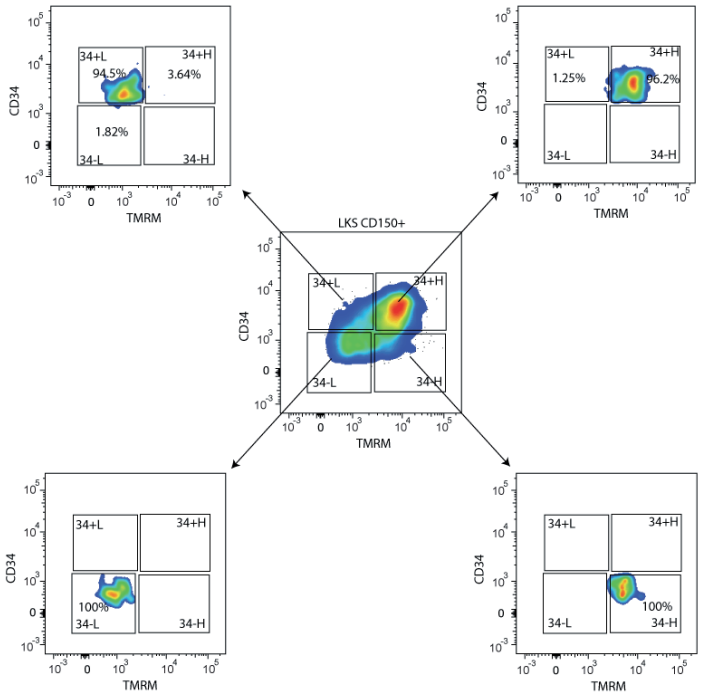

Figure 3.6: Purity analysis on different sorted populations. Four populations were sorted based on CD34 and TMRM staining. Re-sorting of the purified cells clearly shows that they remain exclusively in their respective gates, proving that our metabolically defined populations are distinct and stable.

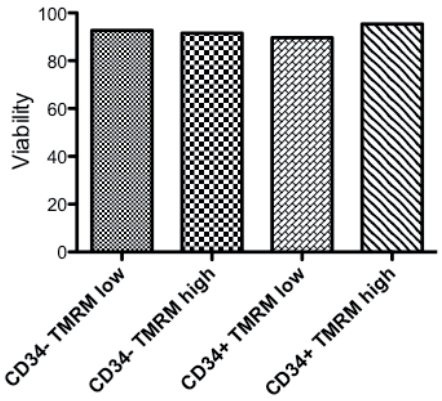

Figure 3.7: Viability analysis of identified populations.
The four indicated HSC populations were sorted and cultured in vitro for 24 hours, followed by Propidium Iodide (PI) staining to assess cell viability. Flow cytometry analysis shows no difference in the PI negative (viable) fraction in the four fractions, ruling out that TMRM staining is related to differences in cell death.

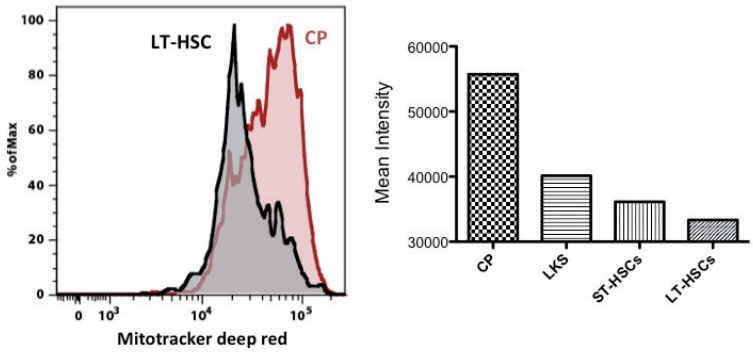

Figure 3.8: MitoTracker® Deep Red analysis of different hematopoietic stem and progenitor compartments. Whole bone marrow cells were stained with MitoTracker® (labeling active mitochondria) and HSC markers, and then analyzed by flow cytometry. MitoTracker® staining shows a similar step-wise increase in intensity from the most primitive to most committed population as already demonstrated for TMRM (Figure 3.1B).

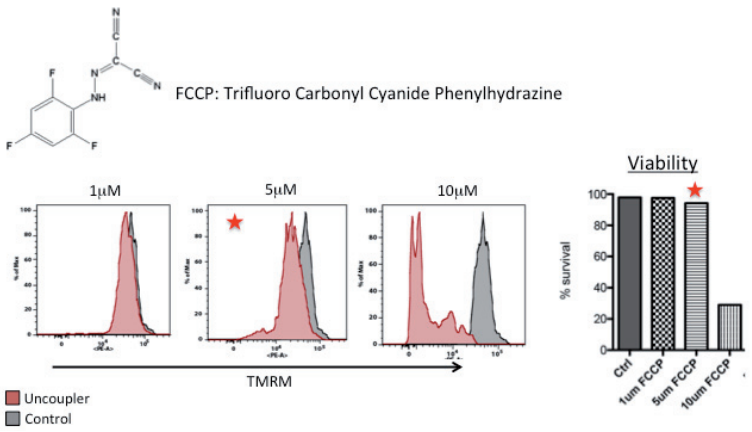

Figure 3.9: Titration of FCCP-mediated uncoupling. Sorted HSCs were cultured in differentiation condition with or without (Ctrl) FCCP for 5 days. FCCP was replenished in the culture media every 24 hours. Concentration-dependent increase in inhibition of mitochondrial activity was seen upon addition of FCCP. 5μM showed lowering of mitochondrial activity without affecting cell viability.

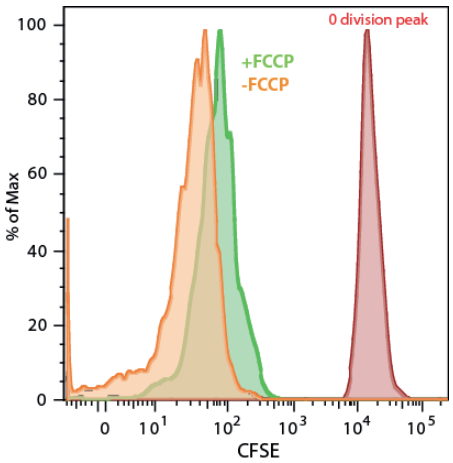

Figure 3.10: CFSE analysis of cultured HSCs in the presence of the uncoupler FCCP. HSCs were isolated from the bone marrow and stained with CFSE followed by a 5-day culture in differentiation condition with or without FCCP. CFSE analysis at the end of culture period demonstrated that all cells had undergone several rounds of division in both conditions, ruling out the presence of quiescent cells in the two conditions.

Figures related to chapter 4

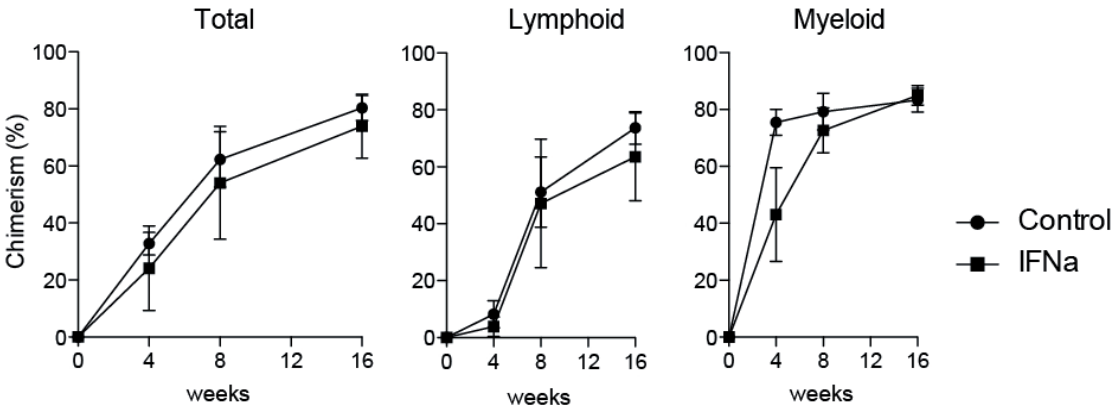

Figure 4.7: IFNa activation does not result in loss of stem cell potential. HSCs isolated from control and IFNa treated mice show similar blood reconstitution levels in irradiated recipients. Moreover, reconstitution was observed in both lymphoid and myeloid lineage.

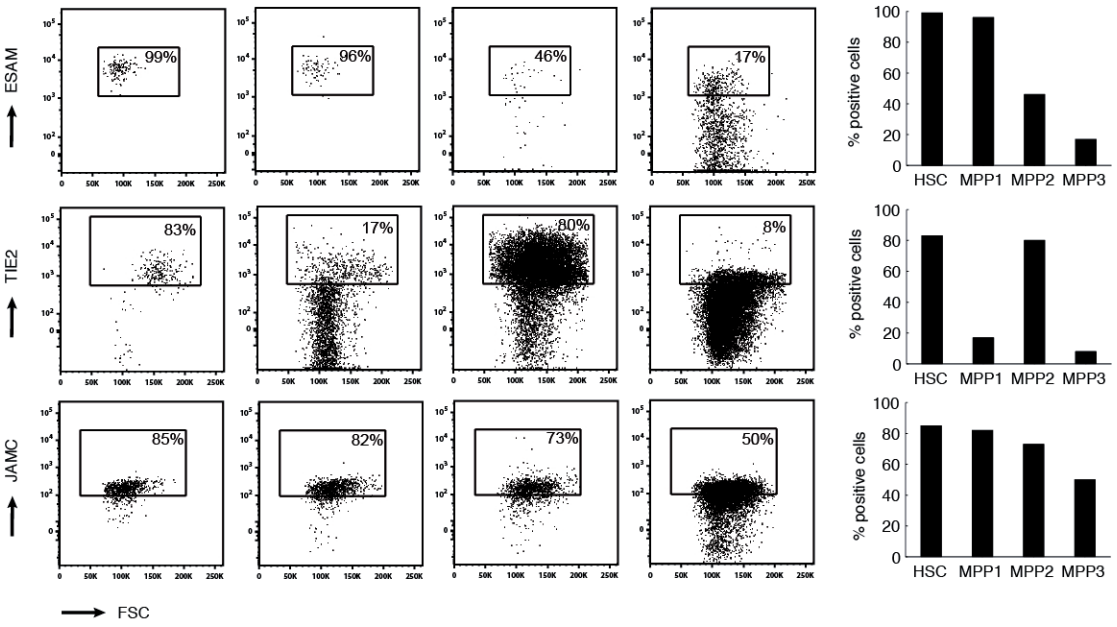

Figure 4.8: Surface expression of Esam1, Tie2 and JamC (same as Jam3) on HSCs and progenitor populations. (Figure from Dr. Aline Roch's thesis). HSCs showed higher expression levels of Esam1, Tie2 and Jam3 as compared to progenitor cells. Different populations were identified with the following cell surface markers; HSC: LKSCD150+CD48-CD34-, MPP1: LKSCD150+CD48-CD34+, MPP2: LKSCD150+CD48+, MPP3: LKSCD150-CD48+

Table 4.2: List of genes tested and their taqman assay IDs

Classification	Gene name	Other name	Taqman assay ID
House keeping gene	HPRT		Mm00446968_m1
ECM proteins	Tgm2		Mm00436987_m1
	Bgn		Mm00455918_m1
Membrane proteins	Esam1	Esam	Mm00518378_m1
	Tie2	TEK	Mm00443243_m1
	Jam3	JamC	Mm00499214_m1
HSC markers	CD150	SLAMf1	Mm00443316_m1
	CD48	SLAMf2	Mm00455932_m1
	CD34		Mm00519283_m1
	C-kit	CD117	Mm00445212_m1
Intracellular adaptors	Grb10		Mm01180443_m1
	ProCR	EPCR	Mm00440992_m1
	Fhl1		Mm03009774_m1
	b-catenin	Cat	Mm00483039_m1
Cell cycle regulators	P57	cdkn1c	Mm00438170_m1
	P27	cdkn1b	Mm00438168_m1
	P21	cdkn1a	Mm00432448_m1
	P130	rab3gap	Mm00618533_m1
	Pten		Mm00477208_m1
Transcription factors	Pbx1		Mm01701537_m1
	Gata3		Mm00484683_m1
	c-myc		Mm00487803_m1
	Hoxb4		Mm00657964_m1
DNA repair	Gadd45		Mm00432802_m1
Glycolysis	HK2		Mm00443385_m1
	PFKFB3		Mm00504650_m1
	Glut1		Mm00441480_m1
	Ldha		Mm01612132_g1
TCA cycle	CS		Mm00466043_m1
	Acly		Mm01302282_m1
	Aco1		Mm00801417_m1
	Suclg1		Mm00451244_m1
	Mdh2		Mm01208232_m1
Oxidative Phosphorylation	Cyt-C		Mm01621048_s1
	NDUFA2		Mm00477755_g1
	COX2	Sdhd	Mm00546511_m1
	ATP5g1	ATP synthase	Mm02601566_g1
	COX1	cyt c oxidase subunit I	Mm04225243_g1
	COX3	cyt c oxidase subunit III	Mm04225261_g1
	COX4	cyt c oxidase subunit IV	Mm01250094_m1
Mitochondrial biogenesis	Mfn2		Mm00500120_m1
	Tfam		Mm00447485_m1
Antioxidants	SOD2		Mm01313000_m1
	Cat		Mm00437992_m1
Fatty acid oxidation	MCad		Mm00431611_m1
	LCad		Mm00599660_m1
	CPT1a		Mm01231183_m1

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Patents

1. Vannini N, **Girotra M**, Naveiras O, Auwerx J, Lutolf MP (2014)
   Methods & Compounds Useful In Hematopoietic Stem Cell Medicine (filed Sept 2014)

Publications

1. Vannini N*, **Girotra M***, Naveiras O, Campos V, Williams E, Roch A, Auwerx J, Lutolf MP.
   Mitochondrial activity determines hematopoietic stem cell fate decisions **(in review).**
   (***Equal Contribution**)

2. Roch A, **Girotra M**, Campos V, Vannini N, Gobaa S, Naveiras O, Lutolf MP.
   Identification of functional artificial niches by single hematopoietic stem cell fate analyses **(in review).**

3. Kobel S, Burri O, Griffa A, **Girotra M**, Seitz A, Lutolf MP.
   Automated analysis of single cells in microfluidic traps.
   Lab Chip. 2012 Aug 21;12(16):2843-9.

4. Sadananda A, Hamid R, Doodhi H, Ghosal D, **Girotra M**, Jana SC, Ray K.
   Interaction with a kinesin-2 tail propels choline acetyltransferase flow towards synapse.
   Traffic. 2012 Jul;13(7):979-91

5. Jana SC, **Girotra M,** Ray K.
   Heterotrimeric kinesin-II is necessary and sufficient to promote different stepwise assembly of morphologically distinct bipartite cilia in Drosophila antenna.
   Mol Biol Cell. 2011 Mar 15;22(6):769-81

Oral and Poster presentations

- Apr 2015 University of Edinburgh, Cancer research UK, Scotland (Oral)
- Mar 2015 Functional Genomics seminar series, Lausanne, Switzerland (Oral)
- May 2014 Stem cell retreat, Basel, Switzerland (Oral)
- May 2014 Functional Genomics seminar series, Lausanne, Switzerland (Oral)
- Sept 2012 Stem Cell Summer School, Hydra, Greece (Poster)
- Oct 2011 Stem Cell Retreat, Charmey, Switzerland (Poster)
- Sept 2009 Young Explorers In Indian Biology, TIFR, Mumbai, India (Poster)
- Sept 2009 Department of Biology (DBS), TIFR, Annual Scientific meet, Mumbai, India (Poster)