CHARACTERIZING A SIGNALING NETWORK

THAT MAINTAINS HEMATOPOIETIC STEM CELLS

Michelle Nguyen-McCarty

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Supervisor of Dissertation

____

Peter Klein, M.D., PhD.

Professor of Medicine

Graduate Group Chairperson

____

Daniel Kessler, Ph.D.

Associate Professor of Cell and Developmental Biology

Dissertation Committee:

Avinash Bhandoola, Ph.D., Senior Investigator, National Cancer Institute

Sarah Millar, Ph.D., Albert M. Kligman Professor in Dermatology

Warren Pear, M.D., Ph.D., Gaylord P. and Mary Louise Harnwell Professor

Nancy Speck, Ph.D., Professor of Cell and Developmental Biology

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ABSTRACT

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Michelle Nguyen-McCarty

Peter Klein

Hematopoietic stem cells (HSCs) are able to self-renew and to differentiate into all blood cells. HSCs reside in a low-perfusion niche and depend on local signals to survive and to maintain the capacity for self-renewal. HSCs removed from the niche can survive if they receive hematopoietic cytokines, but they then lose the ability to self-renew. However, we showed previously that simultaneous inhibition of glycogen synthase kinase-3 (GSK-3) and mammalian target of rapamycin complex 1 (mTORC1) maintains HSC function *ex vivo* without the need for exogenous cytokines. As these experiments were initially done in heterogeneous cell populations, I then showed that purified HSCs can also be maintained under these conditions, demonstrating a direct effect of GSK-3 and mTORC1 inhibition on HSCs. Although Wnt/β-catenin signaling downstream of GSK-3 is required for this response, the downstream effectors of this network remained otherwise undefined. I therefore explored targets downstream of GSK-3 and mTORC1. I found that HSCs express a pro-autophagic gene signature and accumulate LC3 puncta only when both mTORC1 and GSK-3 are inhibited, identifying autophagy as a signature for a signaling network that maintains HSCs *ex vivo*. In contrast, I did not find evidence to support a role for other downstream targets of mTORC1, such as protein translation and mitochondrial biogenesis. I also report a significant reduction in total RNA content in cultured HSCs and describe a method to perform transcriptional profiling of these cells. Together, these findings provide new insight into the relative contributions of various mTORC1 outputs toward the maintenance of HSC function and build upon the growing

body of literature implicating autophagy and tightly controlled protein synthesis as important modulators of diverse stem cell populations.

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CHAPTER 1: INTRODUCTION

Introduction to hematopoietic stem cells (HSCs)

Somatic stem cells are rare populations of cells found in a range of tissues throughout the majority of post-natal life. Distinguished by their unique capacity to both self-renew and give rise to differentiated cell types, stem cells are essential for lifelong tissue maintenance, both under homeostatic conditions and in response to stress or injury. This critical function endows stem cells with vast potential therapeutic value, yet to date their application in the clinic remains limited due in part to an incomplete understanding of the molecular mechanisms and niche interactions that regulate stem cell function.

HSCs, which maintain the blood, are generally considered the best-characterized stem cell population, owing to their relative accessibility and early identification in landmark work by Till and McCulloch (1961) and others during and shortly after World War II. Sixty years later, the HSC transplant (HSCT) is the current standard of care for a variety of congenital and acquired hematological diseases, with over 50,000 performed per year globally (Gratwohl et al., 2010).

However, the therapy remains plagued by rates of complications and mortality as high as 40-50%, depending on factors such as patient age and disease stage at time of treatment (Copelan, 2006; Juric et al., 2016). One major limitation to the success of HSCTs is the limited availability of suitable donor cells. While it is now established that a high level of histocompatibility matching is essential to transplant success, less than 30% of potential transplant recipients have an HLA-matched sibling (Copelan, 2006), and adult bone marrow donor registries currently fail to meet remaining demand. Transplantation of umbilical cord blood (UCB) from unrelated donors has therefore become a strategy to increase the pool of potential donors. An additional benefit of UCB transplantation is that less stringent HLA-matching is necessary between donor and recipient compared to transplantation of adult peripheral blood or bone marrow. However, immune reconstitution is slower following UCB transplant compared to adult HSCT (Juric et al., 2016). In addition, the number of HSCs transplanted directly correlates with the probability of successful engraftment, and a single UCB unit contains a suboptimal number of HSCs to reconstitute the hematopoietic system of a full-sized adult (Hagedorn et al., 2014). Double-UCB unit transplantation accelerates neutrophil engraftment, improves overall engraftment, and post-transplant survival compared to single-unit transplantation. However, this strains cord blood bank resources and increases transplant costs, and ultimately a single unit dominates engraftment (Barker et al., 2005).

The advent of targeted genome editing, such as by CRISPR/Cas9 technology, has made gene therapy a powerful potential application for HSCTs. Gene-corrected HSCs could be used for autologous HSCT in the treatment of monogenic hematopoietic disorders such as sickle cell anemia, Diamond-Blackfan anemia, and severe combined immunodeficiency. However, the relatively low efficiency of gene editing necessitates the availability of more HSCs (Watts et al., 2011). The ability to expand and maintain HSCs *ex vivo* would moreover be a powerful tool for deeper investigation of the molecular regulation of HSCs, as well as for disease modeling and drug screening. There remains thus a tremendous need for the ability to generate expanded numbers of HSCs.

Approaches to expand HSCs

Multiple approaches are being pursued to produce or expand HSCs. The most direct scheme, *ex vivo* expansion of preexisting UCB HSCs, has yielded mixed success. Cytokine cocktails, either alone (Zhang et al., 2008) or in combination with small molecules (Boitano et al., 2010; Delaney et al., 2010; Chaurasia et al., 2014; Fares et al., 2014), appear to achieve expansion of phenotypic and functional HSCs. Non-cell-autonomous feedback signaling likely limits this expansion, as factors inhibiting HSC self-renewal are secreted during UCB cell culture (Kirouac et al., 2010). Zandstra and colleagues (Csaszar et al., 2012) have overcome the impact of this paracrine signaling by demonstrating expansion of serially transplantable UCB HSCs following “fed-batch” culture, in which culture volume is gradually, continuously increased to dilute secreted inhibitory factors. These results are collectively promising, but cytokine-based culture protocols likely promote expansion at the expense self-renewal, multilineage, and/or homing function (Szilvassy et al., 2001; Hofmeister et al., 2007; Chou et al., 2010). How robustly these advances hold true in the clinic remains to be seen.

A fundamental change in potential approaches to expand HSCs occurred when Yamanaka and colleagues reported transcription factor (TF)-mediated reprogramming of mouse and human fibroblasts to induced pluripotent stem cells (iPSCs) (Takahashi and Yamanaka, 2006; Takahashi et al., 2007). Significant effort has since been invested in directed differentiation of embryonic stem cells (ESCs) or iPSCs into HSCs. If successful, this approach would have the additional benefit over UCB HSC expansion of allowing for *de novo* generation of patient-specific HSCs for autologous transplant, thus removing the barrier of finding an HLA-matched donor for prospective transplant recipients.

Endeavors to generate HSCs from pluripotent cells have been heavily guided by current knowledge of embryonic development of the hematopoietic system. In the vertebrate embryo, blood development occurs in two waves, termed primitive and definitive hematopoiesis. The primitive program, which originates in the yolk sac, is transient and gives rise to cells with myeloid, but not lymphoid, potential (Palis et al., 1999). Definitive hematopoiesis is defined by the production of HSCs with full multilineage engrafting function and arises from hemogenic endothelium of the aorticgonado-mesonephros (AGM) after primitive hematopoiesis (Medvinsky and Dzierzak, 1996). Hematopoietic cells derived from iPSCs are transplantable, but chimerism is very low and/or restricted to the myeloid lineage (Lu et al., 2009; Gori et al., 2015). The limited multilineage potential of ESC/iPSC-derived hematopoietic cells indicates a failure to achieve definitive hematopoiesis *in vitro* and thus reveals incomplete recapitulation of complex embryonic developmental signals.

Recent work by Daley and colleagues (Lu et al., 2016) achieves exciting progress on this front. Ectopic expression of HoxB4, a TF important in early embryonic patterning and HSC self-renewal, confers ESCs with long-term, multilineage hematopoietic engraftment potential (Kyba et al., 2002). However, these cells exhibit a myeloid bias, revealing the need for more robust induction of lymphoid potential. As Notch activation provided by the AGM during definitive hematopoiesis promotes HSC specification (Hadland et al., 2015), they added Notch ligand to their differentiation protocol and derived HSCs with robust lymphoid as well as myeloid potential (Lu et al., 2016). This modular approach to generating HSPCs represents a novel strategy in ongoing efforts to engineer clinically usable HSCs.

Another approach to generate HSCs *de novo* has been to reprogram somatic cells to HSCs. Bypassing a pluripotent intermediate would be an advantage because it avoids the risk of generating HSCs contaminated with potentially teratoma-generating cells. Successful direct conversion from one cell type to another has already been reported in numerous therapeutically relevant cell types (Yechoor et al., 2009; Ieda et al., 2010; Son et al., 2011), as well as between hematopoietic cell types (Xie et al., 2004; Laiosa et al., 2006; Taghon et al., 2007). Such approaches have generally been based on combinations of TF expression, miRNA expression, and hematopoietic cytokine exposure (Daniel et al., 2016). This strategy led to early success in reprogramming mouse fibroblasts, revealing expression of Etv6, Fos, Gata2, and Gfi1b is sufficient to induce an HSC-like identity (Pereira et al., 2013).

From a developmental perspective, endothelial cells have been an attractive starting cell type for reprogramming to HSCs due to their close ontogenetic relationship. Enforced expression of four TFs (FOSB, GFI1, PU.1, and RUNX1) in human umbilical-vein endothelial cells reprograms them to a self-renewing HSC-like population capable of multilineage engraftment in serial transplant (Sandler et al., 2014). Importantly, this reprogramming additionally requires co-culture on an endothelial cell type that mimics the embryonic niche. Although the reprogrammed cells possess limited lymphoid potential, the relative success of this approach highlights the essential role of inductive cues supplied by the niche from which HSCs arise during development.

Committed hematopoietic cells have been another promising starting point for inducing conversion to HSCs, as in theory their hematopoietic identity may reduce the epigenetic barriers to reprogramming. Transient expression of six TFs (Hlf, Lmo2, Pbx1, Prdm5, Runx1t1, and Zfp37) imparts multilineage, long-term engraftment potential onto committed mouse myeloid and lymphoid progenitors (Riddell et al., 2014). Importantly, following transduction, the authors transplanted the cells for reprogramming to be completed *in vivo*. This strategy has the dual advantage of circumventing the need to maintain prospective induced HSCs *ex vivo* while also allowing the niche to provide additional inductive cues to further promote reprogramming. One drawback to partially *in vivo* induction, however, is that it impedes further characterization of the reprogramming process.

Many hurdles remain before these substantial advances are translated into the clinic. Each of these TF cocktails for HSC conversion bears little overlap with the others, likely reflecting the different species and starting cell types used in each study. Whether these combinations of TFs are also effective in reprogramming other cell types will be an important question to answer. For example, fibroblasts could be preferable to blood cells as a starting point in autologous transplant of patients with acquired hematological disorders, as this would circumvent the need for corrective gene editing on top of reprogramming to HSCs. Leukemogenic transformation upon reprogramming poses an additional risk, as several of the TFs identified in the above studies are potent oncogenes. Finally, the preservation of HSC function *ex vivo* has posed a notoriously difficult challenge to the field. Maintaining HSCs once stem cell identity is achieved will therefore not be a trivial task. The recent development of a fully-defined recombinant serum that supports HSC culture may reduce experimental variability in these efforts (Ieyasu et al., 2017). Combined, the outstanding questions highlight the continued need for a deeper understanding of the mechanisms behind HSC emergence, reprogramming, and maintenance.

Cellular characterization of the HSC niche

HSCs are capable of massive expansion *in vivo*, with a single stem cell able to reconstitute the hematopoietic system long-term in a mouse (Osawa et al., 1996). The number of transplantable HSCs in mouse bone marrow moreover increases 10-fold upon injection into lethally irradiated recipients (Iscove and Nawa, 1997). Yet despite this enormous potential *in vivo*, robust amplification of HSCs *ex vivo* without loss of self-renewal capacity remains an elusive and challenging goal. The inability to induce their expansion or generation *ex vivo* highlights our insufficient identification of the signals that induce self-renewing proliferation *in vivo*. In-depth characterization of the HSC niche, the local tissue microenvironment in which HSCs reside, will be a major contribution toward defining the molecular network that regulates HSC maintenance and expansion.

Although developmentally HSCs arise from the AGM, the bone marrow is the primary site of postnatal hematopoiesis. Defining the precise niche that directly maintains and regulates HSCs has been challenging due to the difficulty of preserving structural integrity of the bone during sectioning, combined with the extensive panel of markers currently required to identify HSCs. Complex biological interactions between prospective niche cells compound these technical obstacles: genetic perturbation of multiple cell types in the bone marrow alters HSC number, function, and retention (Calvi et al., 2003; Zhang et al., 2003), yet an effect on HSCs does not necessarily indicate a direct relationship. Moreover, the niche is not defined by a single cell type; rather, the niche is the cumulative environment created by the integration of growth factors, cytokines, oxygen tension, and other factors presented directly and indirectly to resident HSCs by a range of neighboring cell types. Recent advances in lineage tracing, high-resolution microscopy, and cell type-specific conditional deletion animal models have contributed significantly to our understanding of the complex cellular and molecular network responsible for HSC quiescence, proliferation, retention, and mobilization *in vivo*.

The bone marrow contains both hematopoietic and non-hematopoietic cells. Bone-forming osteoblasts reside at the endosteum, the interface between bone and bone marrow. Genetic manipulations that increase osteoblast number in mice also increase HSC number (Calvi et al., 2003; Zhang et al., 2003), and HSCs are observed next to osteoblasts that express angiopoietin-1, which promotes retention of quiescent HSCs to the bone marrow (Arai et al., 2004). Although subsequent studies have found these interactions to be indirect (Kiel et al., 2005; Kiel et al., 2007), these early results nonetheless provided initial evidence for an endosteal niche.

The bone marrow is highly vascularized, with a particular enrichment of sinusoids near the endosteum (Nombela-Arrieta et al., 2013). Sinusoids are fenestrated venules that allow passage of cells in and out of circulation. Evidence for a perivascular niche came with the identification of the SLAM family of markers as a means of visualizing *in situ* a population enriched for HSCs (45%) using a simple two-color stain (Kiel et al., 2005). This made it possible to localize most HSCs adjacent to sinusoids, but less frequently near the endosteum (Kiel et al., 2007). A possible link between these two potential niches was suggested by the discovery that most HSCs are in direct contact with cells expressing high levels of the chemokine CXCL12 (Sugiyama et al., 2006), which is required for HSC retention to the niche (Broxmeyer et al., 2005) and for engraftment following transplantation (Peled et al., 1999). Importantly, CXCL12-expressing cells either surround sinusoidal endothelial cells or are near the endosteum, suggesting a common mechanism for HSC retention to two niches (Sugiyama et al., 2006).

As the HSC niche remained poorly defined, Morrison and colleagues (Ding et al., 2012) aimed to identify the cellular source(s) of stem cell factor (SCF), a cytokine required non-cell-autonomously for HSC maintenance *in vivo* (Heissig et al., 2002; Czechowicz et al., 2007). They thus systematically induced deletion of *Scf* from candidate niche cell types and found that loss of *Scf* from hematopoietic, osteoblastic, and Nestin$^+$ mesenchymal cells did not affect HSC frequency or function. HSCs were depleted from the bone marrow, however, when *Scf* was deleted either from endothelial cells or from Lepr$^+$ mesenchymal cells surrounding sinusoids, and nearly all HSCs disappeared when both cell types lacked *Scf* (Ding et al., 2012). Intriguingly, the perivascular mesenchymal cells also expressed *Cxcl12*, supporting previous work reporting contact between HSCs and CXCL12-producing cells surrounding sinusoidal endothelium (Sugiyama et al., 2006). Subsequent identification of Hoxb5 as a highly specific marker of long-term HSCs and *in situ* imaging of these cells in mouse bone marrow further confirmed the existence of a perivascular HSC niche (Chen et al., 2016).

Accumulating evidence suggests that the perivascular niche may not be restricted solely to sinusoids. Silberstein and colleagues (Nombela-Arrieta et al., 2013) used three-dimensional reconstruction of bone sections to perform high-resolution imaging of entire mouse femurs. They acquired high-throughput information on the positioning of HSCs (which authors defined as lineage$^-$cKit$^+$CD48$^-$CD41$^-$) throughout the bone marrow, revealing that most HSCs lie directly adjacent to the bone marrow vasculature. Contrary to previous work, however, these direct *in situ* imaging studies found most HSCs localized near the endosteum (Nombela-Arrieta et al., 2013; Kunisaki et al., 2013). Computer modeling further revealed that the apparently high association of HSCs with sinusoids that had been previously described is statistically random when accounting for the density of the sinusoidal vasculature. Instead, HSCs preferentially associate with endosteal arterioles, which comprise a smaller volume of the bone marrow. Strikingly, quiescent HSCs localize to arterioles, while HSC activation or mobilization promotes their redistribution to sinusoids (Kunisaki et al., 2013). Thus, the arteriolar milieu appears to represent an additional HSC niche, and this work more broadly supports a model in which HSC subsets reside in distinct niches (Boulais and Frenette, 2015).

Multiple biological and technical factors may resolve outstanding conflicting characterizations of the HSC niche. There is an increasing recognition of the functional heterogeneity of HSCs (Goodell et al., 2015). Complex panels of surface markers are moreover still required to enrich for putative HSCs. The relationship between various surface marker combinations and true functional output remains poorly understood, so seemingly conflicting results may reflect real differences between distinct HSC populations. HSCs observed at distinct sites within the bone marrow may moreover represent cells carrying out discrete functions, such as self-renewing versus mobilizing into circulation. The method of observation moreover has clear effects, as contradictory results have been obtained from transplantation (Xie et al., 2009), induced deletion (Ding et al., 2012), and *in situ* visualization (Kunisaki et al., 2013) experiments. The tools available to characterize the niche have until recently been relatively crude, limited in large part by the difficulty in analyzing the rare HSC population among large cell numbers in tissue sections. Such technical limitations likely compound real biological variability.

Despite dense vascularization, the hematopoietic niche is hypoxic (1-5% O_2). Hypoxia limits aerobic respiration and reactive oxygen species (ROS) production and thus protects stem cells and their progeny from oxidative stress (Suda et al., 2011). The lowest oxygen tension has been measured in peri-sinusoidal regions of the bone marrow cavity, while the endosteum is less hypoxic due to a locally higher density of arterioles (Spencer et al., 2014). While local gradients in oxygen tension therefore exist within the bone marrow, it remains a suitably hypoxic setting in keeping with existing general models of the somatic stem cell niche.

An additional factor in interpreting studies defining the HSC niche is that local oxygen tension changes dramatically following irradiation or chemotherapy due to damage to the vasculature (Spencer et al., 2014). Conditioning treatments prior to transplants therefore likely destroy the prospective niches to which HSCs might preferentially home upon transplantation. Such constraints may explain why HSCs are more frequently localized to the endosteum in irradiated transplant recipients but distribute randomly in non-irradiated recipients (Xie et al., 2009). This context-specific niche selection thus illustrates the need to characterize the niche employing assays that induce minimal perturbation of the gross tissue architecture.

Significant questions persist about the HSC niche. The increasingly recognized biological heterogeneity of HSCs (Goodell et al., 2015), combined with remaining technical limitations to identifying pure HSC populations, likely contribute to some of the inconsistent results reported. Distinct cues and microenvironments are likely required to support a range of functional outputs. Therefore, distinct niches may exist for HSCs of different cell cycle status or lineage bias (Ding and Morrison, 2013; Kunisaki et al., 2013). Additional niche-regulatory signaling from other cell types, including macrophages (Chow et al., 2011) and Schwann cells (Yamazaki et al., 2011), remains only superficially characterized. Future development of finer-resolution techniques will help resolve outstanding conflicting reports and further refine current understanding of the HSC niche. Finally, most progress in this aspect of HSC biology has been necessarily driven by studies in mouse. Parallel studies using human cells and humanized mouse models have contributed to our understanding of the human HSC niche, but significant additional work is required to identify the unique regulatory network in this setting and translate it to the clinic.

The regulatory signaling network of HSCs

The role of the niche is to provide the signals to promote HSC quiescence, self-renewal, mobilization, and differentiation as necessary to support lifelong blood production. A complex combination of cytokines, adhesion molecules, and other factors regulates intracellular signaling pathways to direct HSC fate decisions. Thus a thorough understanding of how these signaling pathways converge into a coherent regulatory network would significantly support efforts to drive HSC generation *de novo* or expansion *ex vivo*.

Early efforts to drive HSC expansion *ex vivo* with extrinsic factors focused largely on the addition of combinations of hematopoietic cytokines, commonly including SCF, thrombopoietin, and Flt3-ligand. Multiple cytokine cocktails have been reported to promote expansion of long-term HSCs from human UCB (Conneally et al., 1997; Zhang et al., 2008; Wohrer et al., 2014), but none has yet translated into clinical use. Even currently reported small molecule-based expansion schemes still rely on the addition of a cocktail of cytokines (Boitano et al., 2010; Delaney et al., 2010; Chaurasia et al., 2014; Fares et al., 2014). Such conditions are suboptimal for defining an HSC regulatory network because cytokines have complex, non-additive effects on HSC survival, proliferation, and selection of self-renewal versus differentiation (Knapp et al., 2017a). Different cytokine cocktails correspondingly activate or inhibit distinct signaling pathways (Knapp et al., 2017b). Cytokine culture is thus a complex system in which to deconstruct HSC fate decisions at the molecular level, highlighting the need for in-depth characterization of the influence of individual pathways on HSC fate decisions in a variety of contexts.

In addition to sensitivity to a range of cytokines, HSCs express receptors that can activate developmental signaling pathways, including Notch (Varnum-Finney et al., 1998), TGF-β (Yamazaki et al, 2011), Hedgehog (Bhardwaj et al., 2001), and Wnt (Austin et al., 1997). However, how these pathways integrate into a coherent network that regulates HSC homeostasis remains unclear.

An overview of canonical Wnt signaling

Extensive evidence indicates a role for canonical Wnt/β-catenin signaling in development, adult stem cells, and cancer (Clevers, 2006; Wend et al., 2010; Bhavanasi and Klein, 2016). Wnts are a family of secreted glycoproteins that regulate multiple cell processes, including cell fate, proliferation, and polarity. In the absence of Wnts, a large complex scaffolded by Axin facilitates glycogen synthase-3 (GSK-3) phosphorylation of β-catenin and targets it for proteasomal degradation (Kitagawa et al., 1999) (**Figure 1.1A**). Upon Wnt binding to its cell surface receptor Frizzled (Fzd), GSK-3 is inhibited, allowing unphosphorylated β-catenin to accumulate and translocate to the nucleus, where it activates the TFs TCF/LEF-1 to drive expression of target genes (Behrens et al., 1996) (**Figure 1.1B**). Canonical Wnt target genes include cell proliferation promoters *Ccnd1* (which encodes cyclin D1; Tetsu and McCormick, 1999) and *Myc* (He et al., 1998), as well as negative feedback regulators of the pathway *Axin2* (Jho et al., 2002) and *Dkk1* (Bafico et al., 2001).

Canonical Wnt signaling regulates multiple pathways independently of β-catenin. The same Wnt/Fzd cascade that stabilizes β-catenin also activates the mammalian target of rapamycin complex 1 (mTORC1; Inoki et al., 2006), Hippo (Azzolin et al., 2014), and Wnt stabilization of proteins (Wnt/STOP; Acebron et al., 2014) pathways. These divergent outputs of Wnt signaling are less well characterized, but there is growing recognition of their role in normal and malignant physiology (Bhavanasi and Klein, 2016).

mTORC1 is an evolutionarily conserved nutrient sensor that regulates cell metabolism, growth, and proliferation. GSK-3 enhances the activity of tuberous sclerosis complex 2 (TSC2), a negative regulator of mTORC1. Thus, Wnt inhibition of GSK-3 relieves GSK-3 inhibition of mTORC1, promoting translation and cell proliferation independently of β-catenin-mediated transcription (Inoki et al., 2006). Correspondingly, pharmacologic or genetic inhibition of GSK-3 activates mTORC1 in multiple cell types, including HSCs (Huang et al., 2009) and ESCs (Mansi Shinde and Peter Klein, unpublished). This has particularly significant functional consequences for HSCs, as described below.

Wnt signaling also regulates the Hippo pathway, a conserved regulator of organ size. Like Wnt/β-catenin signaling, Hippo signaling is regulated by the degradation or stabilization of transcriptional activators based on the activity of the destruction complex. Thus upon activation of the Wnt pathway, the Hippo transcriptional activators YAP/TAZ are released from the destruction complex and translocate to the nucleus to drive transcription of Hippo target genes (Azzolin et al., 2014). Aberrant Wnt regulation of Hippo signaling contributes to tumorigenesis in multiple tissues, including small intestine (Cai et al., 2010), breast (Lim et al., 2016), and liver (Kim et al., 2017).

Finally, β-catenin-independent Wnt/STOP has been recently identified as an important mechanism in cell division. Wnt-dependent phosphorylation targets many GSK-3 substrates for proteasomal degradation (Kim et al., 2009; Xu et al., 2009; Taelman et al., 2010). In proliferating cells, Wnt signaling peaks during the transcriptionally silent G2/M phase of the cell cycle (Olmeda et al., 2003). Over 100 candidate proteins have been identified whose polyubiquitylation depends on GSK-3 and that are stabilized upon mitotic Wnt signaling, increasing cellular protein content and size in preparation for division (Acebron et al., 2014). Wnt/STOP is additionally required for mitotic spindle assembly and faithful chromosome segregation (Stolz et al., 2015).

Canonical Wnt signaling in HSCs

Stem cells are defined by their dual capacity to both self-renew and give rise to differentiated cells. The balance between these two processes is essential to lifelong stem cell function, and by extension tissue maintenance and repair. Extensive evidence demonstrates a key role for Wnt/β-catenin signaling in embryonic, somatic, and cancer stem cells (Bhavanasi and Klein, 2016). While many reports support a role for Wnt signaling in the maintenance of HSCs, substantial unresolved contradictory evidence has accumulated, and the true role of Wnt signaling in HSC maintenance remains controversial. Given the range of distinct cascades downstream of canonical Wnt signaling, combined with the variety of approaches employed to investigate its role in HSC function, the key likely lies in a highly context-dependent role for the pathway.

Weissman and colleagues (Reya et al., 2003) demonstrated that Wnt/β-catenin signaling contributes to hematopoiesis with the finding that constitutively activated β-catenin promotes HSC self-renewing proliferation and upregulates expression of *HoxB4* and *Notch1*, both of which had been previously implicated in HSC self-renewal (Antonchuk et al., 2002; Varnum-Finney et al., 2000). Transplantation of wild-type HSCs into *Sfrp*-null hosts (which lack the Wnt antagonist secreted frizzled-related protein 1) leads to a progressive decrease in HSC numbers (Renstrom et al., 2009), demonstrating a requirement for niche-mediated regulation of Wnt signaling. Endogenous HSCs moreover activate a TCF/LEF-1 reporter, indicating that HSCs respond to Wnt signaling *in vivo* (Reya et al., 2003). HSC culture with purified Wnt3a (combined with low doses of cytokines) induces proliferation of cells with multilineage engraftment potential, as demonstrated by competitive transplant (Willert et al., 2003), suggesting potential clinical applications for manipulation of Wnt signaling in HSCs. However, reconstitution was followed only up to six weeks post-transplant, and thus chimerism may have reflected engraftment by progenitors rather than by true HSCs. Nonetheless, these studies collectively provided early evidence for the Wnt pathway promoting HSC self-renewal.

Some corresponding studies inhibiting Wnt signaling have yielded consistent results. *Wnt3a*-knockout mice die at embryonic day 12.5, but have reduced numbers of fetal liver HSCs which possess impaired repopulating function in serial transplant (Luis et al., 2009). Mice with embryonic deletion of *Ctnnb1* (which encodes β-catenin) from the hematopoietic system are viable, but also have HSCs with severely impaired regenerative capacity (Zhao et al., 2007). Osteoblast overexpression of the secreted Wnt inhibitor Dkk1 moreover results in loss of HSC quiescence and a progressive decline in regenerative capacity upon transplantation (Fleming et al., 2008), further supporting a role for Wnt signaling in HSC self-renewal and maintenance.

However, multiple studies have reported no hematopoietic phenotype upon deletion of β-catenin, either alone (Cobas et al., 2004) or in combination with deletion of the functionally related γ-catenin (Jeannet et al., 2008; Koch et al., 2008). Deletion of *Porcn*, which encodes an acyltransferase required for Wnt secretion and function (Tanaka et al., 2000), similarly has no effect on steady-state or regenerative hematopoiesis (Kabiri et al., 2015). Moreover in contrast to Reya et al. (2003), others observe that expression of a constitutively active β-catenin leads to a differentiation block in HSCs (Kirstetter et al., 2006; Scheller et al., 2006). Such findings are difficult to reconcile with a requirement for Wnt signaling in HSC self-renewal.

Potentially resolving some of the reported inconsistencies, Staal and colleagues (Luis et al., 2011) have elegantly proposed that Wnt signaling may regulate HSCs in a dose-dependent manner. They used combinations of two hypomorphic alleles and conditional deletion of *Apc*, a negative regulator of Wnt signaling, to generate varying degrees of Wnt/β-catenin activation. This demonstrated that low levels of Wnt signaling promote HSC self-renewal and expansion *in vivo*, while moderate and high levels of Wnt signaling promote myeloid and T cell differentiation, respectively. Importantly, the highest level of Wnt activation leads to total loss of repopulating function in transplant.

A nuanced review of the existing literature further clarifies some of the conflicting reports. Reya et al. (2003) reported *self-renewal* in HSCs sorted and transduced with a stabilized β-catenin construct. Importantly, to induce cell cycle entry for transduction while limiting differentiation, the authors used HSCs overexpressing the anti-apoptotic factor *Bcl2* (Domen and Weissman, 2000). This study thus employed a tractable but highly artificial system in which the response to Wnt pathway activation may not necessarily reflect a biologically relevant response. In contrast, Scheller et al. (2006), who observed a *differentiation block* with constitutively active β-catenin, induced expression of a stabilized β-catenin on top of endogenous Wnt/β-catenin signaling in adult mice *in vivo*. Careful characterization of the extent of Wnt pathway activation in distinct contexts may thus resolve these conflicting observations and perhaps lend further support to the dose-dependence model of Wnt regulation of HSC function.

An additional factor likely contributing to the divergent observations reported is the developmental time point at which the Wnt pathway is manipulated. A self-renewal defect in HSCs is described when *Ctnnb1* is excised prenatally (Zhao et al., 2007), but not when deletion is induced in adult mice (Cobas et al., 2004; Jeannet et al., 2008; Koch et al., 2008). Importantly, Wnt reporter activity is observed even in the absence of β-catenin and γ-catenin (Jeannet et al., 2008), suggesting a potential catenin-independent mechanism for transduction of Wnt signaling. As β-catenin establishes poised chromatin architecture at Wnt-responsive regulatory elements in development (Blythe et al., 2010), it is possible that β-catenin is required during HSC ontogeny to initially define responsive chromatin architecture, but it is dispensable in adult life provided β-catenin-dependent epigenetic marks are maintained.

Canonical Wnt signaling is implicated in the regulation of self-renewal and differentiation of a variety of stem cell populations during homeostasis, tissue regeneration, and oncogenesis. The extensive literature on the role of Wnt/β-catenin signaling specifically in HSC function demonstrates the need for highly systematic, context-specific investigation and nuanced interpretation of new results. Yet despite this already-detailed characterization of the effects of Wnt pathway manipulation on HSCs, precisely how the Wnt pathway instructs HSC fate decisions remains relatively poorly defined. Identifying the downstream effectors that direct distinct HSC programs will yield important insights into how the Wnt pathway contributes to the signaling network underlying HSC homeostasis.

An overview of mTORC1 signaling

As introduced above, mTORC1 is an essential nutrient sensor and regulator of metabolism that is regulated in part through GSK-3 activation of the TSC1/2 complex. Cells rely on a complex signaling network that includes mTORC1 to integrate nutrient and energy availability with the production or digestion of cellular components such as proteins, lipids, and nucleic acids. mTORC1 regulates transcription of a broad metabolic gene network (Duvel et al., 2010), but plays an especially central role in regulation of cellular metabolism at the level of protein synthesis and catalysis. Dysregulation of this vital process can have disastrous consequences in development, homeostasis, and disease.

In addition to GSK-3, the phosphoinositide 3-kinase (PI3K)/Akt pathway is a major regulator of mTORC1. Signals such as growth factors, nutrients, and appropriate oxygen tension recruit PI3K to the plasma membrane, where it phosphorylates PIP_2 to PIP_3 (Vanhaesebroeck and Waterfield, 1999), which itself recruits PDK1 and Akt to the plasma membrane (Kandel and Hay, 1999). This process is inhibited by phosphatase and tensin homolog (Pten), which dephosphorylates PIP_3 to PIP_2 (Vazquez et al., 2006). PDK1 phosphorylates Akt, promoting Akt inhibition of the TSC1/2 complex (Kandel and Hay, 1999). TSC1/2 is one of the primary inhibitors of mTORC1, and thus PI3K/Akt signaling activates mTORC1 to promote cell survival, growth, and proliferation (**Figure 1.2**).

mTORC1 promotes cell growth largely through stimulation of translation. The best-characterized substrates of mTORC1 are thus the 40S ribosomal protein S6 kinases (S6Ks) and the eukaryotic translation initiation factor 4E-binding proteins (4E-BPs), both important regulators of translation initiation (Hay and Sonenberg, 2004). mTORC1 phosphorylation of S6K promotes S6K activation of multiple translation initiation factors, including ribosomal protein S6 (Isotani et al., 1999). mTORC1/S6K signaling also induces ribosomal gene transcription (Hannan et al., 2003; Xiao and Grove, 2009), further promoting ribosomal biogenesis and increasing translational capacity. Under conditions of low mTORC1 activation, hypophosphorylated 4E-BP competes with eukaryotic translation initiation factor 4G (eIF4G) to bind and inhibit eukaryotic translation initiation factor 4E (eIF4E). Activated mTORC1 phosphorylates 4E-BP residues Thr37 and Thr46. These phosphorylation events are not sufficient to induce 4E-BP dissociation from eIF4E. However, they are required priming events for subsequent additional phosphorylation events that ultimately lead to 4E-BP release, allowing eIF4E-eIF4G interaction and recruitment of the complex to the 5' cap structure of mRNA (Gingras et al., 1999; Gingras et al., 2001).

As mTORC1 activity is coupled to cellular energetic availability and demand, mTORC1 additionally promotes mitochondrial biogenesis and oxidative phosphorylation activity, through both translation-dependent and -independent mechanisms. mTORC1 inhibition of 4E-BP stimulates mitochondrial biogenesis by selectively promoting translation of nuclear-encoded mitochondrial protein genes (Morita et al., 2013). mTORC1 is moreover required for activation of PGC1α, a major transcriptional coactivator of nuclear-encoded mitochondrial genes (Cunningham et al., 2007; Blattler et al., 2012). The mTORC1 inhibitor rapamycin correspondingly reduces mitochondrial gene expression, membrane potential, and oxygen consumption (Paglin et al., 2005; Cunningham et al., 2007).

In addition to promoting cell growth and metabolism under nutrient-replete conditions, mTORC1 supports survival during starvation by activating autophagy. Autophagy also serves as an intracellular quality control mechanism for the turnover of damaged proteins and organelles. The general process of macroautophagy is the cellular recycling mechanism whereby autophagic vesicles (autophagosomes) envelop cytoplasmic proteins and organelles and fuse with the lysosome. Autophagosome contents are thus degraded to free amino acids and lipids to support the ongoing biosynthetic needs of the cell in the absence of exogenous or *de novo*-produced building blocks (Rabinowitz and White, 2010). Selective autophagy of mitochondria, ribosomes, and endoplasmic reticulum (termed mitophagy, ribophagy, and reticulophagy, respectively) has been described, but how specific cargoes are targeted to the pathway remains poorly characterized (Cebollero et al., 2012; Joshi and Kundu, 2013). mTORC1 inhibition of autophagy is twofold: it phosphorylates Ulk1, preventing autophagosome assembly (Jung et al., 2009), and it phosphorylates the master TF of lysosomal biogenesis TFEB, sequestering it in the cytosol and preventing transcription of genes required for autophagy (Roczniak-Ferguson et al., 2012; Settembre et al., 2012).

As translation is considered one of the most energy-consuming processes in the cell (Hay and Sonenberg, 2004), the rate at which it proceeds must be tightly coupled with nutrient availability. Control of mitochondrial biogenesis and lipid synthesis is similarly essential to enacting a stimulus-appropriate metabolic program. Conversely, autophagy serves in part to generate biosynthetic materials in the absence of sufficient extracellular supply. Combined, these major regulatory targets of mTORC1 signaling exert reciprocal control on cellular energy consumption and production.

mTORC1 signaling in HSCs

As described above, HSCs reside in a low-perfusion, reduced-nutrient niche in the bone marrow. This localization underscores nutrient-sensing and more broadly metabolic adaptation to this microenvironment as a vital function. HSCs must additionally maintain a primarily quiescent state to prevent proliferation-induced exhaustion. Consistently, multiple lines of evidence suggest that mTORC1 signaling is low in HSCs under homeostatic conditions. A thorough understanding of mTORC1 pathway regulation of

HSC function and fate decisions will be instrumental in the clinic to induce HSC expansion without concomitant loss of long-term repopulating function.

Consistent with HSC residence in a hypoxic niche (Parmar et al., 2007; Spencer et al., 2014), HSCs stably express the oxygen-sensitive TF HIF1α (Takubo et al., 2010). HIF1α promotes expression of hypoxia-inducible genes, including those related to glycolysis (Semenza, 2013). HSCs correspondingly exhibit a glycolysis-dependent metabolic profile (Simsek et al., 2010), as well as low mitochondrial mass and membrane potential (Mantel et al., 2010) compared to more committed progenitor populations and whole bone marrow. mTORC1 promotes HIF1α translation (Duvel et al., 2010) and may thus partially mediate HIF1α regulation of HSC metabolism. Notably, HSCs retain their distinct metabolic profile for hours after isolation from the bone marrow (Simsek et al., 2010). This suggests that this reduced metabolism may not be simply an adaptation to the niche, but rather an intrinsic property of HSCs.

More direct evidence for low mTORC1 signaling in HSCs at homeostasis comes from loss-of-function studies of multiple negative regulators of mTORC1. Deletion of *Pten* (Lee et al., 2010), *GSK-3* (Huang et al., 2009), or *Tsc1* (Chen et al., 2008; Gan et al., 2008) leads to HSC proliferation, followed by exhaustion and in some cases leukemia (Lee et al., 2010; Guezguez et al., 2016). HSCs expressing a constitutively activated Akt similarly exhibit transient expansion that ultimately leads to impaired engraftment potential and leukemia (Kharas et al., 2010). Loss of *Tsc1* additionally increases mitochondrial biogenesis and intracellular levels of ROS, consistent with mTORC1 activation. *In vivo* administration of an antioxidant, however, preserves HSC number and engraftment potential, indicating that the TSC/mTORC1 pathway maintains HSC function at least in part through restricting ROS levels (Chen et al., 2008).

Although these results collectively establish a clear requirement for low mTORC1 signaling in the maintenance of HSC function, full inactivation of mTORC1 signaling also causes significant hematopoietic defects. Deletion of *Raptor*, a regulatory component of mTORC1 that is required for the complex to function, causes accumulation of immature progenitors and non-lethal pancytopenia. Although HSC frequency is not affected, *Raptor*-knockout cells fail to regenerate the hematopoietic system following transplant or sub-lethal irradiation (Kalaitzidis et al., 2012). *Akt1/2* double-knockout HSCs are consistently more quiescent and have lower levels of ROS than wild-type and fail to reconstitute transplant recipients. Pharmacological restoration of ROS levels rescues the differentiation defect, indicating that some level of Akt activation and ROS is required for HSC function (Juntilla et al., 2010).

Extensive evidence demonstrates a role for mTORC1 signaling in HSC maintenance and regenerative capacity. The defects in hematopoiesis observed in response to either hyperactivation or inhibition of the pathway reveal differential requirements for it at distinct stages of hematopoiesis. Low mTORC1 signaling appears to be required for maintenance of quiescent HSCs, in part through promoting a glycolytic metabolism and restricting mitochondrial content and ROS production in the hypoxic niche. mTORC1 activation may be necessary, however, to promote the extensive proliferation that progenitors must undergo to regenerate the hematopoietic system following injury and to support long-term blood production. The correct level of mTORC1 signaling must therefore be achieved to balance HSC maintenance and proliferation.

Convergence of the GSK-3 and mTORC1 pathways

TSC1/2, a GTPase-activating factor that inhibits mTORC1 (Shaw and Cantley, 2006), is sequentially phosphorylated by AMP-activated protein kinase (AMPK) and GSK-

3 (Inoki et al., 2006). This phosphorylation maintains TSC1/2 suppression of mTORC1 activity, and inhibition of either AMPK or GSK-3 activates mTORC1. Although Wnts inhibit GSK-3, GSK-3 regulation of mTORC1 is independent of β-catenin (Inoki et al., 2006).

The convergence of the Wnt and mTORC1 pathways has considerable implications for stem cells, which must balance signals promoting self-renewal (such as Wnts) and proliferation (mTORC1) to minimize mutation acquisition and exhaustion. This crosstalk between the Wnt and mTORC1 pathways has likely contributed to some of the inconsistent observations reported regarding the effect of Wnt signaling on stem cells, and supports a nuanced characterization of how both pathways regulate stem cell function. Transient GSK-3 inhibition and consequent mTORC1 activation may enhance stem cell function in the short-term. *In vivo* pharmacological inhibition of GSK-3 in murine HSC transplant recipients significantly increases donor-derived chimerism. Notably, this treatment expands the progenitor fraction, but HSC numbers remain constant, and correspondingly no enhanced reconstitution potential is observed in secondary transplant (Trowbridge et al., 2006).

In contrast, persistent mTORC1 activation from longer-term loss of GSK-3 leads to stem cell proliferation followed by exhaustion. Primary recipients of *Gsk3*-depleted HSCs show expansion in the HSC fraction of BM. Secondary recipients, however, exhibit progressive depletion of HSCs (Huang et al., 2009). Importantly, recipient bone marrow shows both stabilization of β-catenin and increased phosphorylation of S6. These results are consistent with a role for Wnt/β-catenin signaling in maintenance of the HSC pool, but also demonstrate that mTORC1 is activated in *Gsk3*-depleted cells. Administration of rapamycin to transplant recipients preserves *Gsk3*-depleted HSCs, confirming that this exhaustion is mTORC1-dependent. This two-step phenotype has also been demonstrated in epidermal stem cells (Castilho et al., 2009), suggesting that the mechanism may exist more broadly in additional stem cell populations.

Proposed model for GSK-3 and mTORC1 regulation of HSC maintenance

These results indicate a dual role for GSK-3 in HSC homeostasis: GSK-3 inhibition of Wnt/β-catenin inhibits self-renewal, while GSK-3 inhibition of mTORC1 inhibits lineage commitment. Inhibition of GSK-3 alone is therefore predicted to activate both pathways, yielding the observed initial expansion and ultimate depletion of HSCs. The Klein laboratory has proposed a model in which simultaneous inhibition of GSK-3 and mTORC1 may inhibit lineage commitment while promoting self-renewal (**Figure 1.3**). If this is the case, then this model deconstructs the aggregate network that maintains HSCs down to the minimal signals required to sustain HSC function. This would be a significant advance, as the complex and incompletely defined niche in which HSCs reside in the bone marrow has constrained efforts to define this network *in vivo*. The development of a model system that could maintain HSCs *ex vivo* under defined conditions would allow further identification of specific targets that mediate HSC maintenance. More precise definition of the signaling network that maintains HSCs will moreover provide rationale for ongoing efforts to expand HSCs *ex vivo* for both research and clinical applications.

Conclusion

The clinical potential of HSCs has been recognized for over a half century, yet substantial barriers remain before this full potential is achieved. One such barrier has been an incomplete characterization of the signaling network that generates, maintains, and expands HSCs throughout life. The Wnt/β-catenin and mTORC1 pathways are central components of this network, although their functional consequences are complex and context-specific. More precise identification of the targets of these pathways in the regulation of HSC function will yield important insights toward expanding their successful use in the clinic.

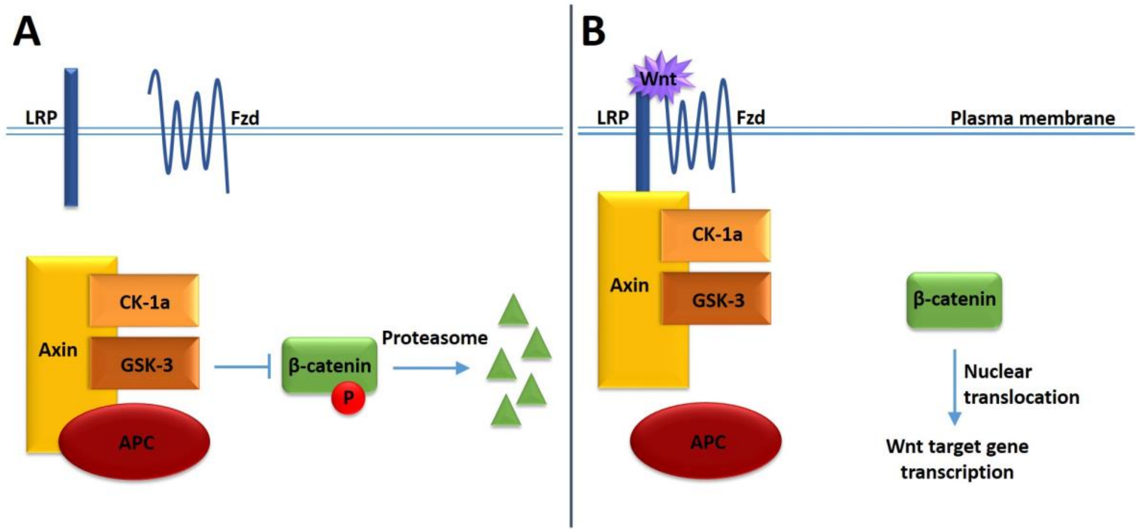


Figure 1.1: The Wnt/β-catenin pathway. (A) In the absence of Wnts, Axin, CK-1a, GSK-3, and APC form the β-catenin destruction complex. Sequential phosphorylation of β-catenin by CK-1a and GSK-3 in the destruction complex targets β-catenin for proteasomal degradation. (B) On Wnt binding to the Fzd/LRP co-receptors, Axin is recruited to LRP and the complex is inactivated. Unphosphorylated, stabilized β-catenin accumulates and translocates to the nucleus, where it activates TFs TCF/LEF to promote transcription of Wnt target genes.

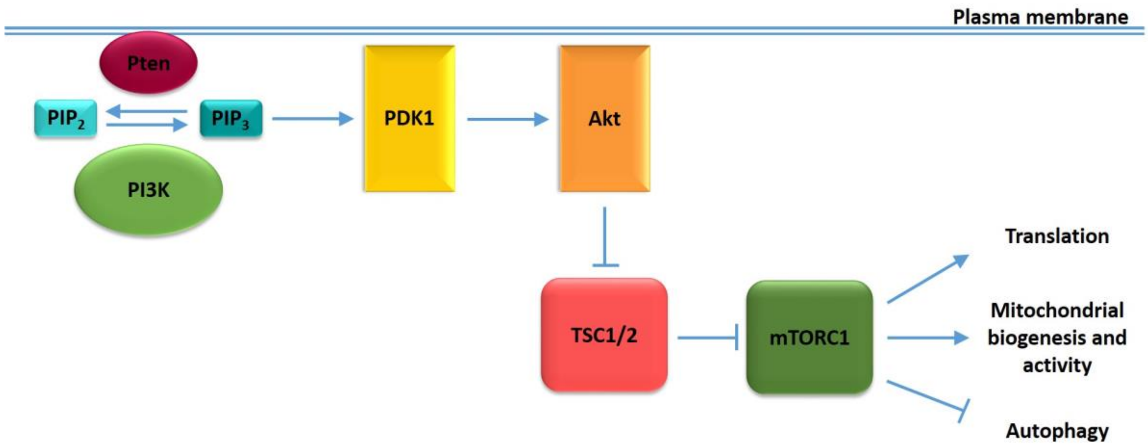


Figure 1.2: The PI3K/Akt/mTORC1 pathway. Signals including growth factors and nutrients recruit PI3K to the plasma membrane. PI3K phosphorylates PIP_2 to PIP_3, which recruits PDK1 and Akt to the plasma membrane. Pten inhibits this process by dephosphorylating PIP_3 to PIP_2. PDK1 activation of Akt promotes Akt inhibition of TSC1/2, relieving TSC1/2 inhibition of mTORC1 signaling. mTORC1 promotes cell growth and metabolism through a variety of effector pathways.

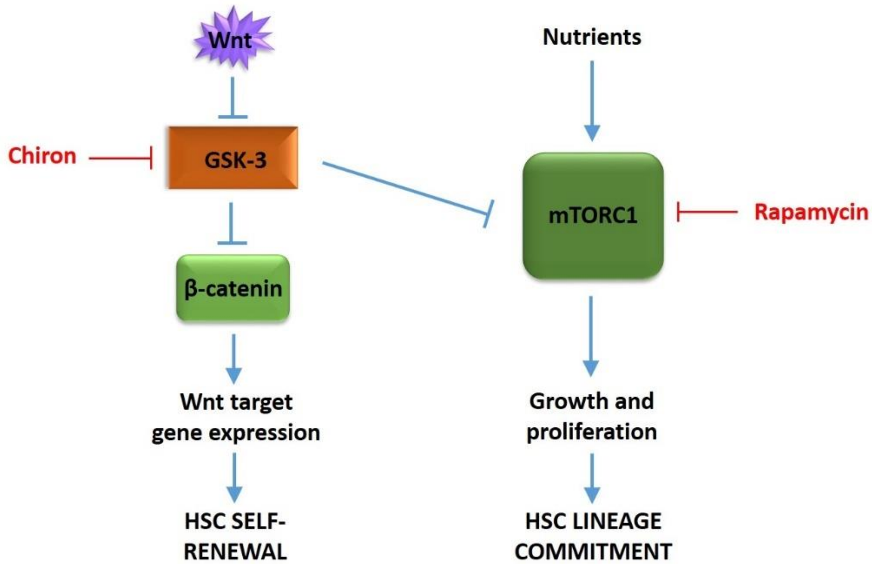


Figure 1.3: Proposed model for GSK-3 and mTORC1 regulation of HSCs. GSK-3 regulates two antagonistic pathways in HSCs. GSK-3 inhibition of Wnt/β-catenin signaling inhibits self-renewal, while GSK-3 inhibition of mTORC1 inhibits lineage commitment. Inhibition of GSK-3 activates both pathways. Simultaneous inhibition of GSK-3 and mTORC1 may therefore block HSC lineage commitment while promoting self-renewal.

CHAPTER 2: HSC MAINTENANCE VIA GSK-3 AND MTORC1 INHIBITION

The data presented in this chapter were published in *Nature Medicine* (Huang et al., 2012).

Introduction

HSCs support lifelong maintenance of the blood at homeostasis and following injury. They are an important model system in the study of stem cells and widely used in the clinic to treat hematopoietic malignancies and bone marrow failure (Purton and Scadden, 2007). Extensive effort has been invested in promoting HSC expansion *ex vivo*, both to facilitate further characterization in basic research and to improve clinical outcomes and broaden potential therapeutic applications. However, HSCs *in vivo* rely on a panoply of signals from their microenvironment to sustain their function. An incomplete understanding of the signaling network that maintains HSCs has significantly constrained the ability to maintain or expand HSCs beyond the niche (Lymperi et al., 2010).

HSCs reside in a hypoxic, low-perfusion niche (Parmar et al., 2007; Spencer et al., 2014), emphasizing the importance of nutrient-sensing in HSC homeostasis. The evolutionarily conserved nutrient sensor mTORC1 integrates diverse signals including nutrient availability, mitogenic stimuli, and oxygen tension, and the mTORC1 signaling pathway is correspondingly vital to HSC maintenance. Loss of function of multiple negative regulators of mTORC1, including *Pten* (Lee et al., 2010), *Gsk3* (Huang et al., 2009), and *Tsc1* (Chen et al., 2008), leads to HSC proliferative exhaustion and in some instances leukemia (Lee et al., 2010; Guezguez et al., 2016). These observations identify low mTORC1 signaling as an essential component of the network that maintains HSCs.

Extensive literature additionally implicates the Wnt/β-catenin pathway in the regulation of HSCs, particularly in promoting self-renewal (Reya et al., 2003; Willert et al., 2003), although this role remains controversial. Embryonic deletion of *Wnt3a* (Luis et al., 2009) or of *Ctnnb1* (which encodes β-catenin) (Zhao et al., 2007) impairs HSC self-renewal. However, deletion of *Ctnnb1* in adult mice produces no hematopoietic phenotype (Cobas et al., 2004; Jeannet et al., 2008; Koch et al., 2008), and expression of a constitutively active β-catenin causes a differentiation block (Kirstetter et al., 2006; Scheller et al., 2006). The effect of Wnt/β-catenin signaling on HSCs is thus context-dependent.

Interpretation of the effect of Wnt signaling on HSCs is complicated in part by crosstalk with other pathways. For example, Wnt signaling activates mTORC1 independently of β-catenin (Inoki et al., 2006). The dual activation of β-catenin and mTORC1 by Wnts may therefore explain why GSK-3 inhibition leads to transient expansion (Trowbridge et al., 2006) followed by mTORC1-dependent depletion of HSCs (Huang et al., 2009). The Klein laboratory therefore tested the hypothesis that activating Wnt/β-catenin signaling and mimicking restricted nutrient availability would together promote the self-renewal pathway while blocking the lineage commitment pathway. In support of this model, we found that simultaneous inhibition of GSK-3 and mTORC1 supports the maintenance of long-term HSC function *ex vivo* in the absence of serum or exogenous cytokines (Huang et al., 2012). This work identifies a signaling network that maintains HSCs and describes the first method to maintain HSCs beyond the niche under completely defined conditions. This method therefore represents a valuable tool to interrogate the signals that maintain HSC function, as well as a potential platform to screen for compounds that induce expansion *ex vivo*.

GSK-3 and mTORC1 inhibition maintains HSC multilineage potential

Previous work from the Klein laboratory (Huang et al., 2009) reported a two-step phenotype in bone marrow depleted of *Gsk3a/b*: primary recipients of these cells exhibit enhanced donor-derived chimerism compared to wild-type, but secondary and tertiary recipients experience a progressive decline in HSC function. Donor-derived cells display increased S6 phosphorylation, indicating mTORC1 activation in the absence of GSK-3. Administration of the mTORC1 inhibitor rapamycin to secondary transplant recipients preserves HSC function, demonstrating that the progressive hematopoietic failure is mTORC1-dependent. mTORC1 inhibition therefore sustains HSC function in the context of Wnt pathway activation *in vivo* (Huang et al., 2009).

As these studies were performed *in vivo*, we could not rule out the possibility that additional factors from the microenvironment may have contributed to HSC maintenance in the context of dual GSK-3 and mTORC1 inhibition. To address this question, we tested whether combined inhibition of GSK-3 and mTORC1 was sufficient to maintain HSCs *ex vivo* under completely defined conditions. We cultured cKit$^+$ bone marrow cells, which are enriched for hematopoietic stem and progenitor cells (HSPCs), for 7 days in serum-free, cytokine-free medium supplemented only with the GSK-3 inhibitor CHIR99021 and rapamycin (CR), followed by functional assessment (**Figure 2.1A**). Induction of the Wnt target gene *Axin2* in CHIR99021-treated cKit$^+$ cells (with or without rapamycin) confirmed activation of Wnt/β-catenin signaling under these conditions (**Figure 2.1B**).

As a preliminary test of hematopoietic potential after cytokine-free culture, we seeded cKit$^+$ cells at three concentrations onto stromal cells that support hematopoietic differentiation (Holmes and Zuniga-Pflucker, 2009). Each concentration was co-cultured in triplicate wells. After two serial passages over 21 days in this stromal co-culture, we performed flow cytometry for hematopoietic lineage markers. This analysis revealed that

CR-cultured HSPCs retained multilineage potential, while vehicle-cultured cells did not (**Figure 2.1C,D**).

The $cKit^+$ fraction of the bone marrow is a highly heterogeneous population, so we repeated these experiments in $Lin^-Sca1^+cKit^+$ (LSK) cells, which are more highly enriched for HSPCs. We cultured LSK cells for 7 days in CR without serum or cytokines, and then transferred the cells to stromal co-culture for 21 days. Flow cytometric analysis again demonstrated both myeloid and lymphoid potential of CR-cultured HSPCs, which was absent from vehicle-cultured cells (**Figure 2.1E**).

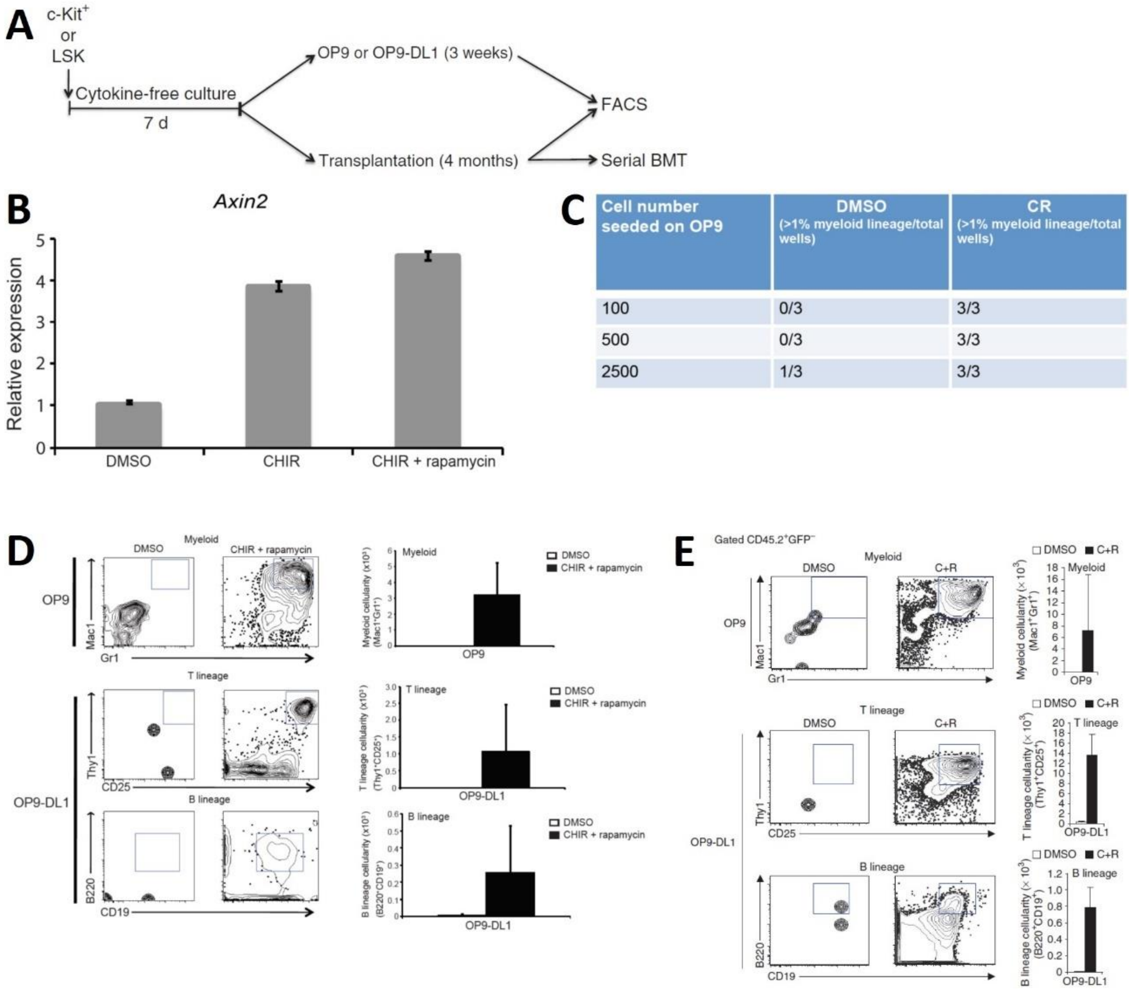
Conclusion

These results provided initial proof of principle that GSK-3 and mTORC1 inhibition is sufficient to sustain at least some hematopoietic function *ex vivo*. CR-cultured HSPCs gave rise to both myeloid and lymphoid cells, indicating multilineage potential. However, stromal co-culture may allow non-physiological lineage commitment of hematopoietic cells (Richie Ehrlich et al., 2011) and is not a definitive test of HSC function. In addition to multilineage differentiation, HSC function must also be demonstrated by the capacity to self-renew. As the current gold standard to confirm these dual properties is bone marrow transplantation, the first author subsequently performed serial transplantation of cultured $cKit^+$ cells into irradiated recipients. CR-cultured HSPCs gave rise to multilineage chimerism and engrafted primary, secondary, and tertiary recipients, demonstrating robust self-renewal capacity (Huang et al., 2012). The first author additionally identified β-catenin as an essential mediator of this signaling network, as *Ctnnb1*-knockout $cKit^+$ cells cultured in CR failed to generate hematopoietic cells in stromal co-culture. Importantly, human UCB $CD34^+$ cells cultured in CR also gave rise to multilineage chimerism in serial transplant, indicating that this signaling network supports both murine and human HSC maintenance. Taken together, this work shows that the combination of Wnt pathway activation and mTORC1 inhibition supports the maintenance of long-term, self-renewing HSCs in defined, cytokine-free conditions.

These findings identify a minimal set of signals required to maintain HSC function beyond the niche. The defined conditions described here thus establish a novel system in which to interrogate the signals that mediate HSC maintenance. Hematopoietic cytokines seem to be essential for the survival of HSPCs beyond the niche, but they likely promote proliferation at the expense of self-renewal (Hofmeister et al., 2007; Chou et al., 2010). Combinations of cytokines additionally interact in often synergistic (non-additive) ways (Ahsberg et al., 2010; Knapp et al., 2017b), confounding interpretations of molecular and functional responses to cytokine treatment. The finding here that GSK-3 and mTORC1 inhibition maintains HSCs in the absence of cytokines provides a novel platform that allows the culture of HSCs under defined conditions, substantially simplifying in-depth characterization of the signaling network that regulates HSC function.

In summary, we reported that simultaneous Wnt activation and suppressed nutrient-sensing by mTORC1 is sufficient to maintain HSC function in the absence of serum or exogenous cytokines. This model culture system identifies a minimal signaling network required to maintain HSCs and lays the groundwork for testing additional compounds that, in combination with GSK-3 and mTORC1 inhibition, might promote HSC expansion while preserving self-renewal. A thorough understanding of the mechanism of GSK-3/mTORC1-inhibition-mediated maintenance will yield substantive insight toward additional pathways to target in the search for conditions that will promote such expansion. Although Wnt/β-catenin signaling is required for this maintenance, defining additional downstream effectors by which GSK-3 and mTORC1 inhibition maintains HSC function is an important goal of follow-up work.

Figure 2.1: Multilineage potential of HSPCs cultured with GSK-3 and mTORC1 inhibitors.



Cell number seeded on OP9	DMSO (>1% myeloid lineage/total wells)	CR (>1% myeloid lineage/total wells)
100	0/3	3/3
500	0/3	3/3
2500	1/3	3/3

Figure 2.1: Multilineage potential of HSPCs cultured with GSK-3 and mTORC1 inhibitors. (A) Experimental design. Mouse $cKit^+$ or LSK cells were cultured in cytokine-free medium with vehicle or CR for 7 days and then either plated on OP9 or OP9-Dl1 stromal cells or transplanted into lethally irradiated mice. Flow cytometry was performed after 3 weeks of co-culture or 4 months after transplantation. Hematopoietic cells were distinguished from OP9 cells (which are GFP^+) by gating on the $CD45.2^+GFP^-$ population. (B) Mouse $cKit^+$ cells were harvested and treated for 12 hours with DMSO, CHIR99021, or CR. Expression of the Wnt target gene *Axin2* was measured by RT-PCR. (C) Effect of CR on $cKit^+$ cells after 7 days in culture. After 7 days of cytokine-free culture, $cKit^+$ cells were serially diluted and plated on OP9 stromal cells. After 3 weeks of co-culture, flow cytometry for hematopoietic cells was performed, and number of positive wells out of total wells was counted. Wells with >1% myeloid lineage out of the total number of viable cells were scored as positive. (D,E) Representative flow cytometry data (left panels) and quantification of myeloid, B, and T lineages (right panels) from $cKit^+$ (D) or LSK (E) cells that were cultured for 7 days, plated on OP9 or OP9-DL1 cells, and assessed for myeloid (OP9), B, and T (OP9-Dl1) lineage markers. Cultured hematopoietic cells were plated on stromal cells in triplicate wells. Histograms represent the mean value from three wells (error bars, S.D.). (The overall experiment was performed twice.)

CHAPTER 3: TRANSCRIPTION PROFILING IN HSC MAINTENANCE

Introduction

HSCs rely on a range of cues from the niche to sustain their function and to instruct cell fate decisions. Parsing out individual contributions of these stimuli *in vivo* to define the signaling network that maintains HSCs has therefore presented a major challenge to the study of HSC biology, moreover compounded by interactions between these stimuli. An incomplete understanding of these cues has furthermore precluded development of controlled conditions *ex vivo* to characterize these pathways. A deeper characterization of this network will inform the investigation of signals regulating HSC homeostasis both *in vivo* and in the context of *ex vivo* expansion for research and clinical applications.

We showed previously that simultaneous inhibition of GSK-3 and mTORC1 maintains HSC function *ex vivo* without the need for serum or exogenous cytokines (Huang et al., 2012). This is the first time HSCs have been maintained *ex vivo* in the absence of cytokines. Cytokines historically have been used to culture HSCs, but at the expense of concomitant lineage commitment (Hofmeister et al., 2007; Chou et al., 2010). The novel culture conditions we described therefore represent a defined system in which to interrogate a signaling network that maintains HSCs. Although β-catenin is required for this maintenance, the mechanism by which GSK-3 and mTORC1 inhibition maintains HSCs remains otherwise unclear. I therefore proposed transcriptional profiling of HSCs preserved under these conditions as a means to characterize this maintenance program in a defined setting, isolated from complex and interacting inputs from the niche. Specifically, I aimed to compare the gene expression profiles of freshly isolated HSCs and of HSCs cultured with vehicle, GSK-3 inhibitor CHIR99021, mTORC1 inhibitor rapamycin, or both (CR). I predicted that CR-maintained HSCs would have a transcriptional profile that was distinct from vehicle- or single-treated cells, and that components of this profile would be required for the maintenance of HSC function.

Reduced RNA content in cultured HSCs

Quiescent (G_0) cells can be identified in part by a lower RNA content compared to actively cycling cells (Holyoake et al., 1999; Challen et al., 2009). HSCs correspondingly contain less total RNA than progenitors (Signer et al., 2014). Quiescent murine HSCs (Huttmann et al., 2001) and satellite cells of the skeletal muscle (Fukada et al., 2007) additionally contain less RNA than their activated counterparts, as measured by staining for the RNA dye pyronin Y. Aged human HSCs, which are less quiescent than young HSCs, are also correspondingly more frequently in the pyronin Y-high fraction than young human HSCs (Pang et al., 2011). Consistent with these reports, I observed that freshly isolated HSCs contain less total RNA per cell than hematopoietic progenitor cells ($Lin^-Sca1^-cKit^+$ [LK]; HPCs), as measured by nanofluidic electrophoresis (**Figure 3.1A**). I additionally discovered that RNA was undetectable in cells after 3 days of culture (as described in greater detail in Chapter 4), regardless of treatment. Importantly, cellular RNA was undetectable despite recovery of up to 70% of an mRNA spiked into each sample at the time of cell lysis (**Figure 3.1B**), indicating that RNA was stable during isolation. CR-cultured cells were moreover viable, as they excluded Trypan blue and engrafted in transplant recipients (as detailed in Chapter 4). I therefore conclude that the lack of RNA recovery from cultured HSCs does not represent a technical limitation, but rather an intriguing biological process associated with hematopoietic cells in cytokine-free culture.

RNA-seq on HSCs maintained *ex vivo*

To overcome the limitation of vanishingly small quantities of RNA in cultured HSCs, I adapted an aRNA amplification protocol designed to permit transcriptome analysis of single cells (Morris et al., 2011). Briefly, polyadenylated RNAs serve as the template to generate double-stranded cDNA into which a T7 RNA polymerase promoter is incorporated. An *in vitro* transcription reaction with T7 RNA polymerase then produces antisense transcripts (aRNA). This process is performed for two to three rounds, with slight technical modifications following the first round to use the aRNA as template. In theory, the method produces linear amplification of polyadenylated transcripts, preserving the relative abundance of components of the transcriptome and generating sufficient material for RNA-seq analysis. With a potential solution to the limited quantity of RNA in cultured HSCs, I performed gene expression profiling in these cells.

I sorted HSCs for RNA isolation either immediately after sorting or following 3 days in culture under defined conditions as described (Huang et al., 2012). Cultured cells were treated with vehicle, CHIR99021, rapamycin, or both. Following RNA isolation, all samples underwent three rounds of aRNA amplification, and aRNA was provided to the Perelman School of Medicine Next-Generation Sequencing Core for library preparation and RNA-seq. I completed five biological replicates of this analysis.

As an initial assessment of the gene expression profiles, I assessed principal component analysis (PCA). Although most samples clustered closely together, PCA showed several experimental outliers (**Figure 3.2A**). I excluded those outliers from further analyses. PCA on the remaining samples revealed tight clustering of uncultured HSCs (**Figure 3.2B**), demonstrating that the starting populations sorted were similar across experimental replicates. Cultured samples formed a second, looser cluster, but they did not generally group by drug treatment (**Figure 3.2B**). This suggested that further analysis may not reveal substantial differences in gene expression between CR- and control-cultured HSCs.

I nonetheless analyzed transcription profiles for genes that fulfilled two criteria: 1) expression level was <1.2-fold different in CR-cultured HSCs compared to uncultured HSCs, and 2) expression level was ≥1.5-fold different in CR-cultured HSCs compared to control-cultured HSCs (with a false discovery rate <25%). One hundred forty-three genes fulfilled these criteria. Based on their reported functions, about half of these genes grouped into recurrent categories (**Figure 3.3A**). Notably, this analysis identified 18 genes related to the ribosome, including 12 ribosomal protein genes. A more systems-level analysis of the full 143-gene list using Ingenuity Pathway Analysis (IPA) suggested enhanced eIF2 or Myc signaling in CR-cultured HSCs could be responsible for the observed expression patterns, primarily due to the presence of the ribosomal genes.

I next attempted to validate some of these RNA-seq results by RT-PCR. I performed RT-PCR for four of the ribosomal protein genes that fulfilled the initial criteria for defining genes of interest. Although neither *Myc* nor *Mycn* was on this list, I assessed their expression levels as well, based on the suggestion from the IPA that Myc signaling may be uniquely enhanced in CR-cultured HSCs compared to controls. In the RNA-seq, *Myc* expression decreased over 10-fold in all cultured cells compared to uncultured (**Figure 3.3B, top panel**). *Mycn* expression was also substantively lower in all cultured cells compared to uncultured, although the magnitude of reduction varied. Two biological replicates of RT-PCR reproduced the reduction in *Myc* expression in cultured cells, but were less consistent in *Mycn* expression (**Figure 3.3B, top panel**). Ribosomal protein gene expression by RT-PCR varied even more from the RNA-seq and revealed a consistent, substantial decrease in detected expression in cultured versus uncultured cells (**Figure 3.3B, middle and bottom panels**). This result was particularly problematic given that I selected these genes based on their similar expression levels in uncultured and CR-cultured cells according to the RNA-seq. Taken together, I conclude that the RNA-seq dataset may be unreliable.

Testing aRNA amplification method

The extensive RNA processing necessary to generate sufficient material for RNA-seq, combined with the inability to confirm RNA-seq results by RT-PCR, led me to test whether any observed differences in gene expression represented true biological differences, or if they might simply be technical artifacts of the amplification technique. In particular, if there were any measurable bias in amplification (such as for short versus long transcripts), then the multiple rounds of amplification required would magnify this bias. I therefore did a serial dilution of total RNA isolated from 650 HPCs, followed by aRNA amplification. If amplification was linear, then I would predict that diluting the input RNA would yield a stepwise decrease in the total amount of RNA at the end of the amplification process. However, more dilute samples yielded only modest decreases in final RNA yield (**Figure 3.4A**). It is noteworthy that the technique was originally optimized for amplification of RNA from single cells (Morris et al., 2011), while the linearity test described here used RNA from hundreds of cells. It is therefore possible that I failed to obtain a reduction in yield at the higher input RNA concentrations (undiluted, 1:5, 1:20) due to saturation of the system. However, the more dilute samples (1:100, 1:500) also failed to yield a magnitude of decrease in aRNA generation that reflects the amount of input material. I therefore conclude that aRNA amplification is only approximately linear at best.

I next tested whether individual transcripts were uniformly amplified across samples subjected to this method. I used a fixed amount of aRNA from each of the serially diluted RNA samples described above for cDNA synthesis and performed RT-PCR for five genes. All samples originated from a single RNA sample, ensuring that the relative abundance of each transcript was equivalent prior to amplification. If amplification was uniform, then these relative abundances would be preserved following amplification. Instead, I observed a distinct effect of initial input RNA dilution on the amount of each transcript detected by RT-PCR (**Figure 3.4B**), indicating that the technique does not reliably preserve relative transcript abundance. Based on the observations that the aRNA amplification method amplified RNA in a manner that was neither linear nor uniform, I conclude that the technique was not an appropriate solution to resolve the significant reduction in cellular RNA content observed in cultured HSCs and thereby perform transcriptomic analysis of these cells.

Normalizing expression data from samples with distinct amounts of RNA

In addition to the exceptionally *small* amount of RNA in cultured cells, the *differing* amounts of total RNA in uncultured and cultured cells (**Figure 3.1**) presented a further challenge in performing expression profiling on HSCs maintained *ex vivo*. Standard practice in global gene expression analysis introduces equivalent amounts of RNA from samples to be compared and normalizes the total signal (Mortazavi et al., 2008). This approach relies on the assumption that the cellular sources being analyzed produce similar amounts of RNA. However, two groups have reported that cells expressing high levels of the transcription factor c-Myc are larger and contain two to three times more total RNA than their wild-type counterparts (Lin et al., 2012; Nie et al., 2012). Rather than defining specific c-Myc target genes, this work therefore identifies c-Myc as an amplifier of the global transcriptional program that is already active in cells. More broadly, however, these findings indicate that the conventional normalization approach is not appropriate for samples that contain different amounts of RNA, as this can both distort apparent relative expression levels across samples and mask true changes in global transcription.

To resolve this limitation, Young and colleagues (Loven et al., 2012) have proposed spiking in a fixed amount of standard RNAs per cell in samples to be subjected to global transcription profiling, permitting normalization to cell number per sample and thus detection of changes in gene expression at the level both of individual genes and of the full transcriptome. Although Loven et al. (2012) spike in the RNA standards following cellular RNA isolation, the method can additionally control for variability in RNA isolation efficiency across samples if the standards are spiked in at the time of cell lysis. In light of these factors, spiking in RNA standards is an attractive strategy to overcome some of the unique challenges presented by my efforts to perform transcription profiling in uncultured and cultured HSCs. This approach will additionally prove important as future gene expression analyses factor in differences in total RNA content under more physiological settings, such as in quiescence (Bulut-Karslioglu et al., 2016), differentiation (Signer et al., 2016), and cancer (Percharde et al., 2017).

Proposed method for transcription profiling in HSCs maintained *ex vivo*

Based on my observations in early attempts to perform gene expression profiling in HSPCs, I present here a detailed workflow describing how such analysis could be completed to define the transcriptional profile of HSCs maintained *ex vivo*. The techniques outlined below account for the obstacles presented by the differing and low RNA quantities encountered in my samples of interest. (The RNA content in cultured cells is so low that simply starting the experiment with more cells is not sufficient to address the obstacles encountered, due to the number of mice that would be required.) This analysis would allow identification of a gene expression signature in cultured HSCs that is specific to cells that retain self-renewal and multilineage repopulating functions.

Due to the panel of multiple markers currently required to isolate high-purity HSCs, flow cytometric sorting of cells is necessary to perform any analysis on HSCs. However, the shear stress of flow cytometric sorting has been reported to modestly but measurably reduce post-sort viability in a variety of cell types (Mollet et al., 2007; Zaitoun et al., 2010). The mechanical stress of sorting additionally influences gene expression (Avvisato et al., 2007; Beliakova-Bethell et al., 2014). Although these factors apply to sorted cells in all samples and the magnitude of these changes is relatively small, they should be minimized where possible. All of the experiments described in this dissertation were performed using HSPCs sorted on a FACSARIA II (BD Biosciences). However, for future experiments involving highly sensitive analyses, such as gene expression profiling, sorting cells on an INFLUX may be a more appropriate choice. Compared to standard sorters such as the FACSARIA, the INFLUX combines a distinct nozzle shape and low sheath pressure to accelerate cells more smoothly and under lower shear stress following stream interception with the lasers, improving cell viability (BD Biosciences) and potentially minimizing changes in gene expression induced by the mechanical force of sorting.

Following HSC isolation, I initially cultured cells in vehicle, CHIR99021, rapamycin, or both inhibitors for 3 days before RNA extraction for gene expression profiling. In choosing this length of culture, I aimed for the shortest period that would yield a transcriptional profile in CR-maintained HSCs that was distinct from that of HSCs cultured in only one or neither inhibitor. In particular, I predicted that rapamycin-dependent transcriptional changes would take time to accumulate, as mTORC1 influences transcription in part through regulating translation and activation of TFs (Laplante and

Sabatini et al., 2013; Showkat et al., 2014). However, Manning and colleagues treated *Tsc1*- or *Tsc2*-null mouse embryonic fibroblasts with rapamycin and observed mTORC1-dependent changes in transcription in as little as 6 hours (Duvel et al., 2010). The dramatic reduction in total RNA content observed in cultured cells by day 3 (**Figure 3.1B**) additionally suggests that a shorter culture period (perhaps 1 day) would be sufficient to identify a unique transcriptional profile in maintained HSCs. This observation moreover reveals a robust effect of culture itself, independent of maintenance of function. A shorter culture period would therefore minimize artifacts of the culture while likely still yielding informative differences in gene expression.

As introduced above, the incorporation of spiked-in RNA standards (Loven et al., 2012) would be essential to appropriate analysis of the gene expression profile of cultured HSCs. Cultured cells contain an undetectably low amount of RNA (**Figure 3.1B**), precluding any controls at the level of inputting a constant quantity of RNA per sample to the library preparation and RNA-seq workflow. RNA standards added to lysates on a per-cell basis at the time of cellular RNA isolation would therefore serve as an important control at normalization. The ability to normalize reads to RNA per cell would additionally allow accurate comparison of cellular transcript abundance between uncultured HSCs and HSCs maintained *ex vivo*, which contain different amounts of RNA. This is a robust phenotype that would be masked by conventional normalization methods. Notably, spiked-in RNA standards compose only ~1-5% of total reads, so conventional normalization methods can still be applied to analyze cellular transcripts. This flexibility of the platform therefore permits detection both of global shifts in transcription (by normalizing to spiked-in RNAs as a surrogate for cell number) and of extreme outlier genes (by conventional normalization to read depth) (Percharde et al., 2017).

Finally, gene expression profiling of cultured HSCs would require RNA amplification to obtain a usable quantity of RNA for library preparation and transcriptome analysis. Although the aRNA amplification method was not reliable in my hands (**Figure 3.4**), the SMARTer Pico PCR cDNA Synthesis kit (Clontech) may be a better tool. The method relies on SMART (Switching Mechanism at 5' end of RNA Template) technology with a proprietary reverse transcriptase that generates double-stranded cDNA from picogram quantities of total RNA. Briefly, oligo(dT) with an added 5' sequence primes reverse transcription of polyadenylated transcripts. Upon reaching the 5' end of the template RNA, the reverse transcriptase adds several nucleotides to the 3' end of each cDNA. A distinct SMARTer oligo then base-pairs to the extended tail and primes the second-strand cDNA synthesis. Standard reverse transcription is prone to interruption by template RNA secondary structure. As cDNAs prematurely terminated in the SMARTer protocol fail to incorporate the 3' SMARTer oligonucleotide, they are not amplified by subsequent PCR. This approach therefore enriches for generation and amplification of full-length cDNAs. The method is moreover compatible with the addition of spiked-in transcripts, as commercially available RNA standards (such as from the External RNA Controls Consortium) are polyadenylated. Notably, the SMARTer kit manufacturer recommends a minimum starting amount of 20 pg/µl (Clontech), but success has been described with as little as 10 pg total RNA (Igor Antoshechkin, personal communication). This technology is therefore ideally suited for the amplification of RNA isolated from cultured HSCs.

Together, the workflow described here presents an informed strategy broadly applicable to gene expression profiling in cells with differing and/or limiting quantities of RNA. This approach will therefore be especially valuable to define a transcriptional program that is specific to HSCs maintained under the Klein laboratory's defined *ex vivo* conditions. Further characterization of this profile may identify both novel regulators of HSC maintenance and rational targets to promote their expansion while preserving function to facilitate molecular characterization and clinical applications of HSCs.

Conclusion

The HSC niche instructs HSCs with diverse signals. A long-standing challenge in the study of HSCs has therefore been characterizing pathways that regulate their function and fate. The Klein laboratory's conditions to maintain HSCs *ex vivo* under defined conditions (Huang et al., 2012) represent a valuable tool to achieve this goal. Transcriptional profiling of these HSCs, and comparison to uncultured HSCs and to HSCs cultured under conditions that do not maintain stem cell function, could yield significant insight into the signaling network that maintains HSCs.

Given how drastically different the HSC niche is from the culture conditions we have reported to maintain HSCs *ex vivo*, it was somewhat unsurprising that the gene expression profile of these cells bore limited overlap with that of uncultured HSCs (**Figure 3.2B**). This prediction was supported by the dramatic decrease in RNA content observed in cultured compared to uncultured HSCs (**Figure 3.1B**). The comparison may nonetheless be informative for two reasons. First, minimal overlap of only a handful of genes between these two populations would significantly filter down the total transcriptional program to just those genes that fundamentally drive retention of long-term HSC function. Second, the comparison of uncultured and cultured HSCs will help define how HSCs maintain their function while adapting to an environment beyond the niche, providing valuable insight as researchers attempt to expand HSCs *ex vivo* to facilitate basic and translational research.

Deep sequencing could reveal a more nuanced level of transcriptional regulation in HSC maintenance by additionally permitting analysis of alternative splicing events. Gazit and colleagues recently described comprehensive mapping of splicing in mouse HSCs, uncovering alternative splicing throughout the transcriptome (Goldstein et al., 2017). They furthermore observed some enrichment for multiple isoforms of genes preferentially expressed in HSCs, such as *Hlf*, *HoxA9*, and *Prdm16*, and some of these isoforms exhibited distinct levels of activity. GSK-3 has been identified as a regulator of alternative splicing (Heyd and Lynch, 2010; Mansi Shinde and Peter Klein, unpublished), suggesting the intriguing possibility that in addition to β-catenin-dependent transcription (Huang et al., 2012), altered RNA splicing could be a pathway through which GSK-3 inhibition contributes to HSC maintenance *ex vivo*.

Although all of the work presented in this dissertation was performed in mouse HSPCs, the ultimate goal is improved characterization and clinical application of human HSCs. The experimental workflow presented in this chapter is readily applicable to human HSCs. The identification of markers such as CD49f (Notta et al., 2011) has enabled isolation of highly purified human HSCs, a technical advance that will promote a more refined definition of the human HSC regulatory network.

Finally, my attempts to perform transcription profiling in cultured HSCs revealed a dramatic decrease in total RNA content, an unexpected but highly robust response of HSCs to serum- and cytokine-free culture. This phenotype was accompanied by a remarkable decrease in cell size (as described in Chapter 4). The reduction in RNA content is consistent with a quiescent state, as has been recently reported in mouse blastocysts pharmacologically diapaused with an mTORC1 inhibitor (Bulut-Karslioglu et al., 2016). Notably, however, I observed this in all cultured cells, regardless of retention of HSC function. It may therefore not be a component of the mechanism of HSC maintenance *ex* *vivo*, but rather an artifact of the culture itself. The limited quantity of RNA in all cultured cells highlights the possibility that HSCs maintained *ex vivo* under these conditions may exhibit only modest differences in expression levels of specific genes compared to control-cultured HSCs. By extension, HSC maintenance may be mediated post-transcriptionally. For example, β-catenin recruits chromatin remodelers (Blythe et al., 2010) as well as promotes target gene transcription, and mTORC1 is a known regulator of translation. Chromatin immunoprecipitation for assessment of histone modifications is now possible starting with as few as 10,000 cells (Adli et al., 2010), enabling the investigation of chromatin remodeling that might occur in HSCs maintained *ex vivo* by CR culture. Ribosomal profiling may also eventually be informative of transcriptome-wide ribosome occupancy, although the technology currently requires orders of magnitude more cells than is possible to perform on such a rare population (Hsieh et al., 2012).

The transcriptional profile of HSCs maintained *ex vivo* under defined conditions would be an informative resource in ongoing efforts to define the regulatory network that promotes retention of HSC function. Although I have not yet achieved this goal, my efforts so far have yielded valuable insight into the technical considerations of such an undertaking, allowing me to propose detailed methodology for how this might be completed in the future. The method described here is moreover broadly applicable to biologically relevant conditions that yield limiting quantities of RNA or differing quantities of RNA between samples.

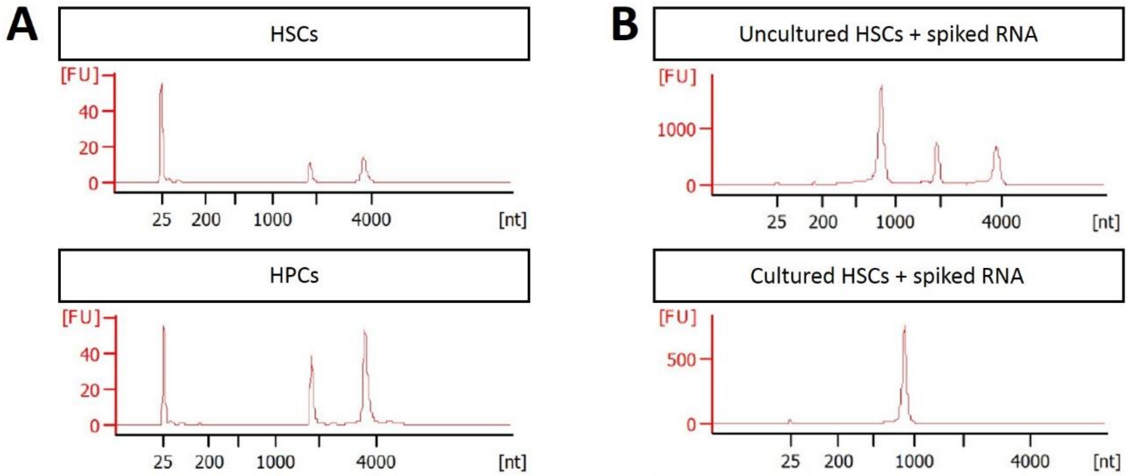


Figure 3.1: Reduced RNA content in cultured HSCs. (A) RNA was isolated from freshly sorted HSCs and HPCs. Total RNA content was measured by nanofluidic electrophoresis. (B) Cellular RNA was isolated with spiked-in YFP mRNA from freshly sorted HSCs and from HSCs following 3 days culture. Total RNA content was measured by nanofluidic electrophoresis. The peak at ~900 nt indicates the spiked-in mRNA, while peaks at 2000 nt and 4000 nt indicate 18S and 28S rRNA, respectively (or lack thereof).

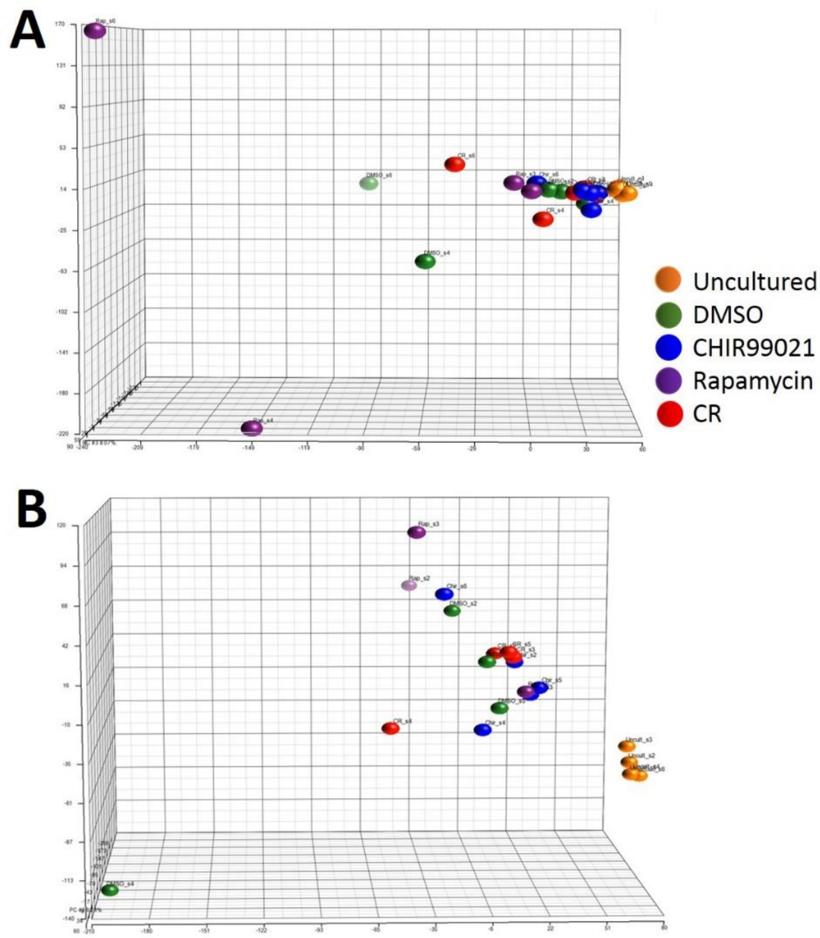


Figure 3.2: PCA of RNA-seq on HSCs maintained ex vivo. (A,B) RNA was isolated from freshly sorted HSCs and from HSCs following 3 days cytokine-free culture in DMSO, CHIR99021, rapamycin, or CR. RNA was amplified and RNA-seq was performed on five biological replicates. PCA was applied to all samples (A) and following exclusion of outlier samples (B). Each sphere represents an individual sample from one replicate.

Figure 3.3: Gene expression profile analysis.

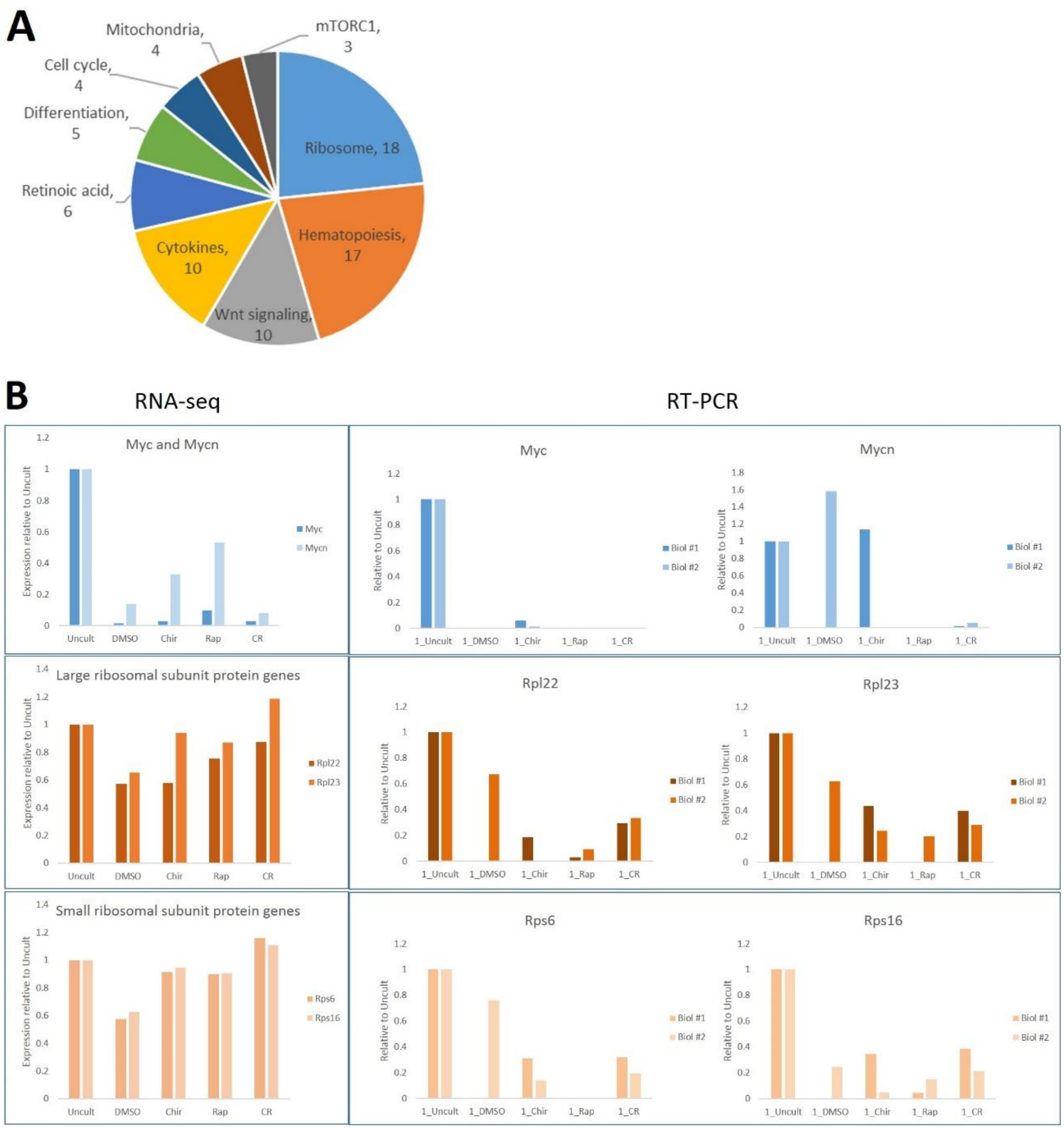


Figure 3.3: Gene expression profile analysis. (A) RNA-seq was performed on RNA isolated from uncultured HSCs and from HSCs following 3 days cytokine-free culture in vehicle, CHIR99021, rapamycin, or both. Genes of interest were identified as those whose expression level was 1) <1.2-fold different in CR-cultured HSCs compared to uncultured HSCs, and 2) ≥1.5-fold different in CR-cultured HSCs compared to control-cultured HSCs. One hundred forty-three genes fulfilled these criteria. Based on their reported functions, about half of these genes grouped into the categories shown. (B) RT-PCR was performed on two biological replicates (right panels) to validate genes of interest identified by IPA of RNA-seq (left panels).

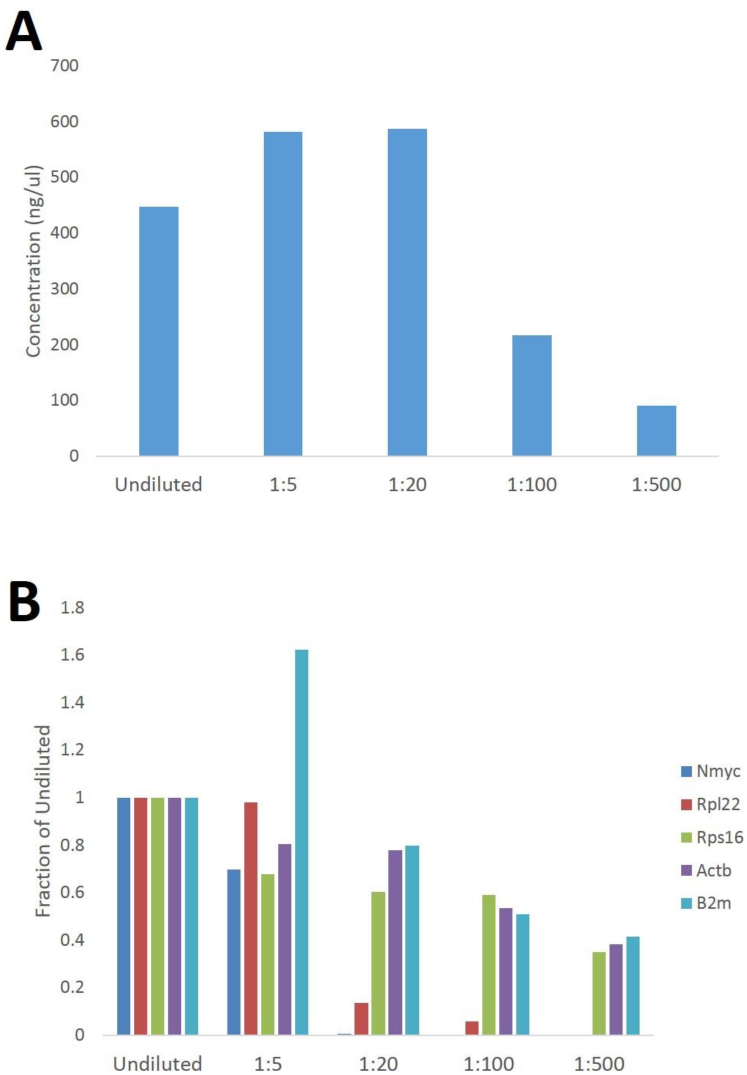


Figure 3.4: Verifying aRNA amplification method. (A,B) Total RNA was isolated from 650 HPCs, serially diluted, and subjected to three rounds of aRNA amplification. aRNA concentration was quantified by spectrophotometry (A), and relative transcript abundance of several genes following amplification was measured by RT-PCR (B).

CHAPTER 4: AUTOPHAGY IS A SIGNATURE OF A SIGNALING NETWORK THAT MAINTAINS HSCS

The data presented in this chapter are in revision at *PLoS One*.

Introduction

Stem cells are defined by the ability to both self-renew and generate differentiated cells, enabling them to regenerate tissues under steady-state conditions and in response to injury. This essential function in lifelong tissue maintenance endows stem cells with exceptional potential for clinical applications. HSCs, for example, are responsible for maintaining the blood, and are used for the treatment of both congenital and acquired hematological disorders (Purton and Scadden, 2007. While tremendous progress has been made toward optimizing therapeutic use of HSCs, this is a rare population. Efforts to expand HSCs *ex vivo*, for example for umbilical cord transplants or for therapeutic genome editing, have been hampered by an incomplete understanding of the signaling networks that regulate HSC fate decisions and subsequent difficulty defining conditions that maintain HSC function beyond the niche (Lymperi et al., 2010).

HSCs reside in a low-perfusion niche (Parmar et al., 2007; Simsek et al., 2010; Spencer et al., 2014), underscoring nutrient-sensing as an essential function. The evolutionarily conserved nutrient sensor mTORC1 antagonizes HSC function, as interventions that activate mTORC1, including loss of the negative regulators *Pten* (Yilmaz et al., 2006), *Gsk3* (Huang et al., 2009), and *Tsc1* (Gan et al., 2008), lead to HSC proliferation followed by exhaustion and in some cases leukemogenesis (Yilmaz et al., 2006; Guezguez et al., 2016). Developing a thorough understanding of mediators of mTORC1 signaling in this context will therefore be a critical step toward expansion of functional HSCs *ex vivo*.

mTORC1 regulates cell growth and proliferation through multiple effectors. The relative importance of each of these effectors to HSC function and fate decisions remains unclear. The best-characterized output of mTORC1 activation is the regulation of translation via activation of S6 kinase (S6K) (Isotani et al., 1999) and inhibition of eIF4E binding protein (4E-BP) (Gingras et al., 1999). HSCs require a precise level of translation: they exhibit a low basal rate of translation compared to more differentiated hematopoietic cell populations, and either increasing or decreasing this rate impairs their engraftment upon transplantation (Signer et al., 2014). mTORC1 additionally promotes mitochondrial biogenesis through both translation-dependent (Morita et al., 2013) and -independent (Cunningham et al., 2007; Blattler et al., 2012) mechanisms. HSCs have been reported to have lower mitochondrial mass and membrane potential than other hematopoietic populations, with increases in these features correlating with loss of self-renewal capacity (Mantel et al., 2010).

mTORC1 also suppresses autophagy, which is both a pro-survival mechanism in the context of nutrient starvation and an important quality control system in the turnover of old or damaged proteins and organelles. Autophagy is therefore a critical component of stem cell maintenance, differentiation, and aging (Guan et al., 2013), and disruption of this pathway has been linked to loss of function of a variety of stem cell populations, including embryonic, epidermal (Salemi et al., 2012), skeletal muscle (Tang and Rando 2014; Garcia-Prat et al., 2016), neural (Vazquez et al., 2012; Wu et al., 2016), and hematopoietic (Mortensen et al., 2011; Ho et al., 2017) stem cells. In the hematopoietic system, loss of the essential autophagy genes *Atg7* or *Atg5* impairs HSC function while promoting proliferation of hematopoietic progenitor cells, leading to bone marrow failure (Mortensen et al., 2011; Watson et al., 2015). Moreover, highlighting the essential role of nutrient-sensing in the reduced-perfusion HSC niche, HSCs activate autophagy to survive cytokine starvation, while progenitors fail to activate autophagy and instead undergo apoptosis (Warr et al., 2013). These findings indicate a unique requirement for autophagy in the function of HSCs as opposed to other hematopoietic cell populations.

Despite the extensive body of literature characterizing these distinct outputs of mTORC1 signaling in HSCs, the role of each in HSC maintenance remains unclear. The complexity of the HSC niche and consequent challenge maintaining HSCs *ex vivo* have constrained efforts to address this question. Previous work from our laboratory showed that HSCs are maintained *ex vivo* in cytokine-free conditions when GSK-3 and mTORC1 are inhibited (Huang et al., 2012). Inhibition of GSK-3 activates downstream Wnt/β-catenin signaling, and β-catenin is required for HSC maintenance in this setting, but the pathway(s) downstream of mTORC1 that contribute to this response have not been identified. We have investigated the complex signaling network downstream of mTORC1 associated with the maintenance of long-term HSCs. We find that activation of autophagy is uniquely associated with conditions that maintain self-renewing HSCs.

Cell-autonomous regulation of HSC function by GSK-3 and mTORC1

We previously reported that simultaneous GSK-3 and mTORC1 inhibition maintains HSC function *ex vivo* in HSPCs ($Lin^{-}Sca1^{+}c\text{-}Kit^{+}$ [LSK]) (Huang et al., 2012). While this fraction is enriched for HSCs, it is a heterogeneous population composed primarily of progenitor cells. To address a potential indirect effect of modulating GSK-3 and mTORC1, we sorted HSCs ($LSK\text{-}CD48^{-}CD150^{+}$ [LSK-SLAM]) and cultured them in serum-free, cytokine-free medium in the presence or absence of the GSK-3 inhibitor CHIR99021 and rapamycin (CR). Cell number did not significantly change during culture, and ~87% of cells remained viable after 7 d of culture (**Figure 4.1A,B**). To assess HSC function, we performed a competitive repopulation assay. $CD45.1^{+}$ HSCs cultured in vehicle or CR for 7 d were injected with CD45.2$^+$ competitor whole bone marrow cells into lethally irradiated recipients. HSCs cultured with CR retained both multilineage and long-term (up to 24 weeks) engraftment potential, while control cultured cells failed to engraft (**Figure 4.1C,D**). These results indicate that GSK-3 and mTORC1 inhibition maintains stem cell function by acting directly on HSCs.

mTORC1 activity and global translation during HSC maintenance

mTORC1 regulates cell growth and metabolism through multiple independent effectors, the best-characterized of which is promotion of translation through activation of S6K and inhibition of 4E-BP. As HSCs require a precise level of translation (Signer et al., 2014), we further explored the regulation of S6 and 4E-BP in cultured HSCs. The canonical readout for mTORC1 activity and regulation of translation is therefore measurement of phospho-S6 (pS6) and phospho-4E-BP1 (p4E-BP1) levels, which we assessed over time in LSK-CD48$^-$ cells. (The CD150 epitope is destroyed during fixation and permeabilization [Kalaitzidis and Neel, 2008].) GSK-3 suppresses mTORC1, and GSK-3 inhibition therefore activates mTORC1 (Inoki et al., 2006; Huang et al., 2009; Huang et al., 2012). Although we did not observe an effect on pS6 or p4E-BP1 using the inhibitors at our standard concentrations for HSC culture, CHIR99021 and rapamycin are typically used at three and 100 times higher concentrations, respectively. A correspondingly higher concentration of GSK-3 inhibitor increases phosphorylation of S6 in LSK-CD48$^-$ cells, as shown by flow cytometric detection with a phospho-specific pS6 antibody (**Figure 4.2A,B**). Rapamycin inhibits S6 phosphorylation, as expected, and attenuates but does not completely inhibit increased S6 phosphorylation when combined with CHIR99021 (**Figure 4.2A,B**). Rapamycin also reduces 4E-BP1 phosphorylation in LSK-CD48$^-$ cells. We moreover observe a trend toward a further decrease in p4E-BP1 in

CR-treated cells, although the difference fails to reach statistical significance (**Figure 4.2C,D**). Simultaneous inhibition of GSK-3 and mTORC1 therefore achieves a level of mTORC1 activity that is distinct from that following inhibition of either alone.

mTORC1 regulates translation in part via S6K promotion of ribosomal gene transcription (Xiao and Grove, 2009). We therefore cultured c-Kit$^+$ cells in vehicle, CHIR99021 (3 μM), rapamycin (5 nM), or both for 24 h and measured the expression of large and small ribosomal subunit protein genes by RT-PCR. CR-treated cells consistently express higher levels of all ribosomal protein genes assessed when compared to control or rapamycin-treated cells, and in most cases compared to CHIR99021-treated cells as well (**Figure 4.2E**). Importantly, ribosomal RNA expression is equal across conditions (**Figure 4.2F**). Together, these results demonstrate a specific enrichment for expression of ribosomal protein genes in CR-treated HSPCs, suggesting an elevated capacity for protein biosynthesis.

The increased expression of ribosomal protein genes in CR-cultured HSCs was unexpected, as we predicted that rapamycin would block mTORC1-dependent activation of translation. However, the effective concentration of rapamycin in our culture system is 100-fold lower than typically used. To address translation directly, we measured the rate of global protein synthesis by O-propargyl-puromycin (OP-Puro) incorporation into nascent polypeptides and fluorescent labeling (Liu et al., 2012) followed by flow cytometric analysis. We find a stepwise increase in the rate of translation as HSCs differentiate into more committed progenitor populations (**Supplementary Figure 4.1A**), as previously reported (Signer et al., 2014). We next measured OP-Puro incorporation in LSK-CD48$^-$ cells after 3 h and 24 h culture with or without inhibitors. Bulk protein synthesis is elevated in CHIR99021-treated cells compared to cells cultured with DMSO or rapamycin alone, consistent with mTORC1 and S6K activation. However, protein synthesis was also increased in CR-treated cells (**Figure 4.2G, Supplementary Figure 4.1B**); while this finding is consistent with the increased pS6 and increased ribosomal protein expression observed in CR, it is not consistent with suppression of global protein synthesis as a mechanism for CR maintenance of HSCs.

Targeting S6K and eIF4E downstream of mTORC1 in HSC maintenance

While these findings argue against suppression of protein synthesis as a mechanism of HSC maintenance *ex vivo*, we could not rule it out completely. We sought to address this possibility further by more specifically inhibiting S6K and/or eIF4E. To achieve targeted inhibition of S6 and 4E-BP, as opposed to broader inhibition of mTORC1, we replaced rapamycin in CR culture with the S6K inhibitor PF-4708671, the eIF4E inhibitor 4EGI-1, or both (**Figure 4.3A**). If rapamycin supports HSC maintenance by inhibiting translation, then an S6K or eIF4E inhibitor should substitute for rapamycin. PF-4709671 effectively inhibits S6K, as measured by reduced S6 phosphorylation in phospho-flow cytometry (**Figure 4.3B, Supplementary Figure 4.1C**), while 4EGI-1 reduces the rate of bulk protein synthesis (**Figure 4.3C, Supplementary Figure 4.1D**). However, it should be noted that the dose of 4EGI-1 that achieves reduced translation also approaches the limit of toxicity (Moerke et al., 2007).

To test whether inhibition of either of these pathways downstream of mTORC1 is sufficient to maintain HSC function, we sorted HSPCs and cultured for 7 d in CHIR99021 plus PF-4708671, 4EGI-1, or both, followed by competitive transplant. While CR-cultured cells engrafted, we detected no chimerism in recipients of any cells cultured without rapamycin (**Figure 4.3D**). We conclude that combined inhibition of S6K and eIF4E downstream of mTORC1 is not sufficient to maintain HSC function. Taken together with our findings that CR increases expression of ribosomal protein genes and the rate of global translation, these data argue against inhibition of protein synthesis as a mechanism for maintaining HSCs in cytokine-free culture.

Mitochondrial mass and activity in maintained HSCs

In addition to translation, mTORC1 is a known regulator of mitochondria. While mTORC1-driven translation contributes to mitochondrial biogenesis (Morita et al., 2013), mTORC1 also regulates mitochondrial membrane potential and oxidative capacity independently of translation (Schieke et al., 2006; Cunningham et al., 2007). As low mitochondrial mass and membrane potential have been correlated with HSC function (Mantel et al., 2010; Sukumar et al., 2016), we explored how GSK-3 and mTORC1 inhibition might affect mitochondria in HSC maintenance. We first measured total mitochondrial mass by MitoTracker Green in the LSK-CD48$^-$ fraction of lineage-depleted bone marrow cells after 16 h culture. Cells treated with CHIR99021 either alone or in combination with rapamycin exhibit an increase in mitochondrial mass (**Figure 4.4A**), consistent with the CHIR99021-mediated increase in translation (**Figure 4.2G**, **Supplementary Figure 4.1B**). However, while mitochondrial mass is generally low in HSCs compared to non-self-renewing progenitors (Mantel et al., 2010; Sukumar et al., 2016), the increase in CR-maintained HSCs argues against a role for mitochondrial mass in the maintenance of HSCs cultured in CR.

In addition to increased mitochondrial mass, increased mitochondrial membrane potential correlates with loss of long-term repopulating capacity of HSCs (Mantel et al., 2010). We therefore assessed mitochondrial membrane potential, which could allow for the observed maintenance of stemness despite elevated mitochondrial mass in CR-cultured HSCs. Following 16 h culture, we measured mitochondrial membrane potential in LSK-CD48$^-$ cells using JC-1, a positively charged dye that accumulates in the negatively charged matrix of polarized mitochondria in a potential-dependent manner (Reers et al., 1991). While heterogeneous lineage-depleted bone marrow cells exhibit a distribution between populations of high and low membrane potential, LSK-CD48$^-$ cells in all culture conditions are overwhelmingly in the low membrane potential fraction (**Figure 4.4B**). Thus CR culture increases mitochondrial mass but maintains low mitochondrial membrane potential, compatible with the maintenance of HSC function.

Reduced cell volume and RNA content in cultured HSCs

mTORC1 integrates nutrient availability and mitogenic stimuli to direct activity of biosynthetic and catabolic pathways, and is thus a major regulator of cell size (Lloyd, 2013). Our culture conditions lack serum or exogenous cytokines, known activators of mTORC1, and maintenance of HSCs depends on addition of the mTORC1 inhibitor rapamycin. We therefore assessed cell size following culture of purified HSCs in vehicle, CHIR99021, rapamycin, or CR. By 3 d in culture, all cells were visibly smaller than freshly isolated HSCs (**Supplementary Figure 4.2A**). We measured cell area on each day of culture using a micrometer and ImageJ software. Mean cell volume decreased 60% in control, rapamycin, and CR-cultured cells and 45% in CHIR99021-treated cells (**Supplementary Figure 4.2B**).

Ribosome biogenesis and protein synthesis are among the most energy-consuming cellular processes. These pathways must therefore be tightly regulated based upon nutrient availability. As cell size generally correlates with ribosome biogenesis (Cook and Tyers, 2007), we measured total RNA content in HSCs by nanofluidic electrophoresis before and after 3 d culture. RNA was readily detected in uncultured HSCs, but undetectable in an equal number of cultured cells (**Supplementary Figure 4.2C**). The low RNA content was confirmed by flow cytometry. We cultured lineage-depleted bone marrow cells in vehicle, CHIR99021, rapamycin, or both. After 3 d, we performed flow cytometric analysis for DNA content by Hoechst 33342 and for RNA content by pyronin Y in the HSPC fraction. While freshly isolated HSPCs display a clear distribution through the cell cycle, cells cultured under any condition are overwhelmingly in G0 with markedly reduced total RNA content (**Supplementary Figure 4.2D**). We therefore conclude that GSK-3 and mTORC1 inhibition maintains HSC function in cells that have entered a quiescent state.

Autophagy as a molecular mark of HSC maintenance

Quiescent HSCs require autophagy to maintain their capacity for self-renewal; loss of *Atg7* stimulates proliferation of hematopoietic progenitors with a parallel loss of long-term HSCs, leading to a marked myeloproliferative state *in vivo* (Mortensen et al., 2011). Furthermore, suppression of autophagy is a major downstream function of mTORC1, and inhibition of mTORC1 with rapamycin activates autophagy, although typically at concentrations 100 times higher than used here. To examine whether activation of autophagy is specifically associated with maintenance of functional HSCs in culture (e.g., cultured in CR), we performed RT-PCR for a pro-autophagic gene expression signature (Warr et al., 2013) in cKit$^+$ cells cultured for 24 h in DMSO, CHIR99021, rapamycin, or CR. Compared to cells cultured under control conditions, cells cultured in CR express significantly higher levels of multiple markers of autophagy, including *Atg4b*, *Bnip3*, and *Gabarap*, with *Prkaa2* and *Sesn2* exhibiting a trend toward higher expression in CR (**Figure 4.5A**). Notably, expression of these markers is higher in CR than in cells treated with rapamycin alone. This gene signature is consistent with unique activation of autophagy under conditions (simultaneous inhibition of GSK-3 and mTORC1) that maintain functional HSCs in culture.

To confirm activation of autophagy in CR-cultured HSCs, we examined the formation of autophagosomes. As a transition from diffuse to punctate staining for LC3 is a hallmark of autophagosome assembly and activation of autophagy (Kabeya et al., 2000; Tang and Rando, 2014), we performed immunofluorescence staining for LC3 puncta in HSCs cultured for 16 h (**Figure 4.5B**). CR-maintained HSCs exhibited significantly more LC3-puncta-positive cells (**Figure 4.5C**) and on average more LC3 puncta per cell (**Figure 4.5D**) compared to vehicle, CHIR99021-, or rapamycin-treated controls. Taken together with the autophagy gene signature, these results demonstrate that activated autophagy is uniquely enhanced in conditions that maintain HSCs.

Conclusion

mTORC1 regulates cell metabolism and proliferation through multiple pathways. Extensive evidence suggests that mTORC1 activation promotes HSC lineage commitment at the expense of self-renewal (Yilmaz et al., 2006; Chen et al., 2008; Huang et al., 2009; Kharas et al., 2009), but the downstream pathways mediating this effect remain undefined. We have investigated multiple downstream mTORC1 targets to assess which pathways are uniquely activated or inhibited when the GSK-3 and mTORC1 pathways are regulated to maintain HSCs. These studies reveal that autophagy strongly and uniquely correlates with conditions that maintain HSC function *ex vivo*.

We identified a pro-autophagic phenotype by a gene expression signature and the appearance of $LC3^{+}$ autophagosomes. While mTORC1 is a well-characterized inhibitor of autophagy, the dose of rapamycin used for these studies (5 nM) is significantly below the 100-500 nM reported to induce autophagy. Consistently, we only observe a significant increase in autophagy-related gene expression in the presence of rapamycin and CHIR99021 (**Figure 4.5**). GSK-3 is moreover an established negative regulator of mTORC1 (Inoki et al., 2006), and GSK-3 inhibition might therefore be predicted to block autophagy. However, several compelling lines of evidence suggest a potential mechanism for synergy between the combined inhibition of GSK-3 and mTORC1 to activate autophagy. Recent work reports that GSK-3 inhibits lysosomal acidification independently of mTORC1, blocking autophagy (Azoulay-Alfaguter et al., 2015). GSK-3 additionally inhibits autophagy independently of mTORC1 via phosphorylation and cytosolic sequestration of TFEB, the master transcription factor of lysosomal biogenesis (Parr et al., 2012; Marchand et al., 2015). Intriguingly, mTORC1 also inhibits autophagy in part through blocking nuclear translocation of TFEB (Roczniak-Ferguson et al., 2012; Settembre et al., 2012). It is therefore possible that combined inhibition of mTORC1 and GSK-3 allows TFEB to translocate to the nucleus and activate autophagy.

These findings additionally build on a growing body of literature supporting an essential role for autophagy in the maintenance, activation, and differentiation of multiple stem cell populations, including embryonic, epidermal (Salemi et al., 2012), neural (Wu et al., 2016), skeletal muscle (Tang and Rando, 2014; Garcia-Prat et al., 2016), and hematopoietic stem cells (Mortensen et al., 2011; Warr et al., 2013; Ho et al., 2016). The current speculative model in the field is that autophagy is a vital mechanism of protein and organelle quality control that maintains low levels of reactive oxygen species and protects stem cells from oxidative stress and DNA damage over the lifetime of the organism (Phadwal et al., 2013; Schultz and Sinclair, 2016). As a primary purpose of autophagy is to degrade cytoplasmic contents and generate an internal nutrient pool to sustain protein synthesis during periods of nitrogen starvation (Yang et al., 2006), autophagic recycling may also serve as an important source of amino acids for stem cells residing in a low-perfusion, reduced-nutrient niche. On the other hand, autophagy can promote stem cell activation and differentiation by degrading multipotency factors and providing biosynthetic materials to support the growth and proliferation required for tissue regeneration (Vazquez et al., 2012; Guan et al., 2013; Tang and Rando, 2014). Stem cell fate decisions may thus be directed in part by the extent or targets of autophagic degradation.

In contrast, our results do not support a role for suppression of translation as a mechanism to maintain HSC function *ex vivo*. HSCs maintained in CR display increased ribosomal protein gene expression and an elevated rate of protein synthesis compared to HSCs cultured in control medium or freshly isolated HSCs (**Figure 4.2G**, **Supplementary Figure 4.1B**). S6K and eIF4E inhibitors correspondingly reduce S6 phosphorylation and the rate of translation but fail to recapitulate *ex vivo* HSC maintenance when combined with GSK-3 inhibition (**Figure 4.3**). HSCs *in vivo* require a tightly controlled rate of protein synthesis that is regulated in part by the translation initiation inhibitor 4E-BP (Signer et al., 2014; Signer et al., 2016). The increase in bulk translation that we observe in our cytokine-free, *ex vivo* HSC maintenance system may therefore represent a homeostatic adjustment when HSCs are deprived of signals from their normal microenvironment. It also remains possible that restricting translation may be important to maintain long-term function when HSCs are stimulated to undergo self-renewing cell divisions. However, taken together, our findings of increased global translation in CR medium argue against a role for suppression of protein translation as a mechanism for maintenance of HSCs in these *ex vivo* conditions.

We additionally observe a GSK-3-specific increase in mitochondrial mass in cultured HSCs (**Figure 4.4A**). Like the elevated rate of translation, this is a surprising result given that increasing mitochondrial mass has been correlated with loss of self-renewing function in HSCs (Mantel et al., 2010). However, mitochondrial membrane potential similarly correlates with HSC function (Mantel et al., 2010), and we find that this is maintained at low levels in functional HSCs *ex vivo* despite the increase in mitochondrial mass. Our results therefore suggest that preserving the low mitochondrial mass observed in uncultured HSCs is not required to maintain HSC function in CR culture.

All tested culture conditions cause a dramatic reduction in total RNA content and cell size (**Supplementary Figure 4.2**), suggesting a global metabolic shutdown in HSCs upon removal from the niche. At the same time, GSK-3 inhibition (alone or in combination with rapamycin) induces an increase in total protein synthesis (**Figure 4.2G**, **Supplementary Figure 4.1B**), consistent with mTORC1 activation, whereas combined GSK-3 and mTORC1 inhibition uniquely activates autophagy. We speculate that autophagy may provide the biosynthetic material required to support the elevated rate of translation induced by CHIR99021. In this model, only cells that achieve the appropriate balance between anabolism and catabolism resolve these antagonistic metabolic cues and thereby retain self-renewing and multilineage repopulating capacity.

Finally, our studies reveal a signaling network that is sufficient to maintain HSCs cell-autonomously. Extensive crosstalk occurs between HSCs and the microenvironment *in vivo* to contribute to regulation of HSC activity. That progenitors are dispensable in this model system indicates that cell-intrinsic regulation of GSK-3 and mTORC1 is sufficient to maintain HSC function. This finding moreover highlights the potential value of this culture system in characterizing the signaling network that regulates HSC fate decisions, as this analysis would not have been possible to perform on HSCs *in situ*. How HSCs respond to the altered environment outside of the niche is an important consideration as researchers seek methods to expand HSCs to improve the efficacy of HSC transplants.

Our investigation assessed the responses of multiple mTORC1 targets in the context of a signaling network that maintains HSCs. We identified autophagy as a key molecular marker of this signaling network. Future studies may reveal specific targets of these pathways in the maintenance of HSC function.

Figure 4.1: Cell-autonomous regulation of HSC function by GSK-3 and mTORC1.

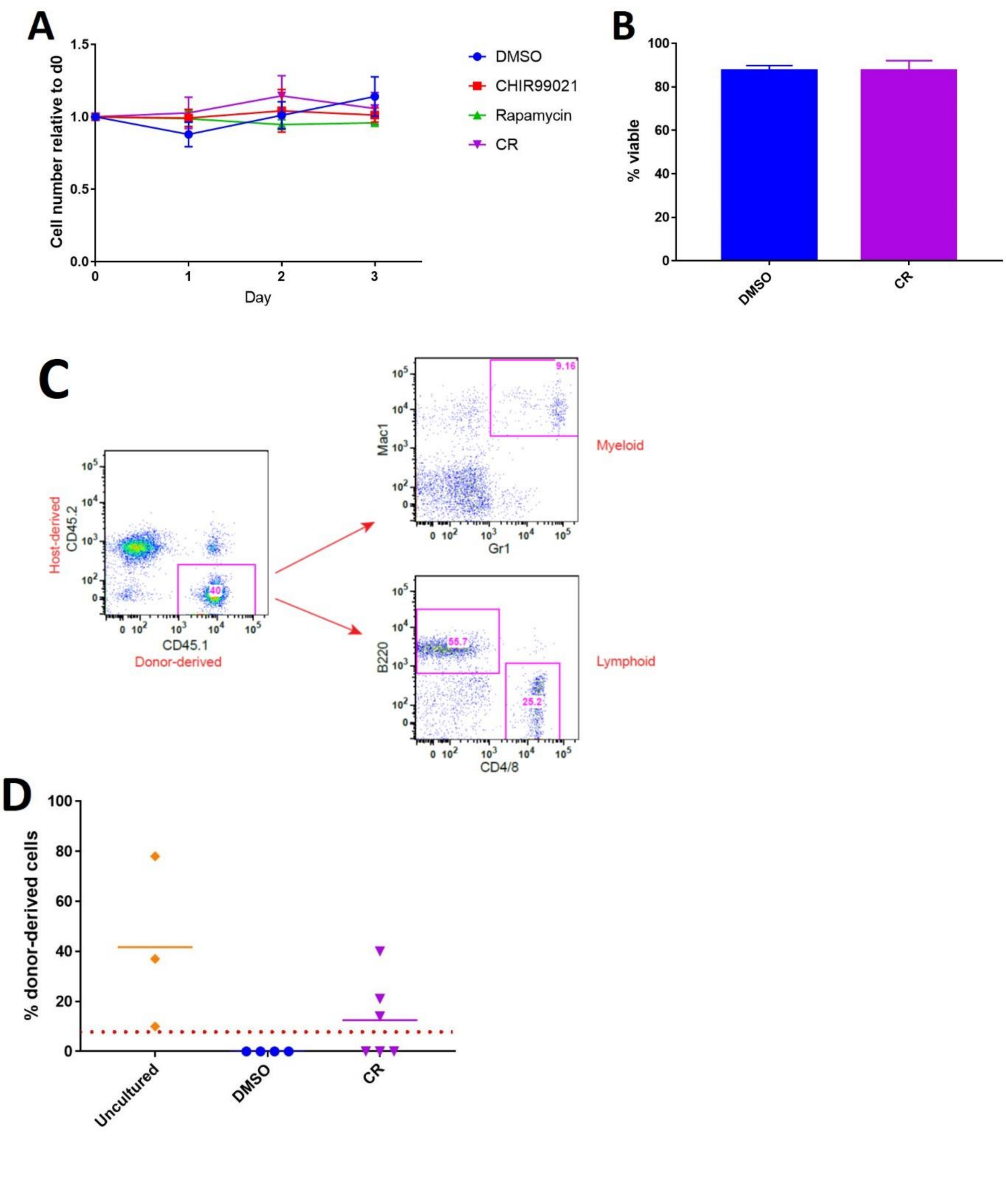


Figure 4.1: Cell-autonomous regulation of HSC function by GSK-3 and mTORC1. (A) HSCs were cultured for 3 d, and cell number relative to day 0 was determined visually. (B) Viability of HSCs cultured in control medium or CR after 7 d culture, determined by Trypan Blue exclusion. (C, D) HSCs were cultured for 7 d and then transplanted with competitor cells into lethally irradiated hosts. Peripheral blood was collected at 24 weeks post-transplant, and multilineage potential of donor-derived ($CD45.1^+$) cells was determined by flow cytometry for lineage-specific markers as indicated (C). Data shown are from one recipient representative of CR-cultured HSCs. Long-term engraftment of freshly isolated HSCs or of HSCs cultured in vehicle or CR was determined by flow cytometry for donor-derived ($CD45.1^+$) cells following competitive transplant (D). Chimerism data shown were collected 24 weeks after transplant. Each symbol represents the results from an individual mouse. Red dotted line indicates 5% threshold defining recipients as positive for donor-derived engraftment. Error bars represent S.E.M.

Figure 4.2: mTORC1 activity and global translation during HSC maintenance.

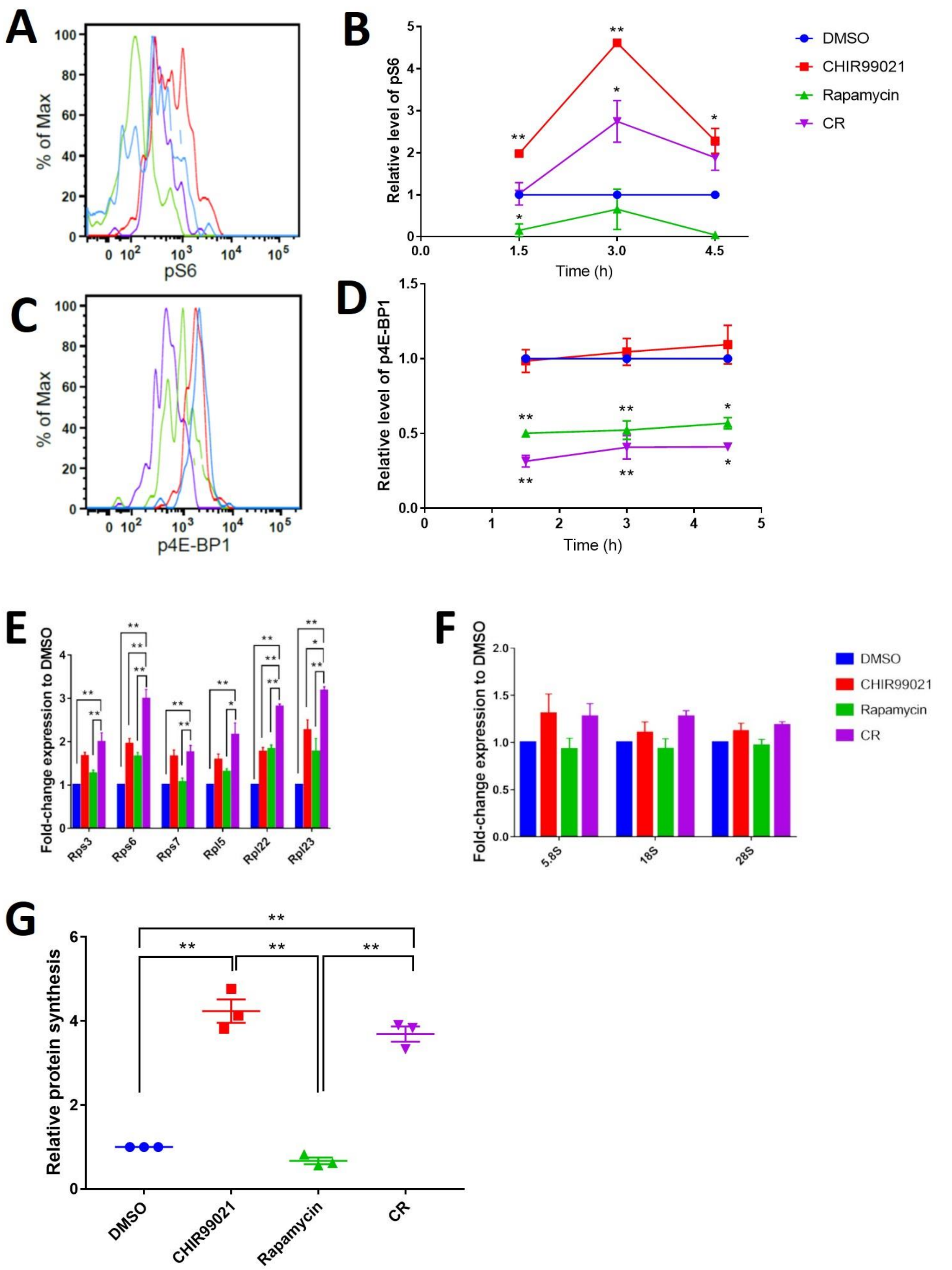


Figure 4.2: mTORC1 activity and global translation during HSC maintenance. (A-D) Representative flow cytometry histograms of pS6 (A) and p4E-BP1 (C) from 1.5 h time point, and their median fluorescence intensities relative to DMSO (B, D), measured by phospho-flow cytometry in the LSK-CD48$^-$ fraction of lineage-depleted bone marrow cells at the indicated times after cells were isolated and placed into culture medium. Asterisks indicate statistical significance for comparison of treatment to DMSO within the same time point. (E, F) cKit$^+$ cells were cultured for 24 h followed by RNA isolation for RT-PCR analysis. RT-PCR for ribosomal protein (E) and ribosomal RNA (F) gene expression relative to DMSO. (G) Lineage-depleted bone marrow cells were cultured for 24 h, and then rate of protein synthesis relative to DMSO was measured by incorporation of OP-Puro followed by flow cytometric analysis of the LSK-CD48$^-$ fraction. Error bars represent S.E.M. Statistical significance for gene expression analyses was assessed using a one-way ANOVA followed by Dunnett's test for multiple comparisons. Statistical significance for pS6 and p4E-BP1 median fluorescence intensities was assessed using a one-way ANOVA followed by Tukey's test for multiple comparisons. * $p < 0.05$, ** $p < 0.005$.

Figure 4.3: Targeting S6K and eIF4E downstream of mTORC1 in HSC maintenance.

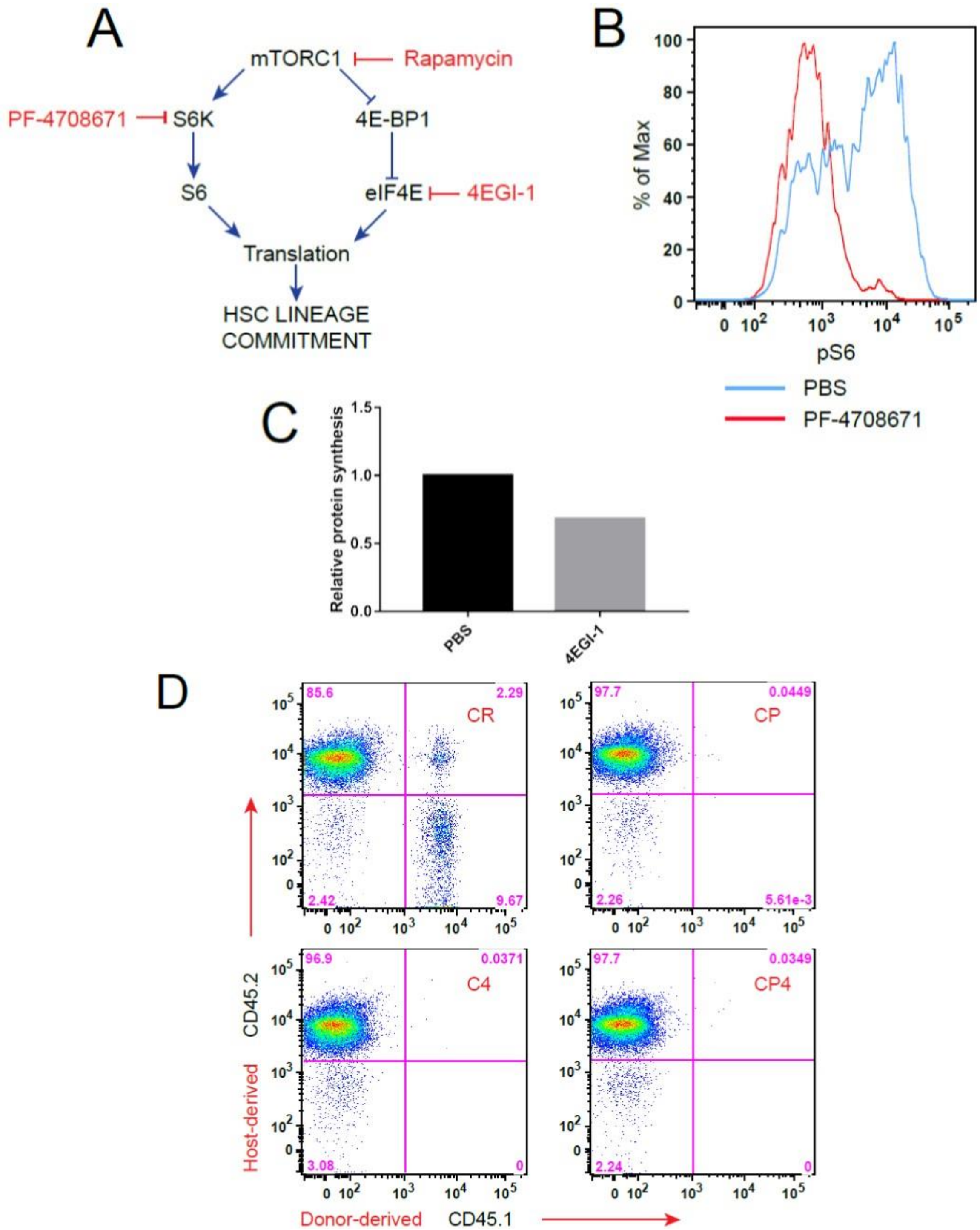


Figure 4.3: Targeting S6K and eIF4E downstream of mTORC1 in HSC maintenance. (A) Strategy to test inhibition of S6K or eIF4E in HSC culture: If the primary effect of rapamycin in HSC maintenance is to inhibit S6K and/or eIF4E, then direct inhibitors of these downstream effectors should be able to replace rapamycin and suppress lineage commitment. (B) Flow cytometric analysis of pS6 in the LSK-CD48$^-$ fraction of lineage-depleted bone marrow cells treated with PF-4708671. (C) Rate of translation relative to vehicle in LSK-CD48$^-$ cells treated with 4EGI-1, measured by OP-Puro incorporation. (D) HSPCs were cultured for 7 d and then transplanted with competitor cells into lethally irradiated hosts. Donor-derived engraftment was determined by flow cytometry for CD45.1$^+$ cells in peripheral blood from recipients of cultured HSPCs following competitive transplant. CP, CHIR99021 + PF-4708671; C4, CHIR99021 + 4EGI-1; CP4, CHIR99021 + PF-4708671 + 4EGI-1. Chimerism data shown were collected 8 weeks after transplant and are representative of 4-5 recipient mice per group.

Figure 4.4: Mitochondrial mass and activity in maintained HSCs.

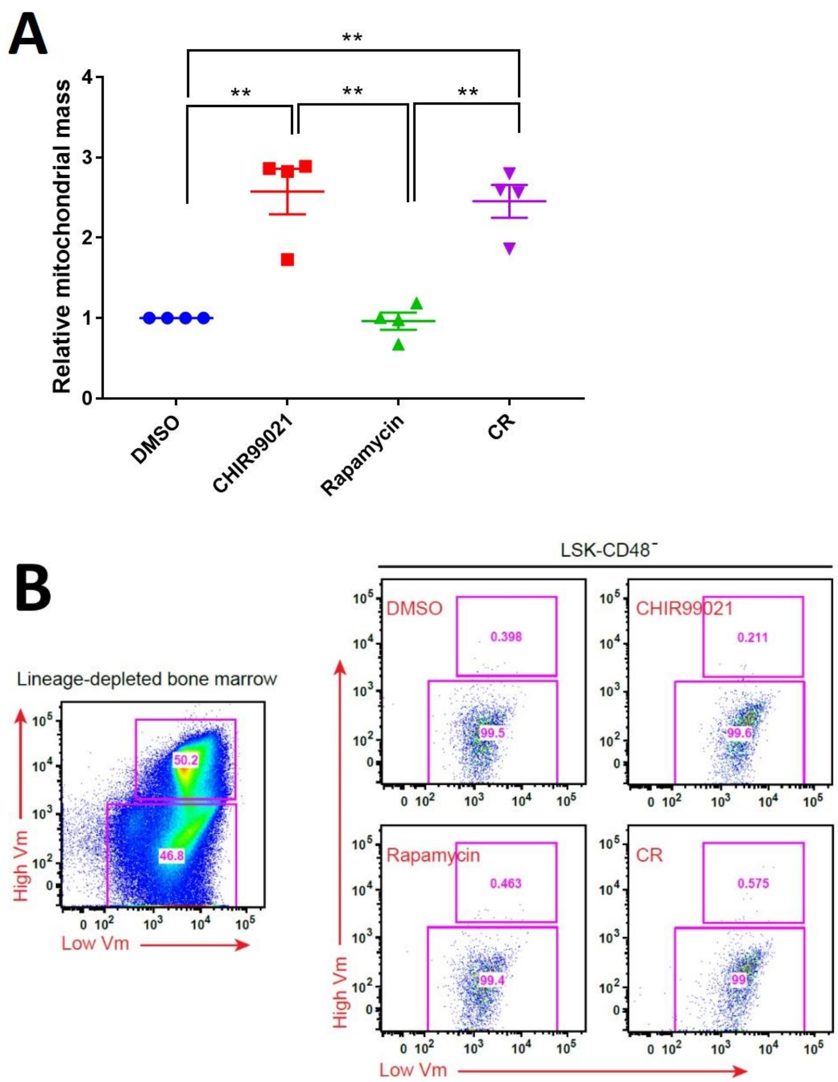


Figure 4.4: Mitochondrial mass and activity in maintained HSCs. (A, B) Flow cytometric analysis of mitochondrial mass (median fluorescence intensity of Mitotracker Green) relative to DMSO (A) and mitochondrial membrane potential (JC-1) (B) in the LSK-CD48$^-$ fraction of lineage-depleted bone marrow cells following 16 h culture. Flow cytometric data shown are representative results of 3 experiments. Error bars represent S.E.M. Vm, mitochondrial membrane potential. Statistical significance was assessed using a one-way ANOVA followed by Tukey's test for multiple comparisons. * $p < 0.05$, ** $p < 0.005$.

Figure 4.5: Autophagy as a molecular mark of HSC maintenance.

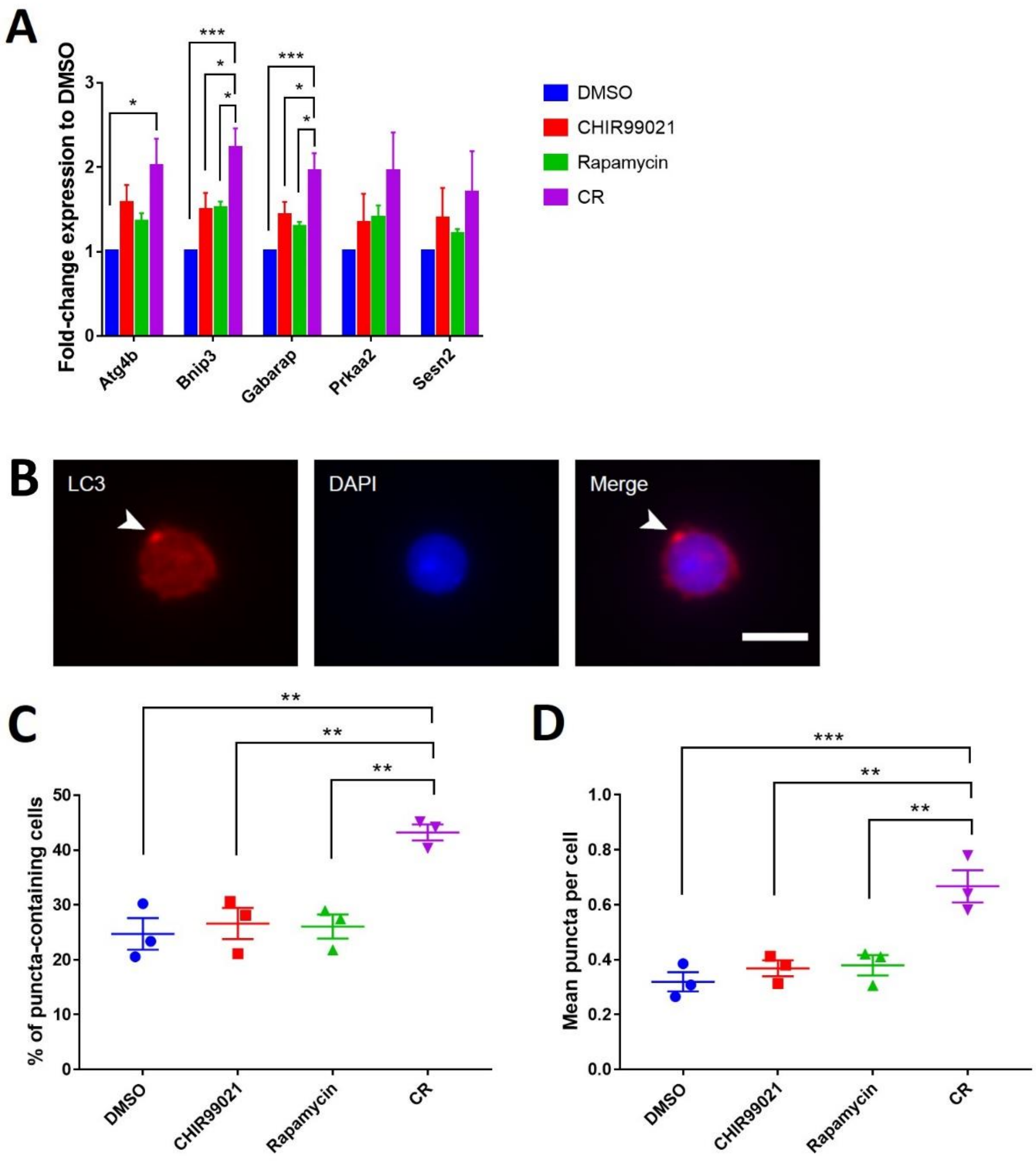
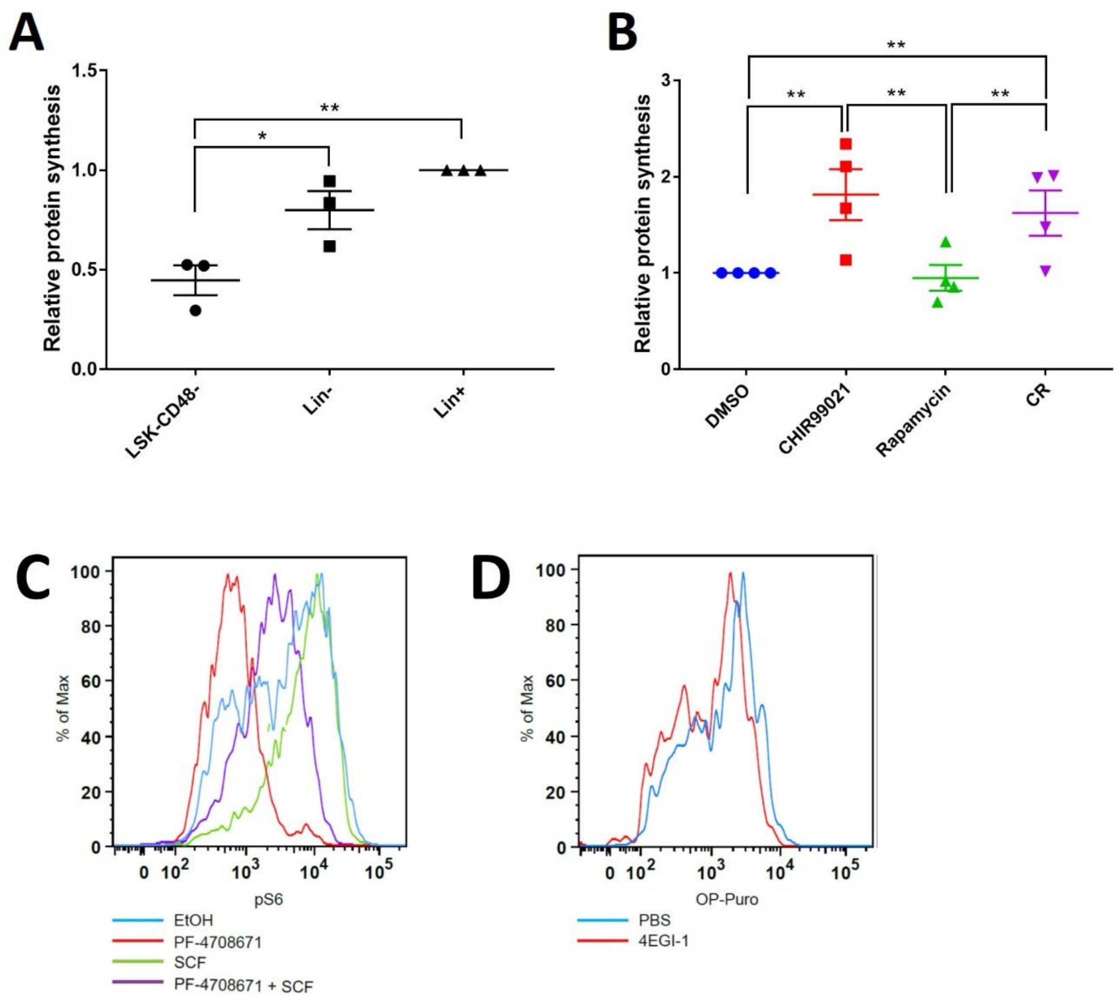


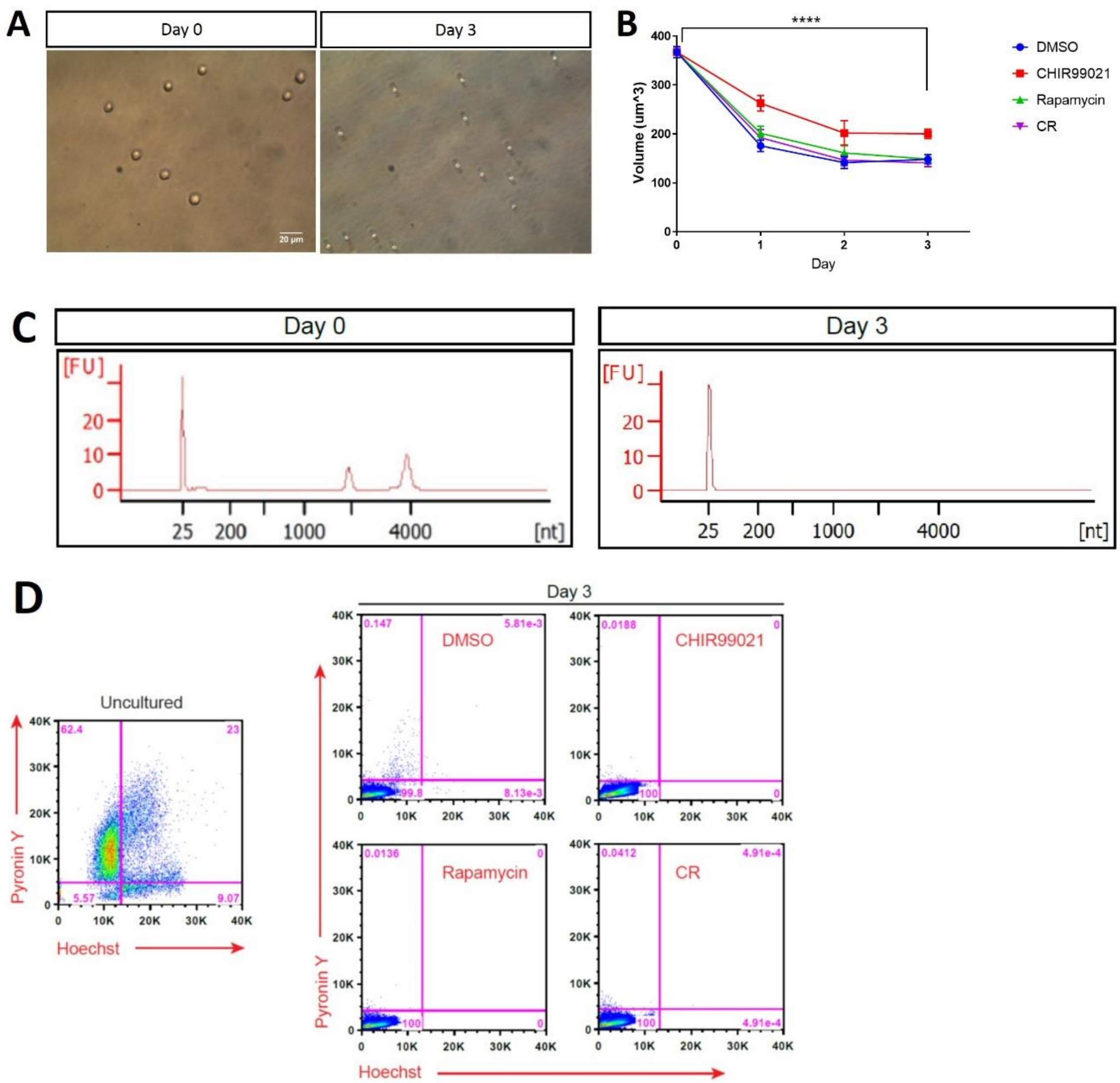
Figure 4.5: Autophagy as a molecular mark of HSC maintenance. (A) RT-PCR for a pro-autophagic gene signature relative to DMSO in c-Kit$^+$ cells following 24 h culture. (B-D) HSCs were cultured for 24 h and then fixed for immunofluorescence analysis of LC3 staining. Sample image of LC3-punctum-positive cell at 400× magnification (B). White arrowhead indicates LC3 punctum. Scale bar, 10 µm. Quantification of percentage of LC3-puncta-positive cells (C) and of average number of LC3 puncta per cell (D). Data shown are average of 3-4 experiments. Error bars represent S.E.M. Statistical significance was assessed using a one-way ANOVA followed by Dunnett's test for multiple comparisons. ** $p < 0.005$, *** $p < 0.0005$.

Supplementary Figure 4.1: HSPC populations display stepwise changes in rate of protein synthesis.



Supplementary Figure 4.1: HSPC populations display stepwise changes in rate of protein synthesis. (A) Rate of protein synthesis determined by incorporation of OP-Puro in freshly isolated HSPC populations relative to Lin^+ cells. (B) Rate of protein synthesis relative to DMSO in the LSK-$CD48^-$ fraction of lineage-depleted bone marrow cells following 3 h culture. (C) Flow cytometric analysis of pS6 in the LSK-$CD48^-$ fraction of lineage-depleted bone marrow cells treated with PF-4708671 and/or stem cell factor (SCF) (100 ng/ml). (D) Flow cytometric analysis of relative rate of protein synthesis in the LSK-$CD48^-$ fraction of lineage-depleted bone marrow cells treated with 4EGI-1. Error bars represent S.E.M. Statistical significance was assessed using a one-way ANOVA followed by Tukey's test for multiple comparisons. * $p < 0.05$, ** $p < 0.005$.

Supplementary Figure 4.2: Reduced cell volume and RNA content in cultured HSCs.



Supplementary Figure 4.2: Reduced cell volume and RNA content in cultured HSCs. (A,B) HSC size by phase contrast microscopy of freshly isolated cells and after 3 d culture (A) and corresponding volume relative to day 0 over time (B). n = 40 – 60 cells counted per treatment group per day; **** indicates $p < 1.5 \times 10^{-18}$ for all conditions compared to day 0 (Student's t-test). Images 400× magnification. Scale bar, 20 µm. (C) RNA was isolated from fresh HSCs and from HSCs following 3 d culture. Total RNA content was measured by nanofluidic analysis with up to 70% recovery of spiked-in RNA. (D) RNA content was measured by flow cytometric analysis in the HSPC fraction of lineage-depleted bone marrow cells that were freshly isolated and after 3 d culture. Data shown are representative results of 3-5 experiments. Error bars represent S.E.M.

CHAPTER 5: CONCLUSIONS AND FUTURE DIRECTIONS

Introduction

HSCs have a long history of use in the clinic for the treatment of congenital and acquired hematological malignancies and disorders, yet their full clinical potential remains constrained by an incomplete understanding of their underlying biology (as reviewed in Chapter 1). Major limiting factors in the thorough characterization of the HSC regulatory network include the complexity of the HSC niche and the rarity of the population, which present significant biological and technical challenges. In this dissertation, I characterized a signaling network that maintains HSCs under defined conditions and identified enhanced autophagy as a signature of this state.

I contributed to the identification of a signaling network that maintains HSCs *ex vivo* through the simultaneous inhibition of GSK-3 and mTORC1 (Chapter 2). I showed that HSPCs cultured with inhibitors of GSK-3 and mTORC1 (CR) in the absence of serum or exogenous cytokines retained multilineage potential, providing initial proof of principle that GSK-3 and mTORC1 inhibition maintains HSC function *ex vivo*. I additionally demonstrated activation of a Wnt target gene by GSK-3 inhibition, consistent with subsequent work by the first author that β-catenin is required for this *ex vivo* maintenance (Huang et al., 2012).

I followed up this work by attempting to perform transcriptional profiling of HSCs maintained *ex vivo* (Chapter 3). Initial efforts to perform this analysis revealed an unexpected massive decrease in RNA content of cultured compared to freshly isolated HSCs. I therefore investigated approaches to overcome this technical hurdle, and presented a detailed method suitable to perform gene expression profiling comparing uncultured and cultured HSCs. If completed, this profile could serve as a valuable resource to identify transcriptional features that mark the state of HSC maintenance. It could moreover reveal how HSCs adjust to conditions beyond the niche, providing insight into how one could uncouple proliferation from differentiation to drive self-renewing divisions. *Ex vivo* expansion of HSCs would both facilitate further molecular characterization of HSCs and accelerate broader clinical use of HSCs, such as for therapeutic gene editing.

Finally, I assessed multiple outputs of mTORC1 signaling to assess which pathways are uniquely activated or inhibited when GSK-3 and mTORC1 activity is suppressed to maintain HSCs. This investigation revealed that although the rate of global translation and mitochondrial mass increase in a GSK-3 specific manner, enhanced activation of autophagy is a unique molecular signature of HSCs maintained *ex vivo* (Chapter 4). This finding is consistent with a growing body of literature describing an essential role for autophagy in stem cell function. As more refined tools to assess autophagic degradation are described, specific targets of autophagy in HSC maintenance may be identified.

Is autophagy required for HSC maintenance *ex vivo*?

The results described in Chapter 4 identify enhanced activation of autophagy as a *marker* of HSC maintenance *ex vivo*, but they do not indicate whether it is *required* downstream of mTORC1. This is a complicated question to address, as knockout of any of several key components of the autophagy machinery leads to rapid myeloproliferation and HSC exhaustion (Mortensen et al., 2011; Watson et al., 2015; Ho et al., 2017). We would therefore predict that *Atg5* or *Atg7* knockdown during CR culture would not be informative: lack of engraftment of such cells upon transplant could indicate a requirement for autophagy in CR-mediated HSC maintenance, but it would also be consistent with autophagy acting in parallel with the mechanism of CR maintenance. However, if *Atg5/7*-knockdown HSCs cultured in CR did reconstitute the hematopoietic systems of transplant recipients, it would show that autophagy is unexpectedly not required for GSK-3- and mTORC1-mediated maintenance of HSCs *ex vivo*.

We have observed that CR-cultured HSCs do not form colonies in colony-forming assays (with Dheeraj Bhavanasi; data not shown), even though they give rise to long-term multilineage engraftment upon transplant (Chapter 4). Accumulating evidence indicates that autophagy promotes HSC quiescence (Mortensen et al., 2011; Watson et al., 2015; Ho et al., 2017). Since CR-cultured HSCs exhibit enhanced autophagy (Chapter 4), it is possible that autophagy maintains cultured HSCs in a quiescent state that renders them incapable of forming colonies. (According to this model, the HSC niche of transplant recipients provides the appropriate cues to suppress autophagy and activate CR-cultured HSCs.) Briefly suppressing autophagy (for perhaps 1-2 days) following CR maintenance may therefore activate quiescent cultured HSCs. Transient inhibition of autophagy could be achieved pharmacologically by inhibitors of the required autophagy proteins Vps34 (Ronan et al., 2014) or Ulk1 (Egan et al., 2015), or genetically by *Atg5/7* shRNA or inducible CRISPR interference (Mandegar et al., 2016). If HSCs cultured in CR and then briefly exposed to an autophagy-inhibiting agent gave rise to colonies, then it would indicate that GSK-3 and mTORC1 inhibition maintains HSCs in a quiescent state that is dependent upon activation of autophagy.

Autophagy is essential to the function of diverse stem cell populations, including embryonic, epidermal (Salemi et al., 2012), skeletal muscle (Tang and Rando, 2014; Garcia-Prat et al., 2016), neural (Vazquez et al., 2012; Wu et al., 2016), and hematopoietic stem cells (Mortensen et al., 2011; Watson et al., 2015; Warr et al., 2013). Accumulating evidence specifically supports a role for autophagy in HSC quiescence (Mortensen et al., 2011; Ho et al., 2017). Autophagy is also upregulated during induction of quiescence in crustacean diapause embryos (Lin et al., 2016). The experiments proposed here will define a role for autophagy in HSC quiescence and activation.

How does simultaneous GSK-3 and mTORC1 inhibition activate autophagy?

mTORC1 inhibits autophagy, and correspondingly, inhibition of mTORC1 by rapamycin is a known inducer of autophagy. However, activation of autophagy requires rapamycin concentrations of 100-500 nM, significantly greater than the 5 nM used in combination with GSK-3 inhibition to maintain HSCs *ex vivo*. Rapamycin at 5 nM was not sufficient to activate autophagy by itself (**Figure 4.5**), and therefore an alternative mechanism is necessary to explain the effects we observe.

GSK-3 and mTORC1 both inhibit autophagy in part by promoting cytosolic sequestration of the lysosomal and autophagy master TF TFEB (Parr et al., 2012; Roczniak-Ferguson et al., 2012). As we only observe enhanced activation of autophagy in HSCs cultured with both inhibitors, it suggests that combined inhibition of GSK-3 and mTORC1 may allow TFEB to translocate to the nucleus and promote autophagy. Immunofluorescence analysis for the subcellular localization of TFEB would address this question, with the prediction that TFEB localization in the nucleus would be enhanced in CR-cultured HSCs compared to vehicle- or single-treated HSCs. This model also predicts that CR-cultured HSCs will express the highest levels of TFEB target genes, including *Atg9b*, *Maplc3b*, and *Sqstm1* (Settembre et al., 2011), which encode components of the core autophagy machinery.

Finally, hepatocytes express higher levels of TFEB target genes in response to treatment with the mTORC1 inhibitor Torin 1, but this effect is blunted in *Tfeb*-knockout cells (Settembre et al., 2012). This result identifies TFEB as a main mediator of this response. I have hypothesized that TFEB regulation may be an important mechanism by which GSK-3 and mTORC1 inhibition activates autophagy in HSC maintenance. If this is the case, then *Tfeb* knockdown should block HSC maintenance in CR. Importantly, *Tfeb*-knockout cells in control medium do not express significantly different levels of target genes compared to wild-type (Settembre et al., 2012), suggesting that *Tfeb* knockdown will not affect HSC function except under conditions that induce autophagy.

GSK-3 or mTORC1 regulation of TFEB has been demonstrated in diverse cell types, including HeLa (Settembre et al., 2012), mouse neuroblastoma (Parr et al., 2012), and human pancreatic cancer (Marchand et al., 2015) cell lines. However, potential co-regulation of TFEB by GSK-3 and mTORC1 has not been explored. GSK-3 and mTORC1 are established regulators of multiple stem cell populations (Welham et al., 2011; Ghosh and Kapur, 2016; Tee et al., 2016). Autophagy is also increasingly recognized as an essential process in stem cell function (Guan et al., 2013; Phadwal et al., 2013). If GSK-3 and mTORC1 inhibition promotes HSC maintenance through TFEB-mediated activation of autophagy, a similar mechanism may function in additional stem cell populations. For example, epidermal stem cells with excessive Wnt signaling exhibit transient expansion followed by mTORC1-dependent depletion (Castilho et al., 2009), a phenotype remarkably like that observed in HSCs (Huang et al., 2009). Refinement to the molecular level of regulation of autophagy in stem cells may reveal valuable targets in senescence and disease.

Does selective autophagy contribute to HSC maintenance?

I reported the expression of a pro-autophagic gene signature and the increased formation of $LC3^+$ autophagic puncta in HSCs maintained *ex vivo* (described in Chapter 4). Although this is sufficient to indicate generally that autophagy (macroautophagy) is uniquely enhanced in these cells, additional observations leave open the possibility that forms of targeted autophagy may contribute to HSC maintenance *ex vivo*. The literature on selective autophagy is limited. However, the selective autophagic degradation of ribosomes and mitochondria (ribophagy and mitophagy, respectively) may play important roles in sustaining HSC function.

Cellular RNAs are subject to extensive quality controls, with mRNA turnover mediated primarily by ribonucleases (RNases) following removal of the 5' cap and poly(A) tail (Chen and Shyu, 2011). rRNA, however, which comprises the majority of cellular RNA, is typically highly structured and bound in RNA-protein complexes, rendering it less accessible to basic RNA degradation machinery. A lysosome-dependent increase in total RNA turnover in serum-deprived human fibroblasts (Sameshima et al., 1981) and in amino acid-deprived rat hepatocytes (Lardeux and Mortimore, 1987) provided early evidence that autophagy might promote more large-scale RNA decay during starvation. Further supporting a role for autophagy-mediated RNA degradation, the evolutionarily conserved and broadly expressed T2 family of RNases is primarily localized to the lysosome, with an optimal pH of 4-5 (Irie, 1999). A requirement for the T2 family of RNases in RNA turnover has been described in *Drosophila* (Ambrosio et al., 2014), zebrafish (Haud et al., 2011), and human (Henneke et al., 2009). Evidence of ribophagy has since been reported in organisms from yeast (Kraft et al., 2008) to humans (Kristensen et al., 2008). Notably, ribophagy requires components both of the core macroautophagic machinery and of additional receptors that recognize targeted cargo and link it to autophagosomal membranes (Frankel et al., 2016). Evidence of activated macroautophagy may be therefore be consistent with activated ribophagy.

HSCs maintained *ex vivo* contain less total RNA and exhibit enhanced activation of autophagy. These observations suggest that ribophagy may be a component of the activated autophagy that marks HSCs maintained *ex vivo*. Ribosome biogenesis and protein synthesis are among the most energy-consuming cellular processes, and must therefore be tightly regulated by nutrient availability. HSCs *in vivo* reside in a low-perfusion niche (Parmar et al., 2007; Simsek et al., 2010; Spencer et al., 2014) and require a restricted rate of translation (Signer et al., 2014). Ribophagy is induced upon nutrient deprivation (Kraft et al., 2008; Kristensen et al., 2008), consistent with the Klein laboratory's serum-free culture conditions. Combined, these observations make ribophagy an attractive mechanism by which HSCs might suppress anabolism under conditions of restricted nutrient availability, such as in *ex vivo* maintenance. Although I observed reduced RNA content in all cultured cells, the hypothesis of ribophagy induction is consistent with the enhanced activation of macroautophagy correlating uniquely with conditions that maintain HSCs.

Although ribophagy appears to involve proteins that do not participate in macroautophagy (Frankel et al., 2016), specific markers of mammalian ribophagy have not been identified. When they are identified, it will become possible to study ribophagy dynamics (e.g., through knockdown of ribophagy-specific adaptors) without interfering with macroautophagy. Until then, indirect readouts must be employed. Expression of the T2 family of RNases increases in response to nutrient deprivation (Ambrosio et al., 2014), and its loss leads to accumulation of undigested rRNA in lysosomes (Haud et al., 2011). Increased expression of *Rnaset2* family members in cultured HSCs would therefore be consistent with enhanced ribophagy. In addition, electron microscopy has been used to visualize ribosomes in autophagosomes and lysosomes (Frankel et al., 2016). Although this observation alone can be consistent with macroautophagy without invoking ribophagy, analysis of protein turnover during amino acid starvation reveals distinct kinetics of autophagic degradation of ribosomes compared to other cellular components, suggesting temporal selection of cargo (Kraft et al., 2008; Kristensen et al., 2008). These experiments would not definitively demonstrate activation of ribophagy, but they would support a role for it in the larger context of activated autophagy and reduced RNA content.

Cells also restrict energy needs in the context of nutrient deprivation through the selective autophagic degradation of mitochondria. Mitophagy additionally serves as the key process for mitochondrial quality control and turnover (Okamoto, 2014). Like ribophagy, mitophagy involves both the core autophagic machinery and receptors that mediate specific cargo targeting to autophagosomes, although mitophagy-specific markers have also not yet been identified. Increasing mitochondrial mass has been correlated with loss of HSC function (Mantel et al., 2010). CR culture maintains HSCs *ex vivo* despite inducing an increase in mitochondrial mass (as described in Chapter 4). Given the concomitant increase in autophagy in these cells, mitophagy is an attractive potential component of the mechanism by which CR maintains HSCs. Although CR-cultured HSCs have higher overall mitochondrial mass compared to vehicle, activated mitophagy would selectively degrade dysfunctional mitochondria. The improved health of the remaining mitochondria may therefore permit retention of function in HSCs despite elevated mitochondrial content.

As with ribophagy, mitophagy-specific markers have not yet been identified, so circumstantial evidence for targeted mitophagy would need to be considered in the broader context of nutrient availability and cellular function. Mitochondria must undergo fission prior to engulfment in autophagosomes (Rimessi et al., 2013). The observation that mitochondrial mass is elevated in CR-cultured HSCs was based on flow cytometric quantification of MitoTracker staining, indicating total mitochondrial content but not the size of individual mitochondria. It would therefore be informative to perform immunofluorescence analysis of cultured HSCs to visualize the extent of mitochondrial fission in cultured HSCs. Mitochondria are also readily visualized in autophagosomes by electron microscopy. Finally, immunofluorescence analysis could colocalize the outer mitochondrial membrane protein TOM20 to the lysosome. Combined, these results would be consistent with activation of mitophagy as a marker of HSC maintenance *ex vivo* and suggest improved mitochondrial quality control as a potential mechanism, although mitophagy markers will need to be identified to obtain definitive evidence.

Selective autophagy is a conserved mechanism of organelle quality control and metabolic regulation during development, homeostasis, and regeneration (Okamoto, 2014). One of the most dramatic described examples of this is the elimination of ribosomes and mitochondria during red blood cell maturation, which occurs in a manner dependent on selective autophagy but not macroautophagy (Kundu et al., 2008). Characterization of such targeted autophagic pathways in HSCs maintained *ex vivo* would offer a more refined definition of a metabolic program that HSCs activate to retain their function.

When is restricted translation important for HSC maintenance?

Morrison and colleagues have reported that HSCs require a low rate of translation *in vivo* compared to more differentiated hematopoietic cells, and that this is mediated in part by the 4E-BPs (Signer et al., 2014; Signer et al., 2016). GSK-3 and mTORC1 inhibition reduces 4E-BP phosphorylation and maintains HSCs *ex vivo* despite elevating the rate of translation compared to controls (as detailed in Chapter 4). These findings therefore argue for a context-dependent requirement for restricted protein synthesis in HSCs.

One possibility is that HSCs may rely on low levels of specific proteins rather than on a globally low rate of translation. The extent to which the 4E-BPs restrict translation appears to be context-dependent. The 4E-BPs generally have only a modest effect on the rate of bulk translation (Lynch et al., 2004; Tahmasebi et al., et al., 2014) and specifically restrict translation of mRNAs related to survival, proliferation (Topisirovic and

Sonenberg, 2011), mitochondrial structures, and oxidative phosphorylation (Morita et al., 2013). In HSCs, however, *4E-BP1/2* double knockout leads to ~25% increased global protein synthesis compared to wild type (Signer et al., 2016). As simultaneous GSK-3 and mTORC1 inhibition leads to reduced 4E-BP phosphorylation without a corresponding reduction in the rate of bulk translation, it is possible that HSC maintenance under these conditions is mediated by restricted translation of specific transcripts, but that this is not detectable at the level of total protein synthesis. Immunoblotting for known targets of 4E-BP-restricted translation would help address this question. On a proteomic scale, ribosomal profiling may eventually identify 4E-BP-regulated transcripts in HSCs under physiological and artificial settings, although this technology currently requires orders of magnitude more cells than is possible to perform on such a rare population.

An additional possibility, which is not mutually exclusive with 4E-BP-restricted translation of selected mRNAs, is that a low rate of translation may be important for maintenance of long-term HSC function in the context of proliferation, such as in cytokine culture. In support of this hypothesis, a small molecule screen was recently completed for compounds that, in combination with CR and a limited cytokine cocktail (no serum), could promote UCB HSC expansion *ex vivo* (Dheeraj Bhavanasi and Peter Klein, unpublished). Under these conditions, the inhibitor of eIF4E-eIF4G interaction 4E1RCat induced 8- to 10-fold HSC expansion by immunophenotypic analysis and by limiting dilution analysis in immunocompromised mice. It will therefore be important to measure the rate of translation in these cells to explore what role protein synthesis may play in the maintenance of HSCs undergoing cytokine-induced self-renewing division.

Restricted translation is an important mechanism of maintenance or quiescence in embryonic stem cells (Sampath et al., 2008), satellite cells (Zismanov et al., 2016), epidermal stem cells (Blanco et al., 2016), and diapaused blastocysts (Bulut-Karslioglu et al., 2016). HSCs *in vivo* additionally require a controlled rate of protein synthesis (Signer et al., 2014). My findings do not, however, support a role for suppression of translation as a mechanism to maintain HSCs in cytokine-free culture. Future studies may identify the molecular mediators and functional contexts that determine when restricted protein synthesis affects HSC function.

Revised model for GSK-3 and mTORC1 regulation of HSC maintenance

Autophagy is essential for restricting ROS accumulation and consequent DNA damage, in part through mitochondrial quality control. Maintaining genomic integrity is particularly consequential in quiescent stem cells, given their function in lifelong tissue maintenance and their limited capacity to dilute cellular waste by division (Guan et al., 2013). Loss of autophagy in HSCs leads to rapid accumulation of mitochondria, ROS, and DNA damage, followed by myeloproliferation and loss of regenerative potential (Mortensen et al., 2011). This is accompanied by increased glucose uptake, NADH and ATP levels, and a shift from glycolysis to oxidative phosphorylation, metabolic features indicating HSC activation (Ho et al., 2017). Mice lacking the mTORC1 inhibitor Tsc1 exhibit a similar phenotype (Chen et al., 2008). Antioxidant treatment restores HSC quiescence and function, identifying ROS as an important regulator of HSC quiescence.

Considering these reports, the work in this dissertation describes the *ex vivo* maintenance of HSCs exhibiting a seemingly paradoxical metabolic profile: these cells are smaller and contain less total RNA than uncultured HSCs, consistent with a quiescent state, yet they also translate more mRNA into protein and have higher mitochondrial content, suggesting stem cell activation. I hypothesize that the enhanced activation of autophagy in maintained HSCs resolves these antagonistic metabolic cues, achieving the balance between biosynthetic and degradative processes necessary to preserve stem cell function. GSK-3 (Parr et al., 2012; Marchand et al., 2015) and mTORC1 (Roczniak-Ferguson et al., 2012; Settembre et al., 2012) each inhibit nuclear translocation of TFEB. TFEB is therefore an attractive potential mediator of the autophagy uniquely enhanced in CR-cultured HSCs (**Figure 5.1**). More thorough analyses of the metabolic profile of HSCs maintained *ex vivo*, such as those described above, would provide further evidence that GSK-3 and mTORC1 inhibition maintains HSC function in cells that have entered a quiescent state.

Conclusion

In this dissertation, I investigated a signaling network that maintains HSCs. in attempting to perform gene expression profiling on these cells, I discovered that they exhibit a significant reduction in total RNA content, leading to the description of a detailed protocol that can be used to characterize the transcriptional profile of cells with limited or differing quantities of RNA. My investigations additionally identified enhanced activation of autophagy as a marker of this network. This work both reinforces the essential role autophagy plays in stem cell maintenance and raises compelling questions about the contribution of metabolism to stem cell function.

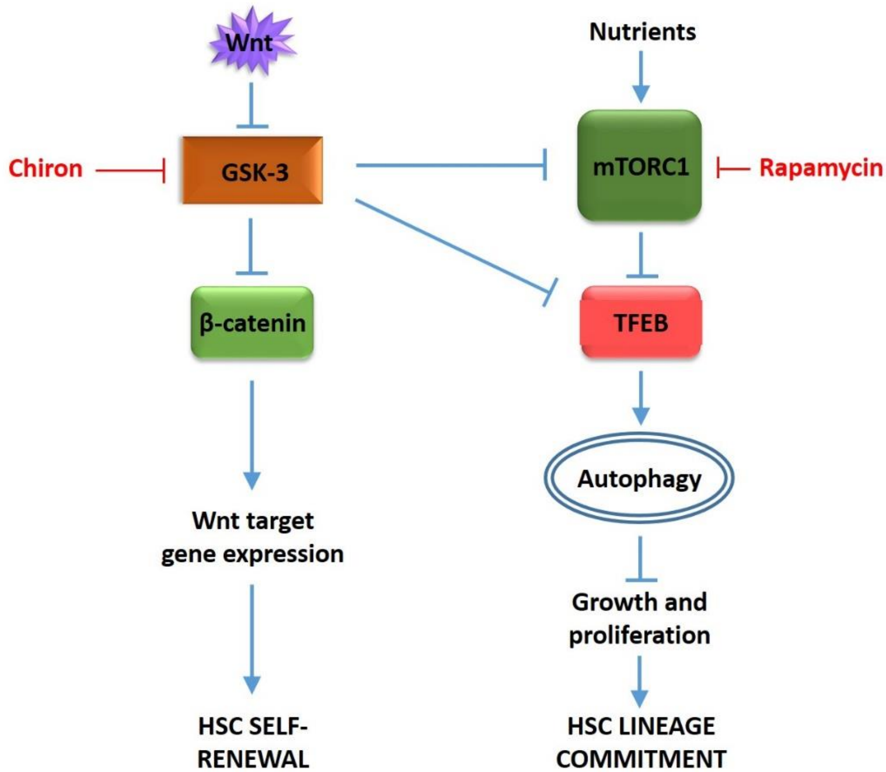


Figure 5.1: Revised model for GSK-3 and mTORC1 regulation of HSCs. GSK-3 regulates two antagonistic pathways in HSCs. GSK-3 inhibition of Wnt/β-catenin signaling inhibits self-renewal, while GSK-3 inhibition of mTORC1 promotes lineage commitment. Inhibition of GSK-3 activates both pathways. Simultaneous inhibition of GSK-3 and mTORC1 may therefore block HSC lineage commitment while promoting self-renewal. GSK-3 and mTORC1 each independently inhibit TFEB. As autophagy activation is uniquely enhanced in HSCs in which both GSK-3 and mTORC1 are inhibited, TFEB may mediate of this effect.

CHAPTER 6: METHODS AND MATERIALS

Mice

C57BL/6 wild-type ($CD45.2^+$) and SJL ($CD45.1^+$) congenic mice were obtained from the Jackson Laboratory. Mice were bred in-house in a pathogen-free mouse facility at the University of Pennsylvania. Transplant recipients were 8- to 10-week-old $CD45.2^+$ females. Donor mice were 6- to 8-week-old $CD45.1^+$ males. Animal experiments were performed in accordance with guidelines approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Pennsylvania (IACUC protocol #80317).

Flow cytometric sorting and analysis of HSCs

Bone marrow cells were flushed from the tibias and femurs of mice with PBS without calcium or magnesium. For detection of LSK cells, whole bone marrow cells were incubated with biotin-conjugated monoclonal antibodies to the following lineage markers: B220 (6B2), CD4 (GK1.5), CD8 (53-6.7), Gr1 (8C5), Mac1 (M1/70), Ter119 (Ter119), and interleukin-7 receptor (IL-7R) (A7R34); FITC-conjugated antibody to Sca1 (Ly6A/E; D7) and APC-conjugated antibody to c-Kit (ACK2). CD48 and CD150 were measured with PE-conjugated antibody to CD48 (HM48-1) and PE Cy7-conjugated antibody to CD150 (TC15-12F 12.2). All antibodies were purchased from eBioscience except for antibody CD150, which was purchased from Biolegend. Biotin-conjugated lineage marker antibodies were detected using streptavidin-conjugated PE Texas Red (ThermoFisher). Antibodies to B220, CD4, CD8, Gr1, Mac1 and IL-7R were diluted 1:550. Antibodies to Ter119 and Gr1 were diluted 1:275. Antibodies to Sca1, c-Kit, CD48, and CD150 were diluted 1:80. Non-viable cells were excluded using the viability dye DAPI (1 $\mu g\ ml^{-1}$). Cells were sorted with a FACSAria (Becton Dickinson) automated cell sorter. Analyses were performed on a

FACSCanto or LSR II flow cytometer (Becton Dickinson). Data were analyzed using FlowJo software (Tree Star).

Cytokine-free HSC and HSPC culture

HSPCs were cultured as described previously [27]. Briefly, mouse bone marrow cells were harvested and distributed into a single-cell suspension by gently drawing through a 22-gauge needle. Red blood cells were lysed in ammonium chloride–potassium (ACK) buffer. Lineage-depleted bone marrow cells were isolated by incubating whole bone marrow with biotin-conjugated lineage marker antibodies indicated above followed by incubation with Dynabeads M-280 Streptavidin (ThermoFisher) and separation with the EasySep magnet (Stem Cell Technologies). c-Kit$^+$ cells were purified with the MACS cell separation kit (Miltenyi Biotec). Isolated HSCs and HSPCs were cultured in X-VIVO 15 (BioWhittaker) supplemented with 1% penicillin and streptomycin (Sigma). CHIR99021 and rapamycin (Cayman Chemical) reconstituted in DMSO were added to final concentrations of 3 µM (CHIR99021) and 5 nM (rapamycin) for all experiments unless otherwise indicated. For inhibition of S6K and eIF4E, cells were cultured in 3 µM CHIR99021 combined with 10 µM PF-4708671 (Tocris Bioscience) reconstituted in ethanol, 50 µM 4EGI-1 (Tocris Bioscience) reconstituted in DMSO, or both. Sorted cells were distributed into 96-well plates with 100 µl medium. One-half volume of medium and full drug volume were replaced every other day.

Long-term competitive repopulation assays

C57BL/6 (CD45.2$^+$) recipient mice were lethally irradiated with a cesium-137 irradiator in two equal doses of 500 rads separated by at least 2 h. SJL (CD45.1$^+$) donor cells were mixed with 3×10^5 competitor C57BL/6 bone marrow cells and injected into the retro-orbital venous sinus of anesthetized recipients. Transplanted mice were given antibiotic-containing water for four weeks following irradiation. Beginning 4 weeks after transplantation and continuing for 24 weeks, blood was collected from the tail veins of recipient mice and analyzed by flow cytometry for the lineage markers Gr1 (8C5), Mac1 (M1/70), B220 (6B2), CD4 (L3T4), CD8 (Ly-3) (eBioscience) to monitor engraftment.

RNA-seq

HSCs were sorted and cultured as described above, and RNA was isolated as described below. Ten microliters of RNA per sample was input to three rounds of the aRNA amplification method (Morris et al., 2011). Following amplification, aRNA product quality and quantity was checked by nanofluidic electrophoresis with the RNA 6000 Pico kit on a 2100 Bioanalyzer (Agilent Technologies). aRNA was delivered to the Perelman School of Medicine Next-Generation Sequencing core for library preparation and RNA-seq. One-hundred-base-pair single read sequencing was performed on a HiSeq4000 (Illumina). Genome alignment and differential expression analysis was performed by the Penn Molecular Profiling Facility.

RNA isolation and RT-PCR

Cultured c-Kit$^+$ or LSK-SLAM cells were centrifuged, lysed in 350 µl RLT Plus Lysis buffer with Reagent DX (Qiagen) and beta-mercaptoethanol (Sigma-Aldrich), and RNA was isolated using the RNeasy Plus Micro kit (Qiagen) as indicated by the manufacturer. For spike-in confirming successful RNA isolation from cultured cells, 150 ng YFP mRNA was added at the time of lysis. RNA concentration was quantified on a NanoDrop 1000 (ThermoFisher). 50 ng total RNA per sample was used for first-strand cDNA synthesis using SuperScript III reverse transcriptase (Invitrogen) as indicated by the manufacturer. Relative gene expression was quantified on a 7900HT Fast Real-Time PCR System (Applied Biosystems) using Power SYBR Green PCR Master Mix (Applied Biosystems).

Expression levels of genes of interest were normalized to *β-actin* expression. RT-PCR primers were designed using NCBI Primer-BLAST, and sequences are available upon request.

RNA content analysis and cell size measurement

Lineage-depleted bone marrow cells were isolated for analysis of RNA content and cell size on day 0 or after 3 d of culture. At the time of analysis, cells were washed with PBS and stained in LIVE/DEAD Fixable Near IR Dead Cell Stain (ThermoFisher) as directed by the manufacturer, washed, and stained for surface markers indicated. For RNA content analysis by flow cytometry, cells were then washed, resuspended in 20 μg ml^{-1} Hoechst 33342 (Cell Signaling Technologies) with 50 μg ml^{-1} verapamil (Calbiochem) in PBS supplemented with 3% fetal bovine serum (FBS; GE Healthcare Life Sciences), vortexed briefly, and incubated covered at 37°C for 45 min. Pyronin Y (Sigma-Aldrich) was added directly to a final concentration of 1 μg ml^{-1} and cells were briefly vortexed and incubated covered at 37°C for an additional 15 min. Cells were washed and immediately analyzed by flow cytometry.

For size measurement, HSCs were sorted and cultured as described above. Each day, cells were imaged on a Nikon Diaphot microscope equipped with a Nikon DS-Fi1 camera and NIS-Elements F imaging software (Nikon). Cell cross-sectional area was measured in ImageJ (NIH) calibrated to a micrometer, and volume was calculated assuming that cells are spherical.

Assessment of pS6 and p4E-BP1

To measure phosphorylated S6 and 4E-BP by intracellular flow cytometry, lineage-depleted bone marrow cells were treated with DMSO, 30 μM CHIR99021, 500 nM rapamycin, or both at 37°C for times indicated. Cells were fixed and permeabilized as indicated by Cell Signaling Technology. Cells were stained for the surface markers indicated and for pS6 conjugated to Pacific Blue (Ser235/236 [D57.2.2E], rabbit mAb, final concentration 1:50, Cell Signaling Technology) and p4E-BP1 conjugated to PE (Thr37/46 [236B4], rabbit mAb, final concentration 1:50, Cell Signaling Technology), then analyzed by flow cytometry.

Assessment of rate of translation

Lineage-depleted cells were cultured as described above for 3 or 24 h, and then O-propargyl-puromycin (OP-Puro; Click-iT Plus OPP AlexaFluor 488 kit, Life Technologies) was added (10 μM) to the medium for an additional 30 min. Cells were washed with PBS and stained with LIVE/DEAD Fixable Near IR Dead Cell Stain (ThermoFisher) as directed by the manufacturer. Cells were fixed in 0.5 ml of 1% paraformaldehyde (Electron Microscopy Sciences) in PBS for 15 min covered on ice. Cells were washed in PBS, then permeabilized in 200 μl PBS supplemented with 3% FBS and 0.1% saponin (Sigma) for 5 min at room temperature. The azide-alkyne cycloaddition was performed using the kit as directed by the manufacturer. After the 30-min reaction, cells were washed in PBS supplemented with 3% FBS, then stained for surface markers indicated and analyzed by flow cytometry.

Mitochondrial analyses

Lineage-depleted cells were cultured as described above for 16 h and stained for surface markers indicated. Mitochondrial mass and membrane potential were then measured using MitoTracker Green FM (20 nM; Life Technologies) and MitoProbe JC-1 Assay Kit for Flow Cytometry (0.2 μM in X-VIVO 15; Life Technologies), respectively, as indicated by the manufacturer and analyzed by flow cytometry.

Immunofluorescence microscopy

LSK-SLAM cells were sorted as described above. Cells were cultured for 24 h as described above, then fixed in 4% paraformaldehyde in PBS on glass slides for 30 min at room temperature. Cells were washed twice in PBS, then permeabilized in 0.5% Triton X-100 (ThermoFisher) in PBS for 10 min at room temperature. Cells were then washed twice in PBS and blocked in 10% bovine serum albumin (BSA; Sigma-Aldrich) in PBS. Cells were stained with rabbit anti-LC3 (1:500; MBL) in 5% BSA in PBS supplemented with 0.1% TWEEN 20 (Sigma-Aldrich) for 1 h at room temperature and then overnight at 4°C. After primary stain, cells were washed four times in PBS with 0.1% TWEEN 20. Secondary stain was donkey anti-rabbit IgG conjugated to Alexa Fluor 555 (1:500; ThermoFisher) in 5% BSA in PBS supplemented with 0.1% TWEEN 20, incubated for 1 h at room temperature and protected from light, followed by four washes in PBS with 0.1% TWEEN 20. Cells were counterstained with DAPI (1μg ml^{-1}) in PBS for 5 min at room temperature and then washed twice in PBS. Slides were mounted with ProLong Gold antifade reagent (Invitrogen) and cured at room temperature for at least 24 h. Slides were imaged on an Olympus IX81 fluorescent microscope setup with a Hamamatsu Camera Controller C10600. Images were recorded and analyzed using the MetaMorph for Olympus Advanced software.

Statistical methods

All data are depicted as mean +/- standard error of the mean (S.E.M.). Statistical significance was assessed by one-way ANOVA, followed by post-hoc Tukey's test or Dunnett's test for multiple comparisons as indicated. For pairwise comparisons, significance was assessed by Student's t-test.

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