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3

Transcriptional Quiescence of Hematopoietic Stem Cells

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1. Introduction

Haematopoietic stem cells (HSC) have the exceptional capacity to undergo continuous self-renewal and differentiation into multiple lineages, which is essential for haematopoietic homeostasis and response to injury. To achieve this life long function, these cells have to be protected from cytotoxic and genetic damage. On the other hand, rapid activation of haematopoietic stem cell proliferation in response to stimuli must be ensured. While cellular quiescence is thought to be the key mechanism underlying this paradoxical nature of HSC, the molecular basis of induction and maintenance of quiescence remains unresolved.

Quiescence is commonly defined as a reversible cell cycle exit. Induction and maintenance of stem cell quiescence has been studied at the level of cell cycle regulation (Orford & Scadden, 2008), cellular metabolism (Tothova & Gilliland, 2007) or interaction with the specific niche (Fuchs *et al.*, 2004). Genome-wide association studies have been performed on a variety of quiescent model systems, such as serum starvation of fibroblasts (Coller *et al.*, 2006), primary lymphocytes (Garriga *et al.*, 1998) or yeast in stationary phase (Patturajan *et al.*, 1998, Radonjic *et al.*, 2005). All of these studies revealed a significant decrease of productive mRNA transcription in these model systems. However, if quiescent adult stem cells share this down regulation of mRNA transcription has never been examined.

Due to their relative ease of isolation, cells of the haematopoietic lineage have been extensively studied. Importantly, several assays for hematopoietic stem cell function have been developed, such as colony forming ability and rescue of lethally irradiated mice. These functional tests are lacking in most other adult stem cell models, with the exception of spermatogonia and mammary gland stem cells (Brinster & Nagano, 1998, Shackleton *et al.*, 2006). Functional assays for HSC ability have provided us with the notion that most defined populations of long term repopulating HSC still contain progenitor cells, which can only transiently contribute to repopulation of the haematopoietic system. This heterogeneity is not only evident in defined cell populations, but also in the in vivo niche for HSC, the bone marrow. HSC in the bone marrow are interspersed with transient amplifying cells and differentiated cells, complicating stem cell identification by spatial organization of the tissue. Other stem cell systems, such as spermatogonia, keratinocyte or crypt stem cells have a clearly defined niche architecture, enabling stem cell identification by location only (Fuchs *et al.*, 2004). In this case, resting stem cells and activated progenitors can be separated by

location and molecular markers can be easily identified. If all adult stem cells share a repertoire of molecular markers, findings from other adult stem cells can be transferred to HSC and should lead to characterization of haematopoietic stem cell subpopulations.

In our previous work, we found that adult melanocyte stem cells exhibit a 10 to 100fold lower level of housekeeping gene mRNA compared to differentiated cells, suggesting a global repression of mRNA transcription (Osawa *et al.*, 2005). We could then show that the largest subunit of RNA polymerase II (RNApII), which is responsible for all mRNA transcription, exhibits a partly phosphorylated C-terminal domain (CTD), characteristic of initiated, but paused mRNA transcription (Freter *et al.*, 2010). In line with this, we found the RNApII kinase CDK9 absent in adult melanocyte stem cells. Inhibition of CDK9 resulted in cellular resistance to withdrawal of essential growth factors, conferring a stem cell-like phenotype to progenitor cells. Interestingly, various other adult stem cells, including keratinocyte, muscle, spermatogonia and also HSC exhibited a similar partial phosphorylation of RNApII (Freter *et al.*, 2010). We concluded that transcriptional quiescence is an early, specific and conserved marker for adult stem cells. This feature can be used to isolate and characterize pure populations of stem cell-like cells from any tissue, enabling a deeper understanding of stem cell biology and recapitulation of the stem cell niche, in order to expand immature stem cells in vitro.

In this chapter I would like to summarize our findings that HSC exhibit a reduction in productive mRNA transcription. I would like to elaborate on the implications arising from transcriptional quiescence of a subset of HSC, both in development and disease. Technical challenges and resulting applications of identifying and isolating transcriptionally quiescent HSC in vitro will be discussed.

2. The mRNA transcription cycle

Regulation of gene expression is essential for all single- and multi cellular organisms. This fundamental process is executed at the level of mRNA transcription by RNApII, typically in distinct transcription steps. The different stages of mRNA transcription, initiation, promoter clearance, elongation, mRNA processing and release of RNApII from DNA are tightly regulated by modifications of the CTD of RNApII (Sims *et al.*, 2004, Fig1). This characteristic domain is in mammalian cells composed of 52 repeats of the consensus sequence YS_2PTS_5PS. During mRNA transcription several posttranslational modifications of the CTD are occurring, most prominently phosphorylation of Serine 5 (Ser5) and Serine 2 (Ser2). These phosphorylation events are requisite for binding of proteins essential for RNA processing, splicing and polyadenylation. Using antibodies specifically detecting these phosphorylation events enables determination of global mRNA transcription activity in single cells in vivo.

Gene expression in mammalian cells has been comprehensively studied at the transcription initiation step that is controlled by cell-specific transcription factors. In fact, it has been thought for long time that assembly of the preinitiation complex and subsequent recruitment on RNApII is the rate-limiting step for gene transcription. However, early results indicated that RNApII is initiated, but paused at Drosophila heat shock genes (Boehm *et al.*, 2003, Ni *et al.*, 2004). More recently, it was observed using genome-wide association studies that initiated but stalled polymerase is not only present on immediate-response or developmentally regulated genes, but also many non-expressed genes,

suggesting transcription elongation as the critical step in gene expression (Guenther *et al.*, 2007, Muse *et al.*, 2007, Zeitlinger *et al.*, 2007).

Transcription initiation requires phosphorylation of Ser5 of the CTD by TFIIH, a heterodimeric kinase consisting of CDK7 and Cyclin H. These phosphorylation events enable binding of the mRNA capping machinery (Ho & Shuman, 1999) and promoter clearance. Typically, a short (~40nt) nascent RNA is then produced by RNApII. However, mRNA transcription is paused at many genes due to the action of negative elongation factor (NELF) and DRB-sensitivity inducing factor (DSIF) (Wu *et al.*, 2003, Yamaguchi *et al.*, 2002).

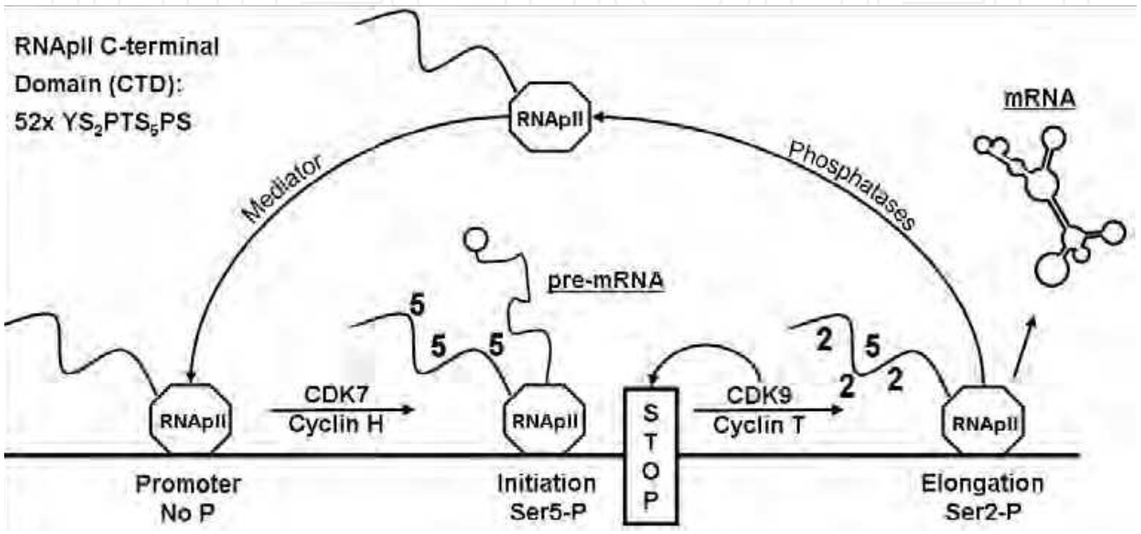

Fig. 1. The mRNA transcription cycle is characterized by phosphorylation of the RNA polymerase (RNApII) CTD. Phosphorylation of Ser5 (5) by CDK7/Cyclin H induces promoter clearance and pre-mRNA capping. RNApII is then halted (STOP), until activation of CDK9/Cyclin T. Phosphorylation of the inhibitory complex and RNA polymerase II CTD Ser2 (2) leads to productive elongation and release of mature mRNA.

Recruitment of positive transcription elongation factor b (P-TEFb), a heterodimeric protein consisting of the kinase CDK9 and one of the regulatory subunits Cyclin T1, T2 or K, to stalled polymerases is required for alleviation of the transcriptional block by NELF and DSIF (Peterlin & Price, 2006, Rahl *et al.*, 2010). P-TEFb phosphorylates RNA recognition motif-containing protein RD, a component of NELF, and Spt5, a subunit of DSIF (Aida *et al.*, 2006, Fujinaga *et al.*, 2004). NELF then dissociates from RNApII, while DSIF remains associated with RNApII and becomes a positive transcription elongation factor (Chen *et al.*, 2009). Importantly, phosphorylation of Ser2 of the CTD by P-TEFb triggers transcription elongation, mRNA processing, and release of mature mRNA (Kohoutek, 2009, Ni *et al.*, 2004).

CDK9 has first been identified as a CDC2-related kinase with a PITALRE motif (Grana *et al.*, 1994). The cyclin partner of CDK9 is Cyclin T1, T2 or K. Unlike other CDK/Cyclin heterodimers, neither P-TEFb levels nor kinase activity is fluctuating during the cell cycle (Garriga *et al.*, 2003, Grana *et al.*, 1994). CDK9 exists in two isoforms, a major 42kD sized peptide and an N-terminal extended peptide, whose transcription starts from an alternative

TATA box upstream of the housekeeping-type promoter of the 42kD isoform (Shore *et al.*, 2003). The expression of both isoforms varies between developmental stages and organs (Shore *et al.*, 2005). For example, the expressed isoform shifts from the longer 55kD form to the shorter 42kD isoform during lymphocyte activation (Liu & Herrmann, 2005). However, the target gene specificity is very similar between the two isoforms (Liu & Herrmann, 2005).

P-TEFb is present in the cell as either a large or small multiprotein complex. The inactive, large complex consists of 7SK non-coding RNA, Hexim1, LARP7 and MEPCE (Li *et al.*, 2005), while the small, active complex is formed by binding of Brd4 to P-TEFb (Yang *et al.*, 2005). Brd4 binds to acetylated histones and may therefore target P-TEFb to actively transcribed genes, when no specific transcription factor is present (Jang *et al.*, 2005) but can also recruit P-TEFb to inducibly acetylated histones (Hargreaves *et al.*, 2009). However, even the large complex contains primarily active P-TEFb, which is sequestered away from the kinase targets. Cellular stresses, such as UV irradiation, cytokines or changes in the microenvironment result in release of active P-TEFb to support quick mRNA transcription to respond to stimuli. Several transcription factors have been shown to interact directly with P-TEFb to stimulate transcription elongation of RNApII. These include NF-kB (Barboric *et al.*, 2001), c-Myc (Eberhardy & Farnham, 2001), CIITA (Kanazawa *et al.*, 2000), GATA1 (Elagib *et al.*, 2008) and Runx1 (Jiang *et al.*, 2005), amongst others. Thus, P-TEFb can be either recruited directly to promoters by specific transcription factors or to acetylated histones by Brd4.

Phosphorylation of Ser2 in the CTD of RNApII and productive transcription elongation is the critical target for eukaryotic gene expression (Bentley, 1995, Chao & Price, 2001). Inhibition of the CTD Ser2 kinase CDK9 by 5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) or Flavopiridol results in degradation of most mRNA (Chao & Price, 2001, Sehgal & Darnell, 1976) and induces apoptosis (Chen *et al.*, 2005, Gojo *et al.*, 2002). Similarly, knockdown of CDK9 in vivo results in complete absence of mRNA synthesis and embryonic lethality (Eissenberg *et al.*, 2007, Shim *et al.*, 2002). Cyclin T1 KO mice exhibit minor immunological defects (Oven *et al.*, 2007), while the Cyclin T2 KO mouse is embryonic lethal (Kohoutek *et al.*, 2009) with an extremely early phenotype before implantation of the blastocyst. This difference in phenotype of CDK9 Cyclin partners can partly be explained by a limited overlap in target genes of these isoforms (Ramakrishnan *et al.*, 2011).

On the other hand, ectopic activation of P-TEFb by ablation of Hexim1 results in embryonic lethality as well, due to hypertrophy of the heart (Huang *et al.*, 2004). Similarly, inactivation of the inhibitory large complex member LARP7 results in aberrant splicing and embryonic death in zebrafish, highlighting the essential role of P-TEFb for pre-mRNA splicing (Barboric *et al.*, 2009). However, overexpression of CDK9 from the endogenous Rosa26 promoter did not result in any phenotype in mice, mainly due to a low level of expression of CDK9 from this promoter (Freter *et al.*, 2010). Limiting P-TEFb activity is necessary for development of germ line cells in both D. melanogaster and C. elegance (Batchelder *et al.*, 1999, Hanyu-Nakamura *et al.*, 2008, Zhang *et al.*, 2003). Ectopic activation of P-TEFb by overexpression or knock down of inhibitors results in misexpression of somatic genes in germ line cells and their subsequent degeneration, resulting in sterile offspring. In summary, levels of P-TEFb and thus the global activity of RNApII need to be maintained within a certain limits, not exceeding and not below a basal threshold.

Most cells, including proliferating, terminally differentiated and senescent cells, actively synthesize mRNA. In these cells, RNApII is phosphorylated on CTD Ser2 and Ser5 independent of the cell cycle (Garriga *et al.*, 2003, Marshall *et al.*, 2005). However, some cells do not display this active phosphorylation pattern. For example, deeply dormant cells, such as primary T and B lymphocytes, exhibit an almost complete absence of RNApII phosphorylation (Garriga *et al.*, 1998, Marshall *et al.*, 2005). Activation of these cells by antigen encounter results in upregulation of Ccnt1 both on mRNA and protein level and subsequent phosphorylation of CTD Ser2 (Marshall *et al.*, 2005).

On the other hand, RNApII in yeast cells in the stationary phase (Patturajan *et al.*, 1998, Radonjic *et al.*, 2005) or on Drosophila heat shock genes (Boehm *et al.*, 2003, Ni *et al.*, 2004) is phosphorylated on Ser5, but not on Ser2. Stimulation of these cells, such as the addition of nutrients or heat shock, ensures rapid activation of gene transcription. Thus, analysis of the specific phosphorylated sites in RNApII can distinguish cells featuring phases of productive mRNA elongation or paused mRNA transcription initiation.

3. mRNA transcription in the hematopoietic lineage

Most of our knowledge of P-TEFb function derived from studies involving HIV replication (Barboric & Peterlin, 2005). Human Cyclin T1 is an essential co-factor of the immediate-early HIV gene product Tat, which recruits P-TEFb to the tar RNA located 5' on HIV genes to activate gene expression (Mancebo *et al.*, 1997, Zhu *et al.*, 1997). In resting lymphocytes, P-TEFb activity is low and thus HIV replication is blocked. Upon stimulation, upregulation of Cyclin T1 results in activation of P-TEFb and transcription of viral genes (Garriga *et al.*, 1998). Human Cyclin T1 (Ccnt1), the major Cyclin associated with CDK9, is characterized by a TAR recognition motif, which is essential for the formation of a ternary complex between tar RNA/Tat and P-TEFb to activate HIV gene transcription in cells (Wei *et al.*, 1998). This motif contains an essential Cysteine, which is required for complex formation with HIV Tat. Mutagenesis of mouse Ccnt1 at this position, which normally contains a Tyrosine in mouse, activates HIV transcription in murine cells (Fujinaga *et al.*, 2002).

P-TEFb is also required for normal hematopoietic development and function. Knock down of CDK9 in zebrafish embryos results in severe defects in definitive erythropoiesis, but no gross developmental defects despite a smaller body size (Meier *et al.*, 2006). Given the ubiquitous requirement of CDK9 for mRNA transcription this surprising observation may be explained by incomplete knockdown using morpholino DNA. Similarly, partial depletion of Ccnt1 in mice results in modest immunological phenotypes, such as appearance of autoimmunity due to impaired negative selection of autoreactive T cells in thymus (Oven *et al.*, 2007). Together, these results suggest that the hematopoietic lineage may be very susceptible for small changes in P-TEFb activity.

Recruitment of P-TEFb by transcription factors to heterochromatin converts this general elongation factor to a repressor of transcription. Runx1 binds the CD4 silencer in thymocytes and leads to active suppression of CD4 transcription during development. Interestingly, despite an engaged RNApII on the CD4 promoter and in the presence of an active CD4 enhancer in these cells, CD4 transcription is silenced (Jiang *et al.*, 2005). This is achieved by binding of Cyclin T1 to Runx1 and sequestering of P-TEFb into inactive chromatin loops (Jiang & Peterlin, 2008). Thus, inactive genes can be loaded with a poised polymerase and

induction of chromatin remodelling complexes result in rapid activation of gene transcription by release of active P-TEFb from adjacent loci (Jiang & Peterlin, 2008). In this sense, assembly of the transcriptional machinery on inactive promoters can be seen as a transcription bookmark, to facilitate future expression.

Activation of hematopoietic gene transcription can also be achieved via binding of P-TEFb to actively acetylated chromatin. Studies on LPS-induced inflammatory gene expression in macrophages revealed that primary response genes have a stalled polymerase at their promoters, already phosphorylated at Ser5 of the CTD (Hargreaves *et al.*, 2009). In response to LPS stimulation, acetylation of H4K5/8/12 recruits Brd4, this in turn engages P-TEFb leading to CTD Ser2 phosphorylation and mRNA transcription (Hargreaves *et al.*, 2009).

Hematopoietic lineages are very susceptible for inhibition of P-TEFb activity and require specific co-factors for their respective differentiation. For example, differentiating macrophages and murine erythroleukemic cells down regulate the 42kD isoform, and up regulate the 55 kD isoform of CDK9 (Liu & Herrmann, 2005). Megakaryocyte differentiation depends on activation of P-TEFb and can be blocked by CDK9 inhibitors or dominant negative CDK9 (Elagib *et al.*, 2008). Erythroid differentiation depends on stabilization of a GATA-1/SCL/LMO2 complex on β-globin chromatin, subsequent association of P-TEFb and RNApII Ser2 phosphorylation in the locus by the ubiquitous enhancer facilitator/chromatin factor Ldb1 (Song *et al.*, 2010). Interestingly, deletion of Ldb1 in vivo results in defects in adult haematopoietic stem cell maintenance and diminished long-term reconstitution potential upon transplantation (Li *et al.*, 2011). However, the authors did not examine activity of P-TEFb in their knock-out mice, which may be reduced and thus result in defects in HSC specification. Certainly, many other genes and cells depend on P-TEFb activity during development and differentiation. Using the hematopoietic lineage as a model system for the basal mRNA transcription machinery will shed light onto many aspects of eukaryotic mechanisms of transcription control.

Conversely, some leukemic cancers are characterized by dysregulation of P-TEFb activity. Several fusion genes of the histone methytransferase MLL1 involved in chromosomal rearrangements leading to myeloid and lymphoblastic leukaemia associate with mRNA transcription elongation factor encoded by ELL or P-TEFb (Benedikt *et al.*, 2011, Lin *et al.*, 2010), suggesting that one major mechanism for leukomogenesis is deregulated transcription elongation (Shilatifard *et al.*, 1996). Indeed, targeting P-TEFb with the specific CDK9 inhibitor Flavopiridol induces apoptosis in chronic lymphocytic leukemic cells by suppression of short-lived anti-apoptotic genes, such as Mcl-1 (Chen *et al.*, 2005). Dysregulation of P-TEFb activity is involved in several other cancer types. For example Hexim1, a negative regulator of P-TEFb activity, was found down regulated in invasive breast cancer samples compared to normal breast tissue (Wittmann *et al.*, 2003). Exploiting the susceptibility of the hematopoietic lineage for disturbance of their mRNA transcription may result in novel targets of cancer therapy.

4. Transcriptional quiescence of hematopoietic stem cells

Hematopoietic stem cells (HSC) have been in the focus of basic and applied research since many decades. Definition of subsets of transplantable HSC and their in vitro culture have advanced considerably in recent years. However, so far no reliable marker for the isolation

of pure HSC exists and our use of transplants is limited by the inability to expand these cells ex vivo. A sensitive marker of HSC quiescence and activation could be useful to isolate unadulterated long-term repopulating HSC and screen for factors that enable stem cell expansion while maintaining their undifferentiated state. It has been known for three decades that HSC down regulate productive mRNA transcription. Low retention of Pyronin Y, an RNA binding dye, can be used to isolate HSC (Shapiro, 1981), suggesting that global suppression of mRNA transcription is a feature of quiescent HSC. However, this observation has not been followed up by analysis of the global status of RNApII activity, which is responsible for all mRNA transcription.

We have previously observed that adult melanocyte stem cells (MelSC) down regulate many housekeeping genes, including ActB, ActG and GapDH, suggesting a global repression of mRNA synthesis in these cells (Osawa *et al.*, 2005). Melanocytes are pigmented cells in the hair follicle and skin, providing melanin granules to differentiating keratinocytes. The MelSC system in mouse hair follicles can serve as a model system for adult stem cell systems. It has the advantage of spatial separation of stem and differentiated cells and a non-lethal but obvious hair graying phenotype if this system is perturbed (Nishimura *et al.*, 2002). We observed that adult MelSC show a complete absence of RNApII CTD Ser2 phosphorylation, while Ser5 was phosphorylated (Freter *et al.*, 2010), suggesting a global down regulation of productive mRNA transcription. In line with this, CDK9 protein and mRNA was down regulated in MelSC as well. This suggests that RNApII is present at many genes in quiescent MelSC, but P-TEFb levels are not sufficient to induce active transcription elongation. Importantly, inhibition of CDK9 in vitro protected melanocyte precursors from stress-induced apoptosis and converted them to a stem cell-like state (Freter *et al.*, 2010).

We then expanded our observation to other stem cell systems, and found CTD Ser2-negative cells in all stem cell systems tested, including keratinocyte, muscle, spermatogonia and hematopoietic stem cells. This suggests that global suppression of mRNA transcription elongation is a conserved feature of adult stem cells. Interestingly, some stem cell systems showed heterogeneity of CTD Ser2 staining. For example, we observed that spermatogonia stem cells attached to the basal lamina are negative for CTD Ser2 phosphorylation, while those detaching up-regulate Ser2 phosphorylation, even though they are still positive for the spermatogonia stem cell marker CD9 (Freter *et al.*, 2010). Attachment to the basal lamina is often a requirement for stem cell function by directing planes of division or maintenance of the undifferentiated state. Thus, the CTD Ser2 negative population seems to be the more stem cell-like population in CD9 positive spermatogonia.

Similarly, murine CD34- c-Kit+Sca1+Lin- (KSL) long-term repopulating hematopoietic stem cells clearly showed two different populations. One population exhibited CTD Ser2 phosphorylation levels as high as short-term repopulating CD34+ KSL cells, while ~27% of all CD34- KSL cells were negative for CTD Ser2 phosphorylation (Freter *et al.*, 2010). Heterogeneity of the HSC pool has been described previously, with a transplantable fraction of 15-25% of CD34- KSL HSC population, also using additional markers (Ema *et al.*, 2005, Foudi *et al.*, 2009, Wilson *et al.*, 2008). Importantly, analysis of transcriptionally quiescent HSC requires isolation of pure subpopulations of cells. We found that adult MelSC exhibit up to 100fold lower levels of total RNA per cell (Osawa *et al.*, 2005), suggesting that one activated stem cell may be sufficient to mask the RNA signal of 100 quiescent stem cells.

In order to identify the transcriptionally quiescent subpopulation of CD34- KSL cells, we performed sorting of bone marrow cells and antibody staining of sorted populations. Unfortunately, this procedure always includes fixation of cells, so they can not be used for assessment of in vivo repopulation ability. In order to validate stem cell function in vivo, it is necessary to convert the negative CTD Ser2 phosphorylation event into a readout which can be measured in living cells. Kinase activity can be measured using fusion proteins of Cyan and Yellow fluorescent proteins (CFP and YFP respectively), separated by a kinase target, a flexible linker and a phosphoprotein binding domain. Phosphorylation events result in binding of the phosphoacceptor to the kinase target and folding of the fusion protein resulting in Foerster resonance energy transfer (FRET) between CFP and YFP (Sato *et al.*, 2007). However, autofluorescence of cellular organelles in low energetic wavelengths, such as CFP, results in low signal to noise ratios, which is particularly difficult for cell sorting of multiparametric cell suspensions, such as bone marrow cells. Furthermore, even a complete lack of FRET by spatial separation results in FRET signal due to high concentration of expressed fluorescent proteins (Nguyen & Daugherty, 2005). Thus, a FRET-based approach can be useful for single-cell based imaging approaches, such as time lapse imaging of individual cells in vitro, but rather not for FACS sorting of heterogenic cell populations.

Recently, the development of circular permuted green fluorescent proteins (cpGFP) has enabled researchers to measure phosphorylation events in living cells using a single wavelength (Kawai *et al.*, 2004). However, the increase in fluorescence was only around 10-15%, which would be too little for separation by FACS. Novel mutations of circular GFP and ratiometric measurement of absorbance at different wavelengths increased the dynamic range up to 16fold between free and saturated forms of cpGFP, at least for measurement of pH or Ca^{2+} in living cells (Bizzarri *et al.*, 2006, Souslova *et al.*, 2007). Yet, if a kinase reporter can be constructed using these advanced cpGFP mutants has still to be shown.

We could observe a specific down regulation of CDK9, the RNApII CTD Ser2 kinase, in adult melanocyte stem cells both on the mRNA and protein levels (Freter *et al.*, 2010). The CDK9 promoter has many features of a housekeeping gene promoter (Bagella *et al.*, 2000, Liu & Rice, 2000), thus down regulation of this promoter may be due to a similar mechanism as other down regulated housekeeping genes in MelSC. Reporters for promoter function, for example GFP, Luciferase or LacZ have been used extensively to isolate or trace specific cells in vivo. However, isolating CDK9 promoter negative cells as transcriptionally quiescent stem cells could be biased by secondary effects on the reporter, such as silencing of reporter constructs or heterogeneity of expression between cells. One solution would be to label all cells with a constitutive reporter, and isolate constitutive promoter positive, CDK9 promoter reporter negative cells. The constitutive promoter has to be carefully chosen, as for example expression from the CMV promoter highly depends on CDK9 (Peng *et al.*, 1998). Given our observation of low activity of CDK9 in HSC, it is not surprising that HSC show limited CMV promoter activity (Salmon *et al.*, 2000). Constitutive promoters, but also promoters used for overexpression of genes in quiescent stem cells thus need to be validated for activity in vivo.

In vitro culture and expansion of immature hematopoietic stem cells could help to achieve better transplantation response in patients, but has not been achieved yet. So far culture of immature HSC leads to almost immediate differentiation and loss of multi-lineage repopulation ability. It has been shown recently that the xenobiotic Aryl receptor is present on HSC (Singh *et al.*, 2009). Activation by ligands results in nuclear translocation,

recruitment of Ccnt1 and activation of hematopoietic gene transcription (Tian *et al.*, 2003). Interestingly, antagonists of the Aryl receptor prevent differentiation of HSC in vitro (Boitano *et al.*, 2010), suggesting that transcriptional quiescence may be beneficial for in vitro stem cell expansion. It would be very interesting to determine if inhibitors of P-TEFb activity have an effect on maintenance of undifferentiated HSC.

Transcriptional quiescence could also be used as a read-out of stem cell function in vitro. Screens of small molecular compounds in in vitro culture of primary HSC for maintenance of the human HSC markers CD34 and CD133 have led to some promising results (Boitano *et al.*, 2010). However, surface proteins may be unstable or unreliable, and may not be an immediate read-out of stem cell function. Transcriptional quiescence could serve as an alternative marker for stem cell identity. The development of fluorescent reporters for this screening is required to evaluate the impact of cytokines or small molecules on maintenance and expansion of HSC in vitro.

We and others have shown that inhibition of P-TEFb activity can be favourable for cell survival during cellular stresses, such as serum or growth factor starvation (Freter *et al.*, 2010, Kanazawa *et al.*, 2003). Down regulation of P-TEFb activity could thus also be advantageous for cancer cell survival during metastasis or therapy. Flavopiridol, a very specific CDK9 inhibitor has been used in BLL with some success (Chen *et al.*, 2005), but failed in most cases as a single agent in cancer chemotherapy (Blagosklonny, 2004). If transcriptionally quiescent cancer cells are present in primary or metastatic tumours, further inhibition of CDK9 activity may not be required. Rather, activation of mRNA transcription in these cells may render them susceptible to therapy and prevent metastasis and relapse. The first step towards this goal would be to identify and isolate transcriptionally quiescent cancer cells from a given tumour using a kinase activity reporter or CDK9 promoter reporter. Next, it would be necessary to determine if transcriptionally quiescent tumour cells have an enhanced tumour forming capacity in vivo or survive treatment with chemotherapeutic agents better than transcriptionally activated cells. Finally, high throughput screens for small molecular compounds which activate transcriptional quiescent cancer cells using the same reporter systems will enable us to activate dormant cancer stem cell-like cells in vivo and improve treatment of metastasis and prevent relapse of cancer in patients.

5. Conclusion

Adult stem cells have the unique capacity to self-renew and give rise to differentiated cells. To fulfil their lifelong function, these cells must be protected from cellular and genetic damage. Most adult stem cells are thought to enter a state of reversible cell cycle quiescence to preserve their role (Orford & Scadden, 2008). Indeed, activation of the cell cycle leads to premature stem cell exhaustion (Cheng *et al.*, 2000, Kippin *et al.*, 2005, Park *et al.*, 2003). However, most cells in the adult body have withdrawn from the cell cycle and can be induced to proliferate again, resulting in their eventual depletion (Bond *et al.*, 2004, Pajalunga *et al.*, 2007). Some somatic cells are able to resume proliferation and even self-renew, for example differentiated T and B lymphocytes (Fearon *et al.*, 2001). It has thus been proposed that adult stem cells are distinguished by other mechanisms rather than cell cycle quiescence (Mikkers & Frisen, 2005). Yet, what kind of defining mechanisms or marker this property integrates, and if it is shared by various adult stem cells, is unclear at the moment.

It has been long known that adult HSC can be isolated by their low retention of Pyronin Y, an RNA binding dye (Shapiro, 1981), suggesting that global mRNA transcriptional quiescence is a hallmark of quiescent stem cells. This observation however, has never been addressed further in terms of the precise molecular mechanism underlying global transcriptional quiescence. Analysis of the distinct phosphorylation patterns of the CTD of RNApII during the different stages of mRNA transcription can reveal initiation, paused transcription or productive elongation. Our analysis of various adult stem cell systems showed that down regulation of productive mRNA transcription elongation is a conserved, specific and early feature of adult stem cells (Freter *et al.*, 2010). In line with this, the CTD kinase CDK9 was absent and its inhibition improved cell survival during cellular stresses, suggesting a beneficial function of transcriptional quiescence for stem cell maintenance and survival. However, we could not induce activation of mRNA transcription by overexpression of CDK9 in vivo. It is thus not clear what the in vivo function of this down regulation is. New animal models with a constitutively active RNApII have to be developed to elucidate if transcriptional quiescence is necessary for adult stem cell maintenance or just a symptom of their quiescence.

Interestingly, we could observe some heterogeneity in terms of mRNA transcription elongation in the HSC pool, where only 27% of cells showed a clear absence of mRNA transcription elongation. Yet, identification and isolation of these transcriptionally quiescent cells depends on the availability of genetically encoded reporters of mRNA transcription. CDK9 activity reporters need to be specific for this kinase, and a large dynamic range has to be provided to clearly separate cell populations. A particular challenge will be to convert the negative observation of CTD Ser2 dephosphorylation into a signal-positive output, in order to avoid false-positive events by untransfected cells.

Taken together, we defined a novel molecular mechanism for adult stem cell quiescence, which may lead to the identification of pure stem cell-like cell populations from various sources, including heterogeneous adult stem cell populations or cancerous tissue. Even though some technical questions and functional tests are still to be answered, transcriptional quiescence is a novel and exciting mechanism to detect, isolate and characterize adult stem cells in an unprecedented purity from various sources.

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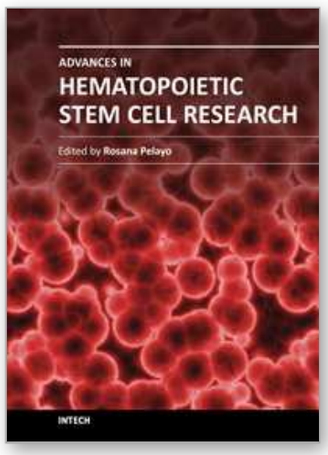

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