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The Role of Hepatic Leukemia Factor in Hematopoiesis

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The Role of Hepatic Leukemia Factor in Hematopoiesis

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Karolina Paulina Komorowska

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Abstract

The development of therapeutic methods based on stem cells (e.g. cell therapy, activating regeneration and organ transplantation), known as regenerative medicine, is still a novel area in the field of life sciences. If successful, such therapies could be applicable to many diseases currently lacking effective treatments, such as Parkinson's disease, diabetes, heart disease or cancer.

Stem cells are cells with a unique potential to self-renew, that is divide into two identical daughter cells having the same capacity as the mother cell, and the potential to differentiate into mature cells. Adult (somatic) stem cells have been identified in many organs such as blood, skin and intestines in adult humans. These stem cells are important for the internal repair system due to their ability to divide essentially without limits to replace the short-lived mature cells. In this way, blood cell production is sustained throughout an individual's lifetime by a few rare hematopoietic stem cells (HSC) residing in the bone marrow (BM).

Blood is a crucial element of the human body since blood cells provide oxygen and nutrients and protect the body against infections. Most of these cells are short-lived and are continuously recreated from HSCs through a gradual process of maturation. Furthermore, HSCs have the capacity to migrate from the BM out to peripheral blood, a process termed mobilization, and to home back to their BM microenvironment called niche.

Due to these abilities, transplantations based on stem cell therapy are today routine clinical procedures as a life-saving treatment in many serious hematopoietic diseases such as leukemia and genetic blood disorders. However, not all patients can be treated in this way due to low HSC yields and the lack of matching donors. Therefore, a long-standing goal for the field has been to establish protocols that would allow for the expansion of HSCs in culture. However, efforts to expand self-renewing HSCs that can engraft efficiently have not been successful as yet due to our limited understanding of their self-renewal mechanisms.

In steady state hematopoiesis, the majority of HSCs are in a dormant, non-dividing, quiescent state residing in the BM niche. However, at each round of cell division, only a subset of stem cells becomes active, suggesting that different states of stem cells coexist within the same niche.

We hypothesize that genetic modification might influence the HSC function during homeostasis, regeneration, aging and leukemia. The transcription factor, hepatic leukemia factor (HLF), as previously demonstrated by us and other researchers, is highly expressed in HSCs. However, the physiological role of HLF is still unknown. This thesis aims to define the role of the HLF in HSC regulation using genetically modified mice lacking the HLF gene.

In **Article I**, we confirmed that HLF is specifically expressed in the hematopoietic stem and progenitor cells and subsequently downregulated during differentiation. We show that HSCs devoid of *Hlf* display increased the cell cycle and decreased HSC quiescence. In line, *Hlf* deficient HSCs demonstrated a poor ability to reconstitute hematopoiesis in serial transplantation assay, indicating a reduced self-renewal capacity. Furthermore, *Hlf* deficient mice were hypersensitive to chemotherapeutic agents, such as 5-FU, that completely eradicated the HSC pool, and *Hlf* deficient mice failed to recover following the treatment. Interestingly, both by immunophenotype and on a transcriptional level, the *Hlf* deficient HSCs resembled more active short-term HSCs than long-term HSCs. Our findings unravel a novel role for HLF as a master regulator of HSC activity, highlighting the important role for HLF in hematopoiesis.

Aging is a general important subject for all of us, and even more serious for people who are exposed to extremely high radiation levels, e.g. cancer patients undergoing chemotherapy or astronauts who work in space. Aging is correlated with reduced cellular fitness, which can be seen in decline in somatic stem cells functions. As such, aged HSCs are associated with altered lineage commitment and a reduced capacity to recreate the hematopoietic system upon transplantation. Still, HSCs are protected by intrinsic mechanisms to ensure a functional stem cell pool over time. Interestingly, our data show that HLF is expressed in the HSC compartment throughout ontogeny, and since we demonstrated in Article I that HLF is crucial to maintain the HSC pool during regenerations, subsequently we studied the role of HLF in aged HSCs and embryonic development. In **Article II**, we showed that, although important for maintaining the HSC pool during regeneration, HLF is dispensable for normal aging, indicating that the mechanism controlling aging differs from the mechanism regulating reconstitution after transplantation.

Another key component of HSC regulation is their ability to home to the niche. In **Article III**, we developed a novel, standardized adhesion assay to investigate the interaction between HSCs and the niche represented by stroma cells. By using an RNA interference screen, we identified that Cytohesin 1 is a critical mediator of the attraction between HSCs and the stroma.

Key words: Hematopoiesis, Hematopoietic Stem Cells, knockout mice, HLF, Transcription Factor, aging

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The Role of Hepatic Leukemia Factor in Hematopoiesis

Karolina Paulina Komorowska

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On the cover: *Sakura,* the Japanese cherry blossom, flying with the wind to create a mouse. The mouse symbolizes the mouse model used in the study of hematopoeitic stem cell (HSC) regulation, which is symbolized by the gear wheels at the bottom of the tree. The background image taken by Talía Velasco-Hernández shows a section of murine bone marrow.
The illustration is inspired by the time the author spent in Japan as part of her MSc study, during the nuclear explosions in Fukushima in 2011. One of the ways to rescue the people from the nuclear power plant accident was to use HSC transplantation. That was the moment when the author decided to dedicate herself to developing the techniques described in this thesis.

Cover photo by Karolina Paulina Komorowska
Back cover drawing by Nadine März with technical assistance of Martin Schubert

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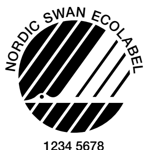

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Preface

How does a biotechnical engineer end up as a medical student?

First, I wanted to be a lawyer, to be able to help people in trouble. At the same time, I loved searching for truth and finding arguments to win people over to my line of thought. Later, I wanted to open my own brewery and study engineering, because mathematics has always been the *queen of all science* for me. Eventually, this led me to the study of Biotechnology. As an engineer, I like to go into details and design things from scratch. These features of my character inspired me to explore in depth the most important cells of our life – stem cells. As cells that create all of our body, they have an amazing power to create as well as destroy – in the case of cancer stem cells. Stem cells are unspecified cells with a remarkable ability to engender all other cell types of our body. I think I am still at the point where I started, helping people being my long-term goal since I was a child – not as a lawyer, though, but as an engineer and biomedical researcher. I hope that this small portion of my research done as part of my PhD studies will help better understand the regulations of those powerful cells that bring hope as well as fear, if not used in the right way. My focus was on blood, and in particular on Hematopoietic Stem Cells (HSC).

It has been a long journey to understand the regulation of HSCs. First, I explored the mechanism of HSC attraction to their microenvironment where they reside, so-called niche. Later, I wanted to find out more about the circadian rhythm in hematopoiesis, which has led me to a very interesting project of a new HSCs regulator – Hepatic Leukemia Factor (HLF).

Understanding the HSC regulation is crucial for successful transplantation, currently a key treatment in hematological disorders such as leukemia. However, there are limitations, which could be possibly reduced by expanding stem cells outside of live organisms or making them more attracted to find a place where they residue and do not get lost on the way, in this way enhancing the engraftment of transplanted cells. As soon as HSCs are isolated from the body, they start to differentiate and lose their stem function. That is why our major goal was to identify HSC regulators. In this thesis, I set out to demonstrate that HLF is one of them.

Przedmowa

Jak to się stało, że będąc biotechnologiem znalazłam się na medycznych studiach doktoranckich?

W dzieciństwie chciałam zostać prawnikiem, żeby pomagać ludziom. Uwielbiałam szukać odpowiednich argumentów, które nie tylko przekonają innych do mojego punktu widzenia, ale też pozwolą dojść do prawdy. Potem myślałam o otwarciu własnego browaru i o ukończeniu studiów inżynierskich, gdyż matematykę zawsze miałam za królową nauk. Ostatecznie zajęłam się biotechnologią. Jako inżynier lubię zgłębiać tajniki wiedzy i projektować rzeczy od zera. Te cechy charakteru zainspirowały mnie do podjęcia badań nad najważniejszymi komórkami w życiu człowieka – komórkami macierzystymi. Komórki te charakteryzują się zadziwiającą zdolnością różnicowania w inne typy komórek naszego organizmu i jednocześnie mają niezwykłą moc tworzenia, ale czasem i niszczenia (w przypadku komórek nowotworowych).

Przedmiotem moich badań w ramach pracy doktorskiej była krew, a w szczególności hematopoetyczne komórki macierzyste (HSC).

Proces zrozumienia regulacji HSC rozpoczęłam od badań mechanizmu przyciągania tych komórek do mikrośrodowiska, w którym przebywają – tzw. niszy. Następnie zainteresowało mnie zagadnienie rytmu okołodobowego w hematopoezie, co doprowadziło do rozpoczęcia prac nad niezwykle ciekawym projektem dotyczącym szczegółowego zbadania nowego regulatora HSC – hepatic leukemia factor (HLF).

Zrozumienie mechanizmu regulacji HSC ma zasadnicze znaczenie dla skuteczności przeszczepów, które w chwili obecnej stanowią podstawową terapię w zaburzeniach krwi takich jak np. białaczka. Istniejące aktualnie ograniczenia być może udałoby się zmniejszyć poprzez hodowlę komórek macierzystych poza organizmem żywym lub zwiększenie ich przyciągania do niszy, w której mogłyby osiąść, nie gubiąc się po drodze w układzie krwionośnym, zwiększając jednocześnie stopień zasiedlenia po przeszczepie. Niestety, HSC od razu po wyizolowaniu z organizmu zaczynają się dzielić i tracą swoją funkcję macierzystą. Z tego powodu ważną kwestią jest znalezienie nowych regulatorów HSC. W niniejszej pracy staram się wykazać, że HLF jest jednym z nich.

Mam nadzieję, że mój doktorat przyczyni się do lepszego zrozumienia mechanizmów regulacji, tych obdarzonych wielką mocą, komórek macierzystych, które rodzą zarówno nadzieję, jak i strach, gdy są niewłaściwie wykorzystywane. Ja zaś nadal pozostaję wierna celowi, określonemu przeze mnie w dzieciństwie, jakim jest pomaganie ludziom – robię to teraz nie jako prawnik, lecz jako badacz i inżynier biomedyczny.

It is only with the heart that one can see rightly;
what is essential is invisible to the eye.

– Antoine de Saint-Exupéry “The Little Prince”

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As this great experience comes to its pinnacle, it is time to reflect beyond the background of all the neat figures and high-impact publications – that is, the people behind the process. Here, I would like to acknowledge those who I encountered, and befriended, on my doctoral path: the people involved in both my scientific and private life. Without your unwavering support, kindness and help, my PhD could not have been completed.

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regardless of the time of day (or night - remember the crazy FACS of BrdU cells on Fortessa?!?). You are the role model of a leader and your passion for science is truly impressive.

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The last, but definitely not the least, thanks goes to all the laboratory animals that are the major contributors to all of our biomedical successes. Here is the Monument dedicated to The Laboratory Mouse. I think we should always bear in mind their enormous invaluable help.

"Mouse knitting a DNA"
Monument at the Institute of Cytology and Genetics, Siberian Branch of the Russian Academy of Sciences in Novosibirsk, Russia.

Popular Abstract

The development of therapeutic methods based on stem cells (e.g. cell therapy, activating regeneration and organ transplantation), known as regenerative medicine, is still a novel area in the field of life sciences. If successful, such therapies could be applicable to many diseases currently lacking effective treatments, such as Parkinson's disease, diabetes, heart disease or cancer.

Stem cells are cells with a unique potential to self-renew, that is divide into two identical daughter cells having the same capacity as the mother cell, and the potential to differentiate into mature cells. Adult (somatic) stem cells have been identified in many organs such as blood, skin and intestines in adult humans. These stem cells are important for the internal repair system due to their ability to divide essentially without limits to replace the short-lived mature cells. In this way, blood cell production is sustained throughout an individual's lifetime by a few rare hematopoietic stem cells (HSC) residing in the bone marrow (BM).

Blood is a crucial element of the human body since blood cells provide oxygen and nutrients and protect the body against infections. Most of these cells are short-lived and are continuously recreated from HSCs through a gradual process of maturation. Furthermore, HSCs have the capacity to migrate from the BM out to peripheral blood, a process termed mobilization, and to home back to their BM microenvironment called niche.

Due to these abilities, transplantations based on stem cell therapy are today routine clinical procedures as a life-saving treatment in many serious hematopoietic diseases such as leukemia and genetic blood disorders. However, not all patients can be treated in this way due to low HSC yields and the lack of matching donors. Therefore, a long-standing goal for the field has been to establish protocols that would allow for the expansion of HSCs in culture. However, efforts to expand self-renewing HSCs that can engraft efficiently have not been successful as yet due to our limited understanding of their self-renewal mechanisms.

In steady state hematopoiesis, the majority of HSCs are in a dormant, non-dividing, quiescent state residing in the BM niche. However, at each round of cell division, only a subset of stem cells becomes active, suggesting that different states of stem cells coexist within the same niche.

We hypothesize that genetic modification might influence the HSC function during homeostasis, regeneration, aging and leukemia. The transcription factor, hepatic leukemia factor (HLF), as previously demonstrated by us and other researchers, is highly expressed in HSCs. However, the physiological role of HLF is still unknown. This thesis aims to define the role of the HLF in HSC regulation using genetically modified mice lacking the HLF gene.

In **Article I**, we confirmed that HLF is specifically expressed in the hematopoietic stem and progenitor cells and subsequently downregulated during differentiation. We show that HSCs devoid of *Hlf* display increased the cell cycle and decreased HSC quiescence. In line, *Hlf* deficient HSCs demonstrated a poor ability to reconstitute hematopoiesis in serial transplantation assay, indicating a reduced self-renewal capacity. Furthermore, *Hlf* deficient mice were hypersensitive to chemotherapeutic agents, such as 5-FU, that completely eradicated the HSC pool, and *Hlf* deficient mice failed to recover following the treatment. Interestingly, both by immunophenotype and on a transcriptional level, the *Hlf* deficient HSCs resembled more active short-term HSCs than long-term HSCs. Our findings unravel a novel role for HLF as a master regulator of HSC activity, highlighting the important role for HLF in hematopoiesis.

Aging is a general important subject for all of us, and even more serious for people who are exposed to extremely high radiation levels, e.g. cancer patients undergoing chemotherapy or astronauts who work in space. Aging is correlated with reduced cellular fitness, which can be seen in decline in somatic stem cells functions. As such, aged HSCs are associated with altered lineage commitment and a reduced capacity to recreate the hematopoietic system upon transplantation. Still, HSCs are protected by intrinsic mechanisms to ensure a functional stem cell pool over time. Interestingly, our data show that HLF is expressed in the HSC compartment throughout ontogeny, and since we demonstrated in Article I that HLF is crucial to maintain the HSC pool during regenerations, subsequently we studied the role of HLF in aged HSCs and embryonic development. In **Article II**, we showed that, although important for maintaining the HSC pool during regeneration, HLF is dispensable for normal aging, indicating that the mechanism controlling aging differs from the mechanism regulating reconstitution after transplantation.

Another key component of HSC regulation is their ability to home to the niche. In **Article III**, we developed a novel, standardized adhesion assay to investigate the interaction between HSCs and the niche represented by stroma cells. By using an RNA interference screen, we identified that Cytohesin1 is a critical mediator of the attraction between HSCs and the stroma.

Streszczenie

Rozwój metod terapeutycznych wykorzystujących komórki macierzyste (np. terapia komórkowa, stymulacja regeneracji i przeszczepianie narządów), zwanych medycyną regeneracyjną, jest wciąż słabo rozpoznanym obszarem w dziedzinie nauk przyrodniczych. Jeśli badania w zakresie medycyny regeneracyjnej okażą się skuteczne, to ich wyniki powinny znaleźć zastosowanie w leczeniu wielu chorób, np. choroby Parkinsona, cukrzycy, chorób serca czy nowotworów.

Komórki macierzyste są komórkami o wyjątkowym potencjale samoodnowy (o niemalże nieograniczonej zdolności do proliferacji), czyli podziału na dwie identyczne komórki potomne o takich samych możliwościach jak komórka macierzysta i do różnicowania się w dojrzałe komórki innych typów. Dorosłe (somatyczne) komórki macierzyste zidentyfikowano w wielu narządach u ludzi dorosłych, takich jak skóra i jelita, oraz we krwi. Komórki te odgrywają ważną rolę w wewnętrznym systemie naprawczym ze względu na ich zdolność do dzielenia się w zasadzie bez ograniczeń, aby uzupełnić krótko żyjące dojrzałe komórki. Dzięki temu wytwarzanie krwinek (proces krwiotworzenia) odbywa się przez całe życie osobnika poprzez niewielką liczbę rzadkich hematopoetycznych komórek macierzystych (HSC) znajdujących się w szpiku kostnym.

Krew pełni kluczową rolę w organizmie człowieka, ponieważ komórki krwi dostarczają tlen i składniki odżywcze do wszystkich tkanek i narządów oraz chronią organizm przed infekcjami. Większość tych komórek ma krótki okres życia i są one w sposób ciągły odtwarzane z HSC poprzez stopniowy proces dojrzewania i różnicowania. Ponadto HSC mają zdolność migracji ze szpiku kostnego do krwi obwodowej w procesie określanym mianem mobilizacji oraz powrotu do mikrośrodowiska krwiotwórczego szpiku, zwanego niszą naczyniową.

Ze względu na te zdolności, przeszczepy oparte na terapii komórkami macierzystymi są dziś rutynowymi procedurami klinicznymi, ratującymi życie w wielu poważnych chorobach krwi, takich jak białaczka czy choroby krwi uwarunkowane genetycznie. Jednak nie wszyscy pacjenci mogą być leczeni w ten sposób, głównie z powodu niskiej wydajności komórek HSC i problemów ze znalezieniem odpowiednich dawców. Dlatego też dalekosiężnym celem w tym obszarze badań jest znalezienie optymalnej metody umożliwiającej szybki wzrost komórek HSC w hodowlach komórkowych *in vitro*.

Jednocześnie dotychczasowe wysiłki w kierunku namnażania komórek HSC, nadających się do przeszczepu, nie zakończyły się powodzeniem z powodu wciąż niewielkiej wiedzy na temat mechanizmów samoodnowy.

W fizjologicznej równowadze hematopoezy, większość HSC znajduje się w stanie uśpionym, nie dzielą się, spoczywając w niszy szpiku kostnego. W każdym cyklu nowych podziałów komórkowych aktywuje się jedynie część komórek macierzystych, co sugeruje, że w tej samej niszy współistnieją komórki macierzyste na różnych etapach różnicowania i rozwoju.

Przed przystąpieniem do badań nad nowymi metodami terapeutycznymi, wykorzystującymi komórki macierzyste, została postawiona hipoteza, że modyfikacja genetyczna może wpływać na funkcje krwiotwórczych komórek macierzystych podczas homeostazy, regeneracji, starzenia się i białaczki.

W niniejszej pracy zostało wykazane, że czynnik transkrypcyjny, hepatic leukemia factor (HLF), jest wysoce i specyficznie ekspresjonowany w hematopoetycznych komórkach macierzystych i progenitorowych (swoistych tkankowo komórkach występujących w narządach dorosłych osobników, służących do regeneracji tych narządów), a następnie jego ekspresja jest wyciszana podczas różnicowania. Zatem celem pracy jest określenie roli HLF w regulacji komórek HSC przy użyciu genetycznie zmodyfikowanych myszy pozbawionych genu HLF.

Ponadto w trakcie badań zostało dowiedzione, że HSC pozbawione genu *Hlf* miały przyspieszony cykl komórkowy oraz skrócony cykl spoczynkowy. Dodatkowo, HSC z nokautem na *Hlf* wykazywały słabą zdolność do odtworzenia hematopoezy w serii testów transplantacyjnych, co wskazuje na zmniejszoną zdolność samodzielnego odnawiania się w przypadku nieobecności tego genu. Ponadto myszy z wyłączonym genem *Hlf* wykazywały nadwrażliwość na środki chemioterapeutyczne takie jak 5-FU, które całkowicie zniszczyły pulę HSC, a myszy nie powróciły do zdrowia po zakończeniu badań. Co ciekawe, zarówno na podstawie immunofenotypu, jak i na poziomie transkrypcji, komórki macierzyste ze znokautowanym genem *Hlf* wykazywały zdecydowanie krótkoterminową aktywność. Uzyskane wyniki wskazują na ważną rolę czynnika transkrypcyjnego HLF jako głównego regulatora aktywności komórek HSC oraz podkreślają ważną rolę HLF w hematopoezie.

Starzenie się jest istotną kwestią dla nas wszystkich, a w szczególności dla osób narażonych na ekstremalnie wysokie poziomy promieniowania, jak np. pacjenci chorzy na raka poddawani chemioterapii lub astronauci, pracujący w kosmosie. Starzenie się jest ściśle skorelowane ze zmniejszoną sprawnością komórkową, co przejawia się w spadku funkcji somatycznych komórek macierzystych. Starzejące się HSC mają mniejszą zdolność do różnicowania w komórki dojrzałe i odtwarzania układu krwiotwórczego po przeszczepie. Jednocześnie HSC

są chronione przez mechanizmy wewnętrzne w celu zapewnienia stałej, funkcjonalnej puli komórek macierzystych. Co ciekawe, niniejsze badania wykazały, że ekspresja HLF jest obecna w HSC przez cały czas ontogenezy. Zbadano zatem rolę HLF w starzejących się HSC jak i w rozwoju embrionalnym. Dowiedzione zostało, że o ile HLF odgrywa ważną rolę w utrzymaniu puli HSC podczas regeneracji, o tyle jest zbędny podczas naturalnego starzenia się, co wskazuje, że mechanizmy kontrolujące starzenie różnią się od tych odpowiadających za regulację odtwarzania komórek po przeszczepie.

Kolejnym kluczowym elementem w regulacji powstawania i utrzymania kultur HSC, jest ich zdolność do powrotu do niszy naczyniowej. W trakcie kolejnych badań został opracowany nowy, znormalizowany test adhezji w celu zbadania interakcji między HSC a niszą reprezentowaną przez komórki stromy. Wykorzystując metodę interferencji RNA zostało stwierdzone, że Cytohezyna1 jest istotnym mediatorem przyciągania między komórkami HSC a stromą.

Populärvetenskaplig sammanfattning

Regenerativ medicin möjliggör stora framsteg inom forskningen, och innebär att man med hjälp av stamceller genererar friska celler eller frisk vävnad för att reparera eller byta ut det som är skadat och återupprätta normal funktion. Potentialen för cellterapi inom sjukvården är mycket stor och skulle kunna ha ett stort genomslag för många sjukdomar som för närvarande saknar effektiv behandling, som till exempel Parkinsons, diabetes, olika hjärtsjukdomar och cancer.

Stamceller har den unika förmågan att kunna självförnya sig, vilket innebär att de kan dela sig i två identiska dotterceller med samma kapacitet som modercellen, samt har förmågan att mogna ut till vävnadsspecifika celler. Idag har forskning identifierat vävnadsspecifika stamceller i många våra organ, som i till exempel blod, hud, tarm och hjärna. De stamcellerna är nödvändiga för att tillgodose det stora behov av nya celler som kroppen dagligen behöver under en individs livslängd, och särskilt då i blodet, tarmarna och huden.

Cellerna i blodet är nödvändiga för att tillgodose kroppen med syre, näring och för att transportera bort restprodukter, men också för att skydda oss mot bakterier och virus. Dessa celler är oftast kortlivade och behöver nybildas hela tiden, vilket sker från ett litet antal celler i benmärgen, de s.k. blodstamcellerna. Blodstamcellerna har förmågan att ge upphov till alla typer av blodceller, och har även potentialen att självförnya sig. Detta innebär att de kan förse kroppen med nästan 1 000 miljarder nya blodceller varje dag livet ut. Dessutom har blodstamcellerna förmågan att migrera från benmärg ut i blod, sk mobilisering, samt hitta tillbaka till sina specifika platser i benmärgen som kallas nisch.

Dessa egenskaper har gjort att transplantation av blodstamceller idag är en rutinbaserad cellterapi som enda botemedlet för patienter med blodcancer (leukemi) och genetiska blodsjukdomar. I mer än 40 år har man genomfört lyckade transplantationer av blodstamceller, men ett centralt problem är att antalet blodstamceller är ytterst få och behovet av donatorer är mycket stort. Om vi kunde lära oss att expandera blodstamcellerna i laboratoriet skulle vi därför kunna förbättra möjligheterna till blodstamcellsbehandling. Trots mycket forskning finns det dock inget säkerställt protokoll som tillåter expansion av blodstamceller utanför kroppen vilket beror på att vi idag inte vet tillräckligt mycket om de mekanismer som styr självförnyelse av humana blodstamceller.

För att säkerställa ett livslångt underhåll av normal blodstamcellsfunktion hålls de flesta blodstamceller i ett vilande (icke delande) tillstånd. Detta skyddar dem mot utmattning och yttre påverkan, och minimerar skada på arvsmassan, vilket kan ge upphov till blodcancer. Blodstamcellerna kan dock snabbt rekryteras för att rekonstruera blodsystemet vid trauma, varefter de återvänder till sitt viloläge.

Vår grundhypotes i denna avhandling är att genom att studera genetiskt modifierade blodstamceller få kunskap om hur blodstamcellsfunktion regleras under homeostas, regenerering, åldrande och blodcancer. Vi hoppas att informationen kan leda till att vi kan expandera stamcellerna utanför kroppen och öka möjligheterna till blodstamcellstransplantation vid t ex brist på stamceller eller donatorer.

Vi och flera andra forskningsgrupper har tidigare identifierat Hepatic Leukemia Factor (HLF) som en möjlig stamcellsregulator för både normal och cancerogen blodbildning. Därför var syftet med denna avhandling att definiera HLF:s roll i reglering av blodstamceller genom att studera genetiskt modifierade möss som saknar HLF-genen.

I **Artikel I** visar vi att HLF-genen specifikt är aktiv i blodstamstamcellerna och därefter stängs av när blodstamcellerna börjar mognar ut. Vidare visar vi att blodstamcellerna som saknar HLF var konstant aktiva och inte längre i sitt viloläge. Detta gjorde vi både funktionellt och genom att analysera vilka gener som är aktiva i blodstamcellerna, och på båda sätten påvisade vi att cellerna var aktivt delande. Detta i sin tur gjorde att blodstamcellerna uppvisade mycket begränsad förmåga att återskapa blodsystemet vid transplantation vilket är bevis på en minskad självförnyelsekapacitet. Vidare fann vi att mössen som saknade HLF-genen inte kunde överleva cytostatikabehandling p g a att alla blodstamceller slogs ut eftersom de var aktiva och inte i sitt viloläge. Utan HLF-genen skyddades alltså inte längre blodstamcellerna mot cytostatika eller mot annan stress, som exempelvis transplantation. Vår forskning visar att HLF-genen är en viktig regulator för blodstamcellsaktivitet och en viktig faktor för att upprätthålla stamcellens förmåga att bilda nytt blod genom att bibehålla blodstamcellerna i vila.

Orsaker som påverkar åldrandet är något som berör oss alla och speciellt viktigt för människor som utsätts för hög strålning, som exempelvis cancerpatienter eller astronauter. Det är känt sedan länge att våra organspecifika stamceller försämras med tiden. I linje med detta har studier visat att blodstamcellernas förmåga att producera celler till vårt immunförsvar försämras med åldrandet och transplantation av ”gamla” blodstamceller uppvisar reducerad förmåga att återskapa blodsystemet. Trots detta skyddas dock blodstamcellerna av inbyggda mekanismer (som man vet väldigt lite om) för att säkerställa en funktionell stamcellspool över tiden.

Intressant nog visar vår data att HLF-genen är aktiv i blodstamcellerna genom hela en individs livslängd, och eftersom vi i Artikel I fann att HLF är avgörande för att upprätthålla blodstamcellernas funktion under återskapandet av blodsystemet undersökte vi i **Artikel II** HLF:s roll i åldrande blodstamceller. I Artikel II fann vi överraskande att möss utan HLF åldras normalt även om blodstamcellernas förmåga fortfarande uppvisade begränsad kapacitet att återskapa blodsystemet efter transplantation. Detta visar att mekanismerna som påverkar åldrandet av blodstamcellerna skiljer sig från mekanismerna som reglerar återskapandet av blodsystemet efter transplantation.

Blodstamcellernas förmåga att hitta hem till sin nisch i benmärgen är väldigt viktig. I **Artikel III** utvecklade vi en ny och effektiv metod som bygger på gravitation för att identifiera de mekanismer som styr interaktion mellan blodstamcellerna och de stromaceller som utgör benmärgsnischen. Genom att systemiskt slå ut geners funktion i blodstamcellen kunde vi med hjälp av denna metod påvisa att Cytohesin1 är en kritisk mediator för interaktion mellan blodstamcellerna och stromacellerna. Dessa resultat visar att vår metod kan användas för att utveckla förmågan hos blodstamcellerna att binda in till benmärgen och därmed förbättra möjligheterna vid blodstamcellestransplantation.

PL: Zdjęcie (po prawej) ilustruje unikalne występowanie komórek macierzystych krwi (tu w kolorze pomarańczowym) w szpiku kostnym i jednocześnie tłumaczy, dlaczego te komórki są istotne dla życia i tak trudne do badania.

The image shows a section of a murine bone marrow stained with c-kit in green, sca-1 in red and laminin in white; blue colour marks nuclei of all cells. This image highlights that HSCs (here in orange) are very rare and difficult to study.

Image courtesy: Talía Velasco-Hernández

List of publications

Article I

Hepatic Leukemia Factor Maintains Quiescence of Hematopoietic Stem Cells and Protects the Stem Cell Pool during Regeneration.

Komorowska K, Doyle A, Wahlestedt M, Subramaniam A, Debnath S, Chen J, Soneji S, Van Handel B, Mikkola HKA, Miharada K, Bryder D, Larsson J, Magnusson M.

Cell Rep. 2017 Dec 19;21(12):3514-3523. doi: 10.1016/j.celrep.2017.11.084.

Article II

Hepatic Leukemia Factor is dispensable for hematopoiesis during aging.

Komorowska K, Miharada K, Larsson J and Magnusson M.

Manuscript

Article III

Cytohesin 1 regulates homing and engraftment of human hematopoietic stem and progenitor cells.

Rak J, Foster K, Potrzebowska K, Talkhoncheh MS, Miharada N, **Komorowska K**, Torngren T, Kvist A, Borg Å, Svensson L, Bonnet D, Larsson J.

Blood. 2017 Feb 23;129(8):950-958. doi: 10.1182/blood-2016-06-720649. Epub 2016 Nov 29.

Hematopoiesis

The word *hematopoiesis* originates from the ancient Greek words “αἷμα” (“haîma”) meaning “blood”, and “ποίησις” (“poíēsis”) meaning “a creation” – and describes the endless and dynamic process of blood cell production during the entire human life span. In adults, hematopoiesis takes place entirely in the bone marrow (BM). The hematopoietic system is highly regenerative and made of several cell types. The most common are red blood cells or erythrocytes, which are responsible for the transport of oxygen from the lungs to all other tissues; white blood cells or leukocytes, which act in the immune defense against infections; and megakaryocyte-derived platelets, or thrombocytes, that facilitate blood coagulation and wound healing.

In order to keep constant blood cell formation throughout life, the hematopoietic system is dependent on a rare cell type population called hematopoietic stem cells (HSC). The turnover of the system is about a trillion of new blood cells every day [1].

Discovery of the Blood System

In the 17th century, shortly after the microscope was discovered, the first component of blood was observed and described as red blood cells by the Dutch microscopist Jan Swammerdam [2; 3]. It took almost two centuries before white blood cells were identified by the French professor of hygiene, Gabriel Andral, in 1843 [4-6]. Although these pioneer findings lay the foundation for our understanding of blood, it was not until World War II that hematopoietic research really started to be well-studied. It was fuelled by the observation that several victims of the nuclear bombings of Hiroshima and Nagasaki who survived the initial nuclear explosion died shortly after due to BM failure caused by gamma irradiation [7]. This finding was the igniting spark to the hematopoietic research field, which has expanded since then. In 1950, research on mice revealed that shielding the spleen with lead would protect the mice from irradiation sickness [8]. Furthermore, two independent studies a year later showed that transplantation using BM or spleen cells could successfully rescue the irradiated mice [9; 10]. In 1959, the first successful human BM transplantations were achieved by

Donald Thomas (Nobel Prize 1990) [11]. In 1960, Till and McCulloch discovered a direct correlation between the survival of irradiated mice and the amount of transplanted BM cells [12]. Just a year after, they also discovered that transplanted BM cells can engraft and form multilineage colonies – CFU-S [13], which can then be transplanted successfully into secondary recipients [14; 15]. Those discoveries showed that the BM and spleen contain proliferating cells that can produce different blood cells after transplantation, and thereby introduced the concept of HSC.

In the 1980s, several excellent studies demonstrated the true existence of HSC by tracking retroviral integration sites. The studies concluded that one single hematopoietic cell could serially reconstitute lethally irradiated mice with all blood cell lineages [16-19]. Shortly afterwards, HSCs were for the first time isolated from the mouse BM using flow cytometry [20]. The existence of long-term, multilineage, repopulating cells was definitely confirmed in mouse studies in the 1990s by showing a single transplanted cell managed to successfully reconstitute an irradiated recipient mouse [21; 22].

Hematopoietic Stem Cells

HSCs differ from all other blood cells by their capability to self-renew and differentiate into all blood cell lineages. Self-renewal proceeds through symmetrical cell division, generating identical daughter stem cells that retain self-renewal capacity, or through asymmetrical cell division resulting in one stem cell and one, more differentiated, progenitor cell. This approach ensures an intact stem cell pool through an individual life span. Somatic (adult) stem cells are crucial for the internal repair system, dividing essentially without limit to replenish dying cells in many organ systems such as blood, skin and intestines.

HSCs supply the human body daily with billions of new blood cells [1; 23]. In adults, HSCs reside in the BM and the majority are quiescent [24; 25]. In fact, they rarely enter the cell cycle, which is believed to keep them protected from stress-induced DNA damage caused by continuous replication and cellular respiration, which could lead to induced apoptosis or cellular transformation [26]. In this way, HSCs, with their unique properties of engraftment in the BM, self-renewal and multilineage differentiation capacity, can sustain blood homeostasis throughout an individual’s lifetime. Importantly, due to these properties, transplantation of HSCs can provide a permanent cure for life-threatening hematological malignancies and genetic blood disorders.

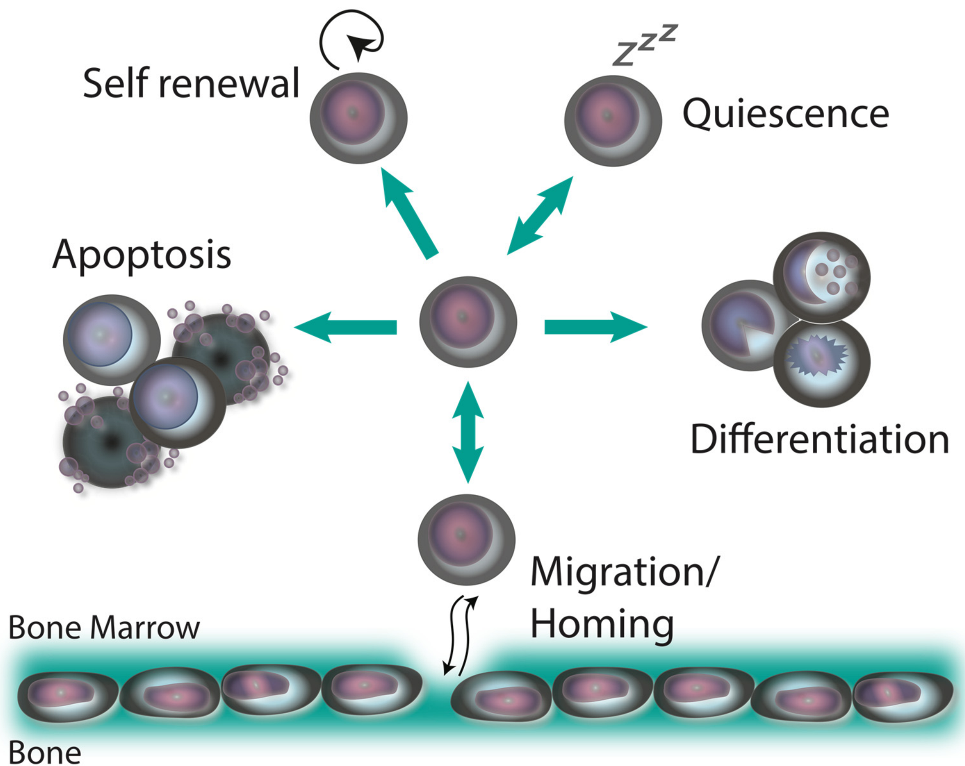


Figure 1. HSC fate options
Schematic overview of different HSC fate choices.

Controlling the fate of HSCs (**Figure 1**) is essential to maintain steady-state hematopoiesis and to rapidly respond to hematopoietic stress caused e.g. by blood loss, infections or injuries. It is a combination of intrinsic (cell autologous) as well as extracellular signals from the BM niche [27-30]. Although the dynamics of HSC contribution to hematopoiesis still remains controversial, most likely it is a hierarchical process with quiescent long-term HSCs (LT-HSCs) on the top. Supporters of this view would argue that these HSCs are indeed true stem cells with an infinite ability to self-renew. They would be followed by short-term HSCs (ST-HSCs) with multipotency and limited self-renewal capabilities [31; 32]. Further downstream in the hierarchy (**Figure 2**), the cells become more differentiated and more restricted in their lineage potential without self-renewal capacity, until they finally become mature blood cells at the bottom of the hematopoietic hierarchy [32-34].

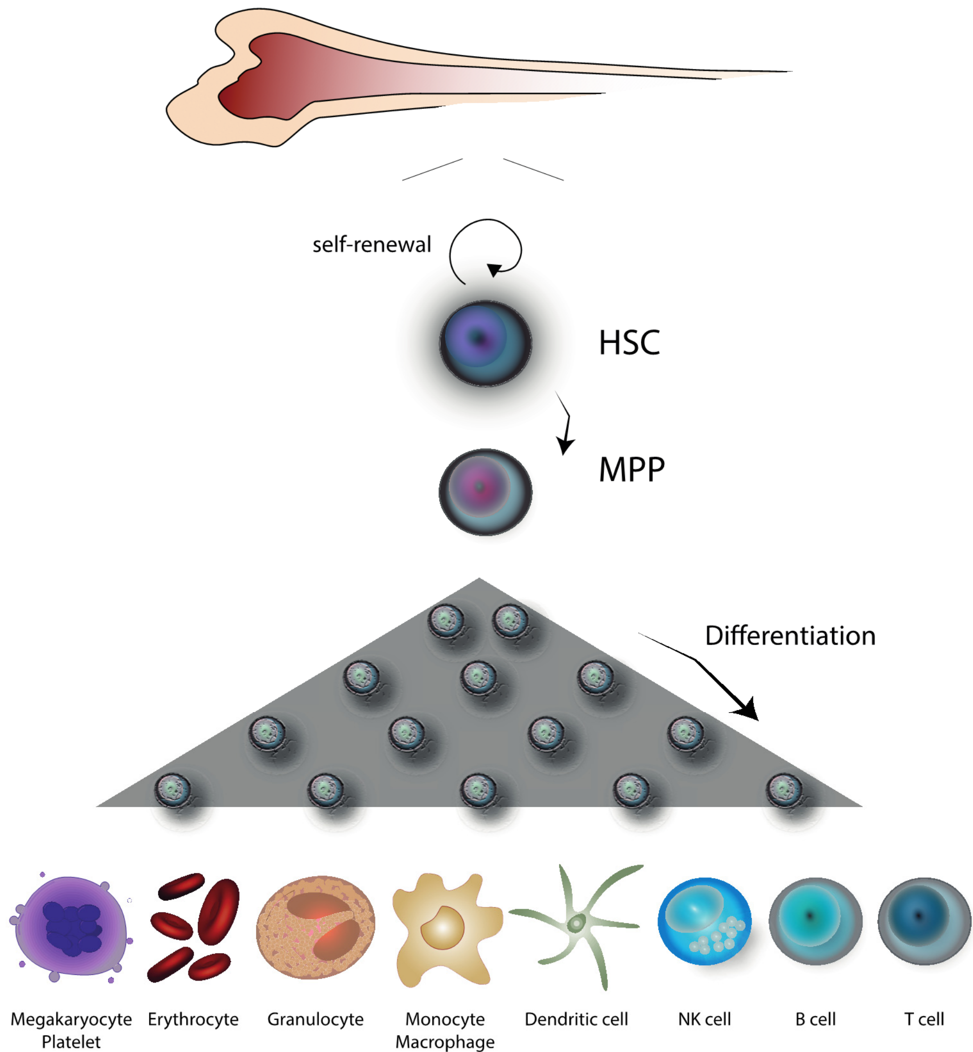


Figure 2. The hematopoietic hierarchy
Simplified schematic overview of lineage determination during hematopoiesis. Grey area represents the still controversial and continuously improved process of differentiation from immature to mature cells. HSC (hematopoietic stem cell), MPP (multipotent progenitor), NK cell (natural killer cell).

Until recently, researchers believed that HSCs were the main contributors to life-long blood production [33; 35]. Indeed, HSCs are crucial during embryonic development in order to establish the hematopoietic system, which may explain why HSCs are very active in fetal and early postnatal life [36]. However, recent data has indicated that once the HSC pool has been established, the LT-HSCs stay mostly quiescent and do not contribute to adult steady-state hematopoiesis.

Instead, they act as a reservoir to restore hematopoiesis in response to cellular stress, insults or after transplantation [37; 38]. In line with this theory, several studies have shown that HSCs undergo a switch from dormancy to self-renewal in response to injury, and once homeostasis has been re-established, they return to a quiescent state [39; 40]. Sun and colleagues. suggest that the ST-HSCs, (which appear downstream of LT-HSCs and have a limited self-renewal capacity but lack any engraftment capability), are the main drivers of steady-state hematopoiesis during adulthood [37]. This is supported by an additional study that also indicates ST-HSCs as sufficient for maintaining steady-state hematopoiesis on an everyday basis [41]. This suggests that ST-HSCs are the main source of the hematopoietic system. In contrast, Sawai and colleagues described that the LT-HSC are the main source of hematopoiesis [42]. However, due to technical limitations concerning the different lineage tracing systems, the question still remains whether steady state hematopoiesis is derived from HSCs or from a committed progenitor. In addition, it may also be a reflection of the heterogeneity that has been shown to exist within this highly purified cell population [43]. Thus, additional research needs be performed to determine the answer to this open question.

The emergence and development of HSCs

During embryogenesis, three distinct germ layers are formed – ectoderm, which develops into the nerve system and skin; endoderm, which becomes lung, gut, and intestine; and mesoderm, which gives rise to blood. The nomenclature comes from the Greek words, *ektos* (outer), *endon* (inner), *mesos* (middle) and *derma* (skin) [44].

The emergence of HSCs and the hematopoietic system is a very complex procedure involving at least three waves in a variety of organ sites (**Figure 3**). In the first wave, known as primitive hematopoiesis, primitive nucleated erythrocytes containing fetal hemoglobin and primitive macrophages are developed in the yolk sac, at embryonic day 7.5 of the mouse (E7.5) [45-49]. So far, the transplantation of these cells into irradiated recipients has not produced successful engraftment, clearly indicating that they are not definitive HSCs yet [49]. However, they are needed to sustain the growing embryo with oxygen [50]. The first wave is rapidly followed by the second wave containing definitive hematopoietic progenitors that emerge in the yolk sac at E8.25. These progenitors have myeloid-erythroid potential and can differentiate into blood cells with adult characteristics. Therefore, they are called definitive, but they still lack the potential for generating lymphoid cells as well as for self-renewal. It is not until in the third wave, at E10.5 in mice, that the first definitive HSCs (transplantable with multi-lineage potential) emerge from a precursor in the endothelium, in a process

called endothelial-to-hematopoietic transition (EHT) [51; 52]. The process takes place in the dorsal floor of the aorta-gonads-mesonephros (AGM) region [49; 53]. In addition, it was also shown that HSCs emerge in the placenta [54; 55] and yolk sac [56]. At E11 in mice, HSCs migrate to the fetal liver (FL) [36; 57; 58]. This is where the HSCs mature and dramatically expand to produce sufficient numbers of HSCs to sustain adult hematopoiesis throughout the entire life span [36; 59] before they migrate to the BM, at E16.5 [60], which becomes the main site of hematopoiesis in adulthood. Four weeks after birth and onwards, HSCs become more and more quiescent [61] and the switch from fetal to adult hematopoiesis takes place [62-64].

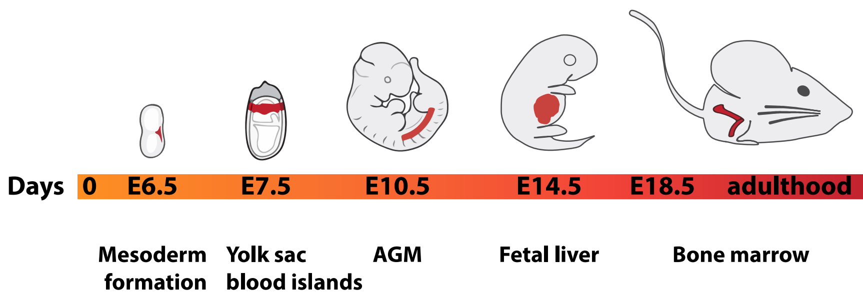


Figure 3. Hematopoietic ontogeny
Schematic overview of early hematopoietic development in the murine embryo over time. E (embryonic day), AGM (aorta-gonad-mesonephros).

Understanding the mechanism and the signalling pathways regulating the emergence of HSCs has been a long-standing goal in order to generate HSCs *de novo* from pluripotent stem cells. Many pathways, such as Notch, BMP, Wnt, Hedgehog as well as inflammatory signals, has shown to play a role, but how they are co-ordinated in the EHT is as yet not fully understood [65; 66].

The HSC Niche

The first concept of the HSC niche was introduced by Schofield in 1978, who posited that a niche is a specialized microenvironment which surrounds HSCs with different kinds of supportive cells within the BM [67]. In humans, the HSC niche is represented by a number of different cells and proteins [68; 69]. The function of the niche is to promote HSCs' survival and self-renewal, as well as to regulate their proliferation, migration and differentiation [67]. It includes all the factors that are necessary for HSC maintenance *in vivo* [67; 70-72].

Extrinsic HSC regulation by the niche

The environment surrounding HSCs in the BM is hypoxic (has less oxygen) in order to protect HSCs from damage induced by reactive oxygen species (ROS) [73-75]. It is important since HSCs interact with their microenvironment to maintain normal hematopoiesis and to control their fate, which is partly determined by complex bidirectional interaction with various cell types within the niche. In addition, the interaction between HSC and the niche is crucial for the regulation of HSCs to migrate in (homing) and out (mobilization) of their niches [76; 77]. There are several adhesion molecules and receptors mediating cell-cell and cell-matrix interaction to retain the HSCs in their niche.

Initially, two types of HSC niches in the BM have been proposed, although the subject is still controversial. It was suggested that the endosteal or osteoblastic niche is supporting HSCs' quiescence, and a perivascular niche supports HSCs' division and/or differentiation. Both niche locations were shown to maintain the proper HSC function *in vivo* [78].

With the development of new technologies, recent data indicate that the majority of HSCs reside in perivascular niches that are mainly associated with sinusoidal blood vessels in adult BM [79]. Still, several types of cells have been described to be a part of the HSC niche (**Figure 4**), such as osteoblasts (OB), osteoclasts (OC), endothelial cells, mesenchymal stromal cells, adipocytes, neurons, Schwann cells, megakaryocyte and monocytes, as well as the extracellular matrix [68; 69; 79].

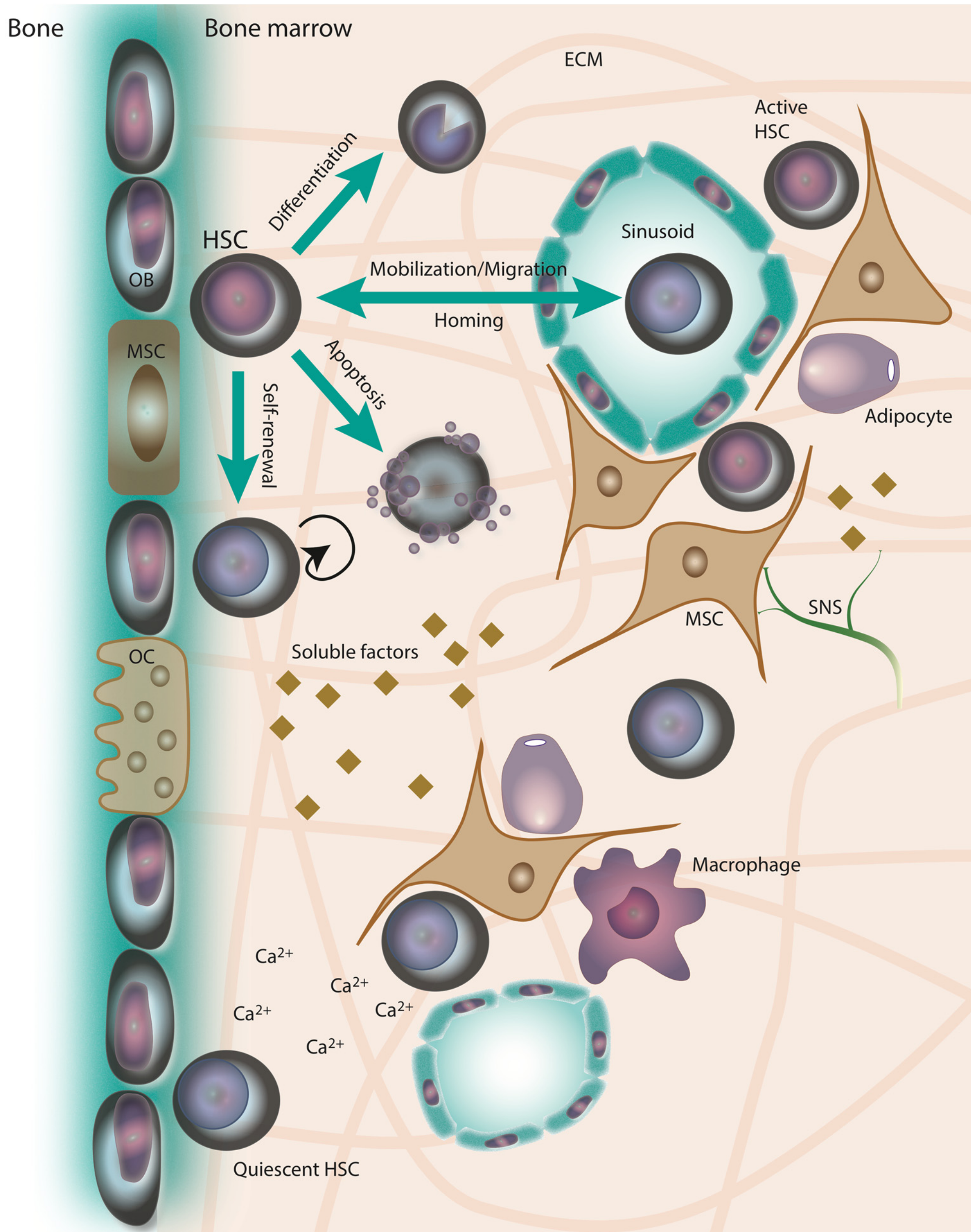


Figure 4. The hematopoietic niche
Schematic overview of HSC fate decisions in the niche. Quiescent HSC residue in the endosteal niche, close to the bone lining, active HSCs are in close contact with vascular sinusoids. HSC (hematopoietic stem cells), ECM (extracellular matrix), MSC (mesenchymal stem cells), OB (osteoblast), OC (osteoclast), SNS (sympathetic nervous system).

Several HSC regulatory factors have been identified within the niche, such as angiopoietin1 (ANG1), known to be expressed by OB, binding to the TIE2 receptor expressed on HSCs, which promotes HSCs' quiescence [80; 81]. It was also shown that an increased number of OBs correlates with increased HSC numbers [81; 82]. However, ablation of the OBs had no immediate effect on the HSC frequency [83].

Another important regulator is the stem cell factor (SCF), expressed on vascular endothelial cells, which binds to c-kit receptor on HSCs [84]. It contributes to self-renewal and maintenance of HSCs *in vivo*, and knockout studies report a reduction of HSC numbers [85; 86]. Also, the SCF, together with chemo-attractant stromal derived factor 1 (SDF-1/CXCL12), are important for HSC migration [87; 88]. SDF-1 interacts with the CXCR4 receptor expressed on HSCs during homing after transplantations [89-91].

Mesenchymal stroma cells (MSC) express high levels of SDF-1 [89; 92]; it was identified that *ex vivo* stimulated HSCs show a higher affinity to MSCs [93; 94], which suggests that adhesion molecules, such as integrins, fibronectin-1 (FN1), vascular cell adhesion molecule-1 (VCAM-1), and SDF-1 regulate the HSCs interacting with their niche.

It was also shown that the deletion of integrins in HSCs restricts their competitive reconstitution ability and self-renewal after transplantation [95]. In particular, integrin β1, was shown by knockout studies in mice to have a role in HSC engraftment *in vivo* [96; 97]. Similarly, it was shown on human HSCs *in vitro* that using a blocking antibody for integrin β1 significantly reduced the HSC adhesion capacity to the stroma cell [98].

HSC adhesion regulators

The family of known HSC adhesion molecules includes integrins, specifically α4β1, very late antigen-4 (VLA-4) in association with VCAM-1 [93]. It has been shown that integrins are essential for engraftment after transplantations [95].

Integrins are synthesized as an 86 kDa polypeptide followed by glycosylation. The mature form of Integrin β1 (CD29) has a mass of 116 kDa, whereas the non-mature one has a mass of 140 kDa. Cell surface expression of mature integrin β1 is important for cell-cell interactions within the niche. In its absence, HSCs are released from their niches. The immature form of CD29 does not play a role in cell adhesion since it is not expressed on the cell surface. Instead, it may work as a reservoir that can be rapidly transferred to the cell surface if needed [99; 100]. Several adhesion molecules belonging to integrin-signaling pathways are expressed by stroma cells known to support HSCs [98]. The fact that primitive

stem cells express high levels of integrin β1 [101] suggests that CD29 is one of the most extensive adhesion receptors to the niche.

Medical application of HSC – niche interaction

The limited number of stem cells isolated from the donor samples, especially from umbilical cord blood (UCB), is the major reason for developing strategies to expand HSCs *in vitro*. It would improve the use of UCB transplantation, and also increase the chances for gene therapy and gene editing to correct genetic disorders. A different strategy would be to enhance the capacity of transplanted HSCs to engraft. The latter gives hope for successful transplantations despite the low number of required donor cells. During the *ex vivo* incubation of HSCs, additional growth factors and cytokines meant to stimulate proliferation are required due to the lack of BM niche components such as stromal cells [102; 103].

The engraftment of injected HSCs in the BM can be divided into three phases [104]. Homing, as a first step, which is the migration from the blood stream into the marrow. Next, lodgment, which involves selective navigation within the marrow towards HSC specific niches. In this second phase, HSCs migrate along two specific gradients where the concentration of ionic calcium (Ca^{2+}) and oxygen tension plays a very important role [105]. In the last phase, the HSCs respond to signals within the niche to reconstitute the hematopoietic system and return to quiescence.

Intrinsic regulation of hematopoiesis

Hematopoiesis is strictly regulated by extrinsic elements such as epigenetic regulators, and intrinsic elements such as transcription factors (TF). They regulate the cells' fate by either enhancing or diminishing specific cell functions.

Transcription factors

TFs are known to bind to specific DNA sequences to control gene transcription, functioning either alone or with other proteins in a complex, by blocking or promoting (repressing or activating) the recruitment of RNA polymerase. By binding to either a promoter or an enhancer region of the DNA, TFs can either up- or down regulate the transcription of specific genes. Many TFs are involved in the development of organisms or in the regulation of the cell cycle. For instance, MYC oncogene regulates cell growth and apoptosis. Furthermore, TFs have been shown to regulate the capacity of HSCs to proliferate and self-renew in order to maintain the HSC pool and to respond to external cues to activate the differentiation program into the committed hematopoietic cell. Additionally, TFs regulate apoptosis, a process necessary to prevent the HSC population expanding to a physiologically dangerous size and to prevent leukemogenesis.

By using gene targeting approaches, many TFs have been identified as important regulators of HSCs [34]. For example, RUNX1 [106; 107], SCL [108; 109], GATA2 [110; 111] and LMO2 [112] were all found to be critical to establish hematopoiesis and HSCs during development.

Other TFs such as GFI-1 [113; 114] and MEIS1 [115; 116] have been identified to be important regulators of HSC quiescence, and their deficiency resulted in increased HSCs cycling, which subsequently led to reduced reconstitution potential. Additionally, transcriptional repressors BMI1 [117; 118], RAE28 [119] or IRF2, generally known to regulate inflammatory responses [120], were all identified to determine HSC proliferation and their self-renewal capacity.

HOX genes are the group of TFs known in regulating mammalian embryogenesis [121] and have been shown to be expressed throughout hematopoiesis [122]. Indeed, several studied have identified HOXA9, HOX10 and HOXA5 as positive

regulators of HSC proliferation [123-126]. Interestingly, an ectopic expression of HOXB4 in mouse HSCs resulted in a 40-fold increase in HSC numbers [127; 128].

TFs have also been shown to control the HSC pool size by regulating HSC apoptosis. Studies using a conditional knockout mouse model of MCL1 resulted in cell death and depletion of the HSC pool [129], while overexpression of BCL2 clearly improved HSC survival in mice [130].

In addition, some TFs have also been shown to regulate the movement of HSCs out of their niches. For example, cMYC and EGR1 were shown to be important in regulating the release of HSCs from the BM niche [131; 132]

Other TFs have been shown to be specific for controlling HSCs differentiation. PAX5 was shown to be essential for B cells development [133], GATA3 for T cell lineage restriction [134] and GATA1 for erythroid commitment [135], whereas CEBPα for the myeloid lineage [136].

Epigenetic regulators

Epigenetics is a broad field which regulates changes in overall DNA and chromatin structure without affecting the DNA sequence itself and is important for maintaining cell identity [137]. The field started to rapidly grow with the work of Shinya Yamanaka, who was a pioneer in the reprograming of somatic cells and in 2012 was awarded the Nobel Prize for his research [138]. Yamanaka and co-workers showed that an overexpression of four embryonic stem cell TFs – OCT4, SOX2, KLF4 and cMYC, could re-specify somatic fibroblast cells into pluripotent stem cells with the potential to differentiate into all other cell types [139]. A year later the team generated human induced pluripotent stem cells (iPS) [140]. In the same year, it was demonstrated in mice that iPS cells can be used for autologous correction of anemia [141]. By the end of 2008, iPS cells were generated from patients with many genetic diseases, including Gaucher disease, Parkinson disease or Down syndrome [142].

Epigenetic modifiers have also been proposed to play a major role in HSC regulation [143]. Studies have shown that overexpression of EZH2, a histone methyltransferase and part of the PRC2 complex, resulted in increased HSC numbers [144]. In addition, Wang and co-workers demonstrated that Histone H2A Deubiquitinase MYSM1 is required to maintain the quiescence and pool size of HSC by regulating GFI [145]. Furthermore, the work of Goodell's group on the methyltransferases DNMT3a and DNMT3b demonstrated the importance of these epigenetic regulators in regulating HSC fate decisions [146; 147].

Reprogramming of iPS cells to HSCs is a very promising tool for regenerative medicine. However, the cells produced so far still lack efficient engraftment capacity [148]. Nevertheless, the field has opened up many new possibilities and currently many studies focus on epigenetic patterns to achieve successful reprogramming of somatic cells to a pluripotent state [149; 150]. For example, studies from Rossi's lab, using the combination of RUNX1T1, HLF, LMO2, PRDM5, PBX1, and ZFP37, successfully reprogrammed mature blood cells into HSC-like cells *in vivo* [151]. Also, Daley's lab recently published a paper demonstrating for the first time the generation of transplantable HSC-like cells from human embryonic stem cells (ESC) by overexpressing ERG, HOXA5, HOXA9, HOXA10, LCOR, RUNX1 and SPI1 [152]. Reprogramming is a very powerful tool for medicine, but it is still highly controversial due to safety concerns and certainly needs to be studied further.

Aging

Since the beginning of mankind, humans have been looking for possibilities to stay young forever. However, nature is cruel and offers us an unstoppable, mysterious process of aging. Aging is a time-dependent, slow process of malfunction of organismal functions. It is associated with a decrease in the quality of life owing to reduced capacity in maintaining normal hemostasis, leading to a gradual failure of tissues and organs. In addition, tissues and organs display difficulties in returning to normal conditions after stress and have a higher risk of disease such as cancer [153].

In the 1950s, it was proposed that aging is a process of accumulated mutations [154]. Later, theory proposed that it is related to pleiotropic genes, advantageous at early life, but disadvantageous with age [155]. Aging is related to both, intrinsic (metabolic and replicative stress) [156] as well as extrinsic factors (aging environment within the niche and radiation) [157]. Natural selection wants organisms to be fit for reproduction, and once this process is over, it is no longer beneficial to keep them fit anymore [157].

As the organism ages, the hematopoietic system becomes skewed towards myeloid cells [158]. Transplantation experiments showed a myeloid skewing reconstitution pattern in favour over the lymphoid compartment [158-161]. This phenomenon was not observed when transplanting adult HSCs into an aged recipient, indicating that myeloid skewing is intrinsic to aged HSCs [158]. It was also confirmed transcriptionally, as aged HSCs express increased levels of myeloid regulators [158] as well as have an increased frequency of myeloid progenitors, resulting in reduced numbers of lymphoid precursors [158; 162; 163] as shown in **Figure 5**. Several studies have reported on epigenetic changes in aged HSCs that may contribute to the aging phenotype such as altered DNA methylation of genes regulating HSC differentiation and maintenance [164].

Additionally, aging is also associated with a significant increase in HSC frequency, [158; 159; 165-168] presented in **Figure 5.** Although the HSC pool increases, the cells remain quiescent and perform worse in competitive transplantation settings on comparison to adult HSCs [158; 160; 161; 168], indicating that the increased number of HSCs may be a way to compensate for the reduced function. The reduced function can be explained by several mechanisms, such as for example accumulated DNA damage due to a compromised

DNA damage response in aged HSCs [169]. However, recent data demonstrated that aged HSCs repair the DNA damage as efficiently as adult HSCs [170]. Another explanation could be that aged HSCs have higher levels of ROS than adult HSCs [171]. This is interesting, since HSCs with low ROS levels have increased the reconstitution capacity and may therefore explain the reduced function in aged HSCs [172]. Nevertheless, further studies need to be undertaken to further clarify the role of ROS in the aging of HSCs.

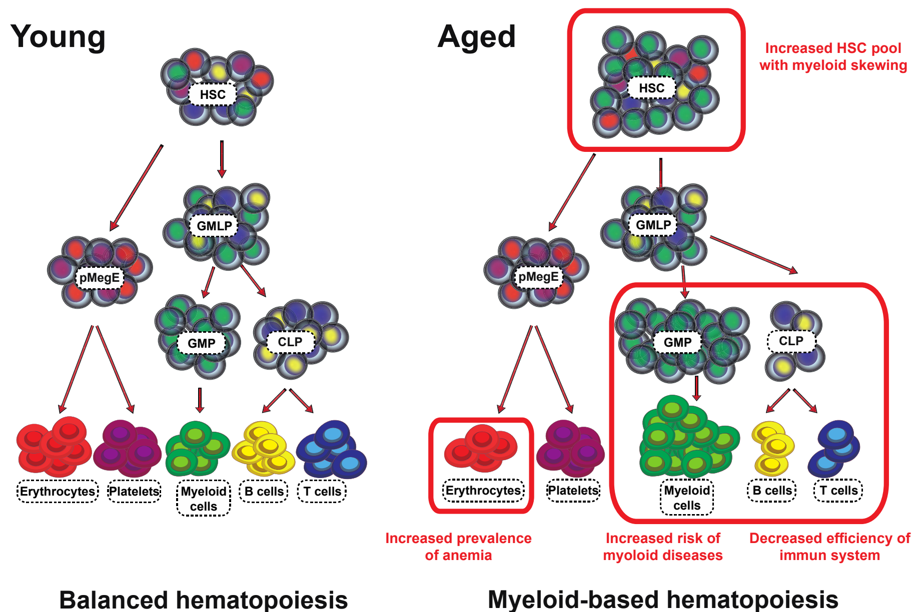


Figure 5. Age-related alteration of hematopoiesis
Simplified schematic overview of young vs aged modification in the hematopoietic system. HSC (hematopoietic stem cell), GMLP (granulocyte-monocyte-lymphoid progenitor), pMegE (pre-megakaryocyte and erythroid), GMP (granulocyte/macrophage progenitor), CLP (common lymphoid progenitor).

Interestingly, thanks to measuring the telomere length, it might be possible to track the division memory of each cell and provide a molecular explanation to the mitotic clock since with every division of the cell the telomere gets shortened [173; 174].

To sum up, aging causes an alteration in the HSC pool that results in a changed composition of the hematopoietic system. It is important to dissect the mechanism regulating aging in order to better understand age-related diseases.

The circadian rhythm

The circadian system, which is composed of many individual tissue-specific clocks, ensures that the organism can adapt to environmental changes. The molecular clock was first identified in the 1970s in *Drosophila* [175] and later found to be important in the mammalian systems as well [176]. It became now a hot subject, as the Nobel Prize in 2017 in medicine was awarded for the discovery of the molecular mechanism controlling the circadian rhythm [177]. Sleep and activity cycles, heartbeat frequency, body temperature, blood pressure, endocrine functions, renal plasma flow, intestinal peristalsis, metabolism, and other physiological aspects as well as behaviours of an organism are all controlled by a timekeeping system to maintain the homeostasis of the body [178; 179].

Using the circadian clock fluorescent reporter mouse model, it was shown that the dormant stem cell niche in the skin contains two populations of stem cells at opposite phases of the clock. Unbalancing this equilibrium of active stem cells, through the conditional deletion of a core clock component, resulted in long-term, progressive accumulation of non-responsive stem cells, premature impairment of tissue self-renewal and a significant reduction in the development of squamous cell carcinomas [180].

Although it is already known that the hematopoietic system is affected by circadian rhythms [181; 182], the regulation of stem cells by clock genes is not fully understood. The role of molecular clocks in the HSC function during homeostasis, regeneration, aging and cancer may have a significant influence in the regulation of hematopoietic mechanisms.

Interestingly, a study from the Franette's lab has indicated that hematopoietic progenitor cells follow a physiological rhythmic release from the niche to the blood stream, regulated by CXCL12 – a chemokine previously reported to be responsible for the migration of HSCs [87]. The rhythmic HSC release may be one way to improve the yield of harvested stem cells, which is of importance in clinical transplantations.

The molecular work of the circadian clock is based on transcriptional-translational feedback loops with use of transcriptional activators CLOCK, BMAL1 and NPAS2 as well as negative acting elements [183]. Interestingly, the proline- and acidic amino acid- rich basic leucine zipper (PAR bZip) protein family members

D box binding protein (DBP), thyrotroph embryonic factor (TEF) and hepatic leukemia factor (HLF), are directly regulated by the clock gene and expressed in a circadian pattern in the liver [184]. Strikingly, mice which are deficient of all three family members, died from epileptic seizure possibly due to an altered circadian rhythm.

It has also been suggested that the core clock genes are involved in the development of cancer. Ebert's group recently found that BMAL1 and CLOCK is expressed on leukemic stem cells (LSC) and necessary for the growth of the AML [185], demonstrating the importance of a correctly regulated circadian program in normal and malignant stem cells.

Methods to study HSCs

One of the most powerful methods that have changed the entire field of hematopoiesis was the discovery of fluorescence-activated cell sorting (FACS). This potent method combines physical properties of the cell, like size and granulocity, with using cell-specific surface antibodies that detect, bind and label cell antigens with conjugated fluorescent markers [7; 186].

Isolation of HSCs

HSCs are an extremely rare cell population estimated to represent approximately 0.004% of the total BM [22]. Up to this day, there is no single cell surface marker to isolate HSCs, but a combination of different surface markers has to be used instead in order to identify this rare population within the BM. The cell surface proteins used for isolating HSCs are different for humans and mice, as well as for adults and foetuses, however they all do not express specific lineage surface markers. Murine adult HSCs express stem cell antigen-1 (*Sca-1*) and tyrosine kinase receptor (*cKit*). The population Lin^- $Sca1^+$ $cKit^+$ (LSK) is quite heterogeneous and contains both HSCs as well as progenitor cells [20; 186-188]. It is estimated that, within the LSK population, about one in thirty cells is an actual stem cell. Therefore, to further purify HSCs from the LSK population, additional surface markers have to be used. The first successful protocol identifying HSCs within the LSK population used the CD34 and Flt3 markers (LSK $CD34^-$ $Flt3^-$) where HSCs lack both the markers [22; 189]. However, a more recent protocol uses the signaling lymphocyte activation marker (SLAM) receptors to isolate HSCs. HSCs were found to possess the CD150 marker and lack the CD48 [70]. At the moment, the best purification can be done by combining all the antibodies together (LSK $CD34^-$ $Flt3^-$ $CD48^-$ $CD150^+$), which results in a very high purity of transplantable HSCs at a single cell level [190].

Additionally, HSCs have been shown to express highly conserved membrane pumps that are known to be expressed on stem cells and have the unique capacity to efflux drugs and chemical agents [191; 192]. This activity is illustrated by the DNA dye Hoechst 33342 and mitochondrial binding dye Rhodamine 123 [191]. Analyzing the level of Hoechst 33342 with two-emission wavelength

simultaneously allows to purify HSCs by identifying the so-called side population [193]. Additionally, several other studies have identified cell surface proteins to decrease the heterogeneity of the LSK population [194] such as CD201, CD11a, CD9 or ESAM1 [195-198], but so far there is no better, universal strategy to isolate murine HSCs.

Human HSCs still cannot be as purely isolated as murine HSCs. One of the commonly used cell surface markers to enrich for human HSC and progenitor cells is CD34, originally called My-10 and identified already back in the 1980s [199]. In contrast to adult murine HSCs that lack CD34, human HSCs and progenitors highly express CD34. However, this population is still highly heterogeneous, which explains a number of current studies that have been trying to further purify human HSCs. It has been found that CD38 is expressed on progenitors, but not on HSCs, so the exclusion of this marker substantially enriches for human HSCs [200]. More recently, it was found that with the addition of CD90 and CD49f surface markers ($CD34^+$ $CD38^-$ $CD90^+$ $CD49f^+$) as much as 1 in 10 cells, could engraft and reconstitute human hematopoiesis in the long term [201].

Although a lot of research has been done to identify the best markers for mouse and human HSCs, this subject still remains controversial and highly interesting.

Functional studies of HSCs

Only immunophenotypical properties of HSCs have been described above, but the true HSC function can only be confirmed functionally by the reconstitution of the hematopoietic system in a new host after transplantation.

HSCs transplantations

Prior to transplantation, the recipient will require conditioning with irradiation or meyoblation to ensure space for the transplanted cells [202] (**Figure 6**). The recipient will – if not transplanted shortly after irradiation – suffer from internal bleeding as the lethal dose of irradiation will eradicate all blood cells, including platelets [203]. After transplantation, a process called homing occurs, when the donor's HSCs migrate to their niche in the BM. The two most common transplantation approaches for evaluating the HSC function include either transplanting the unfractionated BM containing all cells including HSCs, or transplanting sorted HSCs mixed with support cells [59; 204].

When cells are transplanted in competitive settings, HSCs are challenged to compete for their space in the niche [205]. This kind of competition challenges

HSCs and helps to evaluate the long-term and multilineage reconstitution potential of donor cells against a fixed number of co-transplanted cells. The multilineage capability of HSCs can be detected after 12 weeks from the transplantation day. To read out the specific effect of HSCs and not only progenitors, BM is isolated and analysed 16 weeks post transplant [16].

It has been well documented that HSCs are required for long-term survival [10], but they are not able to produce mature blood cells rapidly enough to ensure the recipient's survival. This is why, in order to maintain sufficient levels of specified hematopoietic cells, purified HSCs need to be supported with co-transplantation of another donor's mature blood cells.

To truly prove their self-renewal capability, HSCs needs to be successfully transplanted into a secondary host. Distinguishing the donor cells from competitor/support or remaining recipient cells can be achieved through the use of congenic mice that are genetically identical except one locus, Ly5/CD45.1 or Ly5/CD45.2 [206]. These different isoforms can be then distinguished using FACSs. However, CD45 is only expressed on leukocytes, which is why the chimerism of erythrocytes and platelets is neglected.

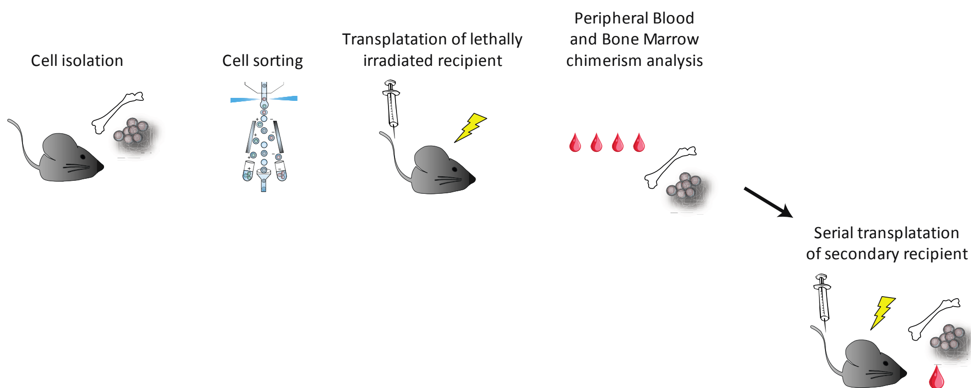


Figure 6. Experimental settings
Schematic overview on HSC transplantations followed by evaluation of their key characteristics – long-term multilineage potential (chimerism analysis) and self-renewal (secondary transplantation).

To estimate the frequency/number of functional HSCs, so-called competitive repopulating unit (CRU), donor cells are transplanted in a limited dilution together with a fixed number of competitor cells. Then, after 16 weeks, mice are scored as positive for the engraftment if the chimerism of the donor cells is higher than 1% in both myeloid and lymphoid cells [207]. The frequency of CRUs can be

calculated using the online mathematic formula for extreme limiting dilution analysis (ELDA) [208].

Nowadays, new technologies such as "barcoding" of HSCs allow for a better evaluation of the reconstitution capacity from a single cell [209; 210] and are a new hope in order to study HSC reconstitution patterns at single cell resolution.

The mouse as a model organism for studying hematopoiesis

The house mouse, *Mus Musculus*, has long served as an experimental model of human biology and disease because of its phylogenetic resemblance and physiological similarity to humans. Genomic studies have shown striking genetic homologies between the two species [211]. Along with that, the ease of maintaining and breeding these mice in the laboratory setup makes it possible to study diseases and complex physiological processes *in vivo*. The mouse genome can be relatively easy to manipulate to induce diseases that do not naturally develop in mice. Presently, thousands of inbred strains and genetically engineered mice exist for either study the gain of function (knock-in model) or to study the loss of function (knock-out model) [212]. Specifically, to study hematopoiesis, mouse models have been an inimitable tool for defining characteristic stem cells and define the hierarchy based on the capacity for long-term repopulation in a recipient.

Knock-in mouse models

To appraise the role of a "novel" gene in the hematopoietic system, the overexpression of a gene of interest is a valuable method. This can be achieved by using lentiviral vectors to introduce a stable integration of a transgene into the genome of a HSC, which results in the transgene being overexpressed in every progeny of the entire hematopoietic system [213]. However, the use of strong transgene promoters can cause a massive increase of the transgene product. This is one of the major drawbacks of the knock-in system as it could lead to non-physiologically high levels of the protein being produced, which could result in effects unrelated to the normal function of the protein.

Knock-out mouse models

Approaches to evaluate the loss-of-function of a gene include the use of knockdown or knockout (KO) mice, which can provide valuable information regarding the normal function of a gene. Conventional KO models are built based on the functional elimination of the targeted gene in every cell of the mouse, whereas conditional KO models permit for a tissue-specific gene deletion and chronological control of induction. In the scenario where the gene of interest is indispensable during development, a conventional KO model leads to embryonic

lethality; however, a conditional KO approach based on inducible Cre-loxP can be used instead. The system allows studies of the loss-of-gene function in an adult system. The Cre-loxP technique is constructed on the specific excision of DNA segments, flanked by two short sequences known as loxP sites, by the induction of Cre recombinase [214].

Xenograft models

Due to variances in mouse and human biology, human studies corresponding to mouse experiments are required to develop relevant therapies. The 1980s made a new beginning for human hematopoiesis in the functional studies, when the humanized mouse model was developed [215], allowing injections of human hematopoietic cells into the immunodeficient mouse model in *in vivo* xenograft assays. The method eliminates such problems as immune rejection due to species differences. The first xenograft mouse model was the severe combined immune-deficient (SCID) mouse model, deficient in B cells and T cells. To support higher levels of human engraftment, it was later crossed with the non-obese diabetic (NOD) mouse model [216; 217]. The major drawback of the NOD-SCID mouse model is that mice have a relatively short life span and the residual of NK-cells limit the human engraftment potential, making it difficult to evaluate long-term HSC engraftment [217]. To avoid these problems, the NOD-SCID mice strain was crossed with NSG mice that lack the IL-2 receptor common γ chain (IL2RG), which leads to the loss of B, T and NK cell activity and thus enhanced levels of human engraftment [217-219].

RNA interference assay

RNA interference (RNAi) is a natural mechanism occurring in post-transcriptional gene silencing. The discovery of the gene silencing mechanism opened the possibility for a selective knockdown of molecular targets that are essential for individual effective treatments by target specific RNAi agents.

The application of RNAi involves two types of molecules: the chemically synthesized double-stranded small interfering RNA (siRNA) and the vector-based short hairpin RNA (shRNA) [220]. Both approaches can be applied to achieve similar functional effects. siRNA and shRNA have a different life span within the cell and only shRNA can give a stable knockdown. Also, the efficiency of siRNA is ten times lower than that of primary transcribed shRNA [220].

The biggest disadvantage of using RNAi is its unintended effects on gene expression, so-called off-target effects. Specific off-target silencing is induced by a partially complementary sequence of the RNAi construct to mRNAs other than the designated target. Unlike specific off-target silencing, the nonspecific

off-target effects are induced by a wide range of immune and toxicity related effects, which are fundamental to the RNAi construct itself or to its delivery vehicle and not the result of the direct interaction between the RNAi construct and an mRNA transcript [221].

Lentiviral vector as a tool to deliver shRNA

The lentivirus vector can introduce new genetic material into cells, such as shRNA, without any negative side effects caused by the integration [222]. The main advantage of lentiviral vectors over gammaretrovirus is that they can infect non-cycling cells such as HSCs. The negative regulation of a gene by shRNA is induced through post-transcriptional gene silencing by the degradation of sequence-specific mRNA [223].

Lentiviral vector is derived from the human immunodeficiency virus-1 (HIV-1) vector pseudotyped with vesicular somatic virus glycoprotein G (VSV-G). Lentivirus can easily transduce HSCs without influencing their fundamental properties [224]. The HIV-1 vector cassette is based on the strategy of segregating the cis-elements in the HIV-1 genome, which are important for its packaging, reverse transcription, import and integration. Unlike the vector cassette, the packaging cassette encodes trans-elements of the vector resulting in the production of the vector in target cells as well as efficient infection of host cells. The envelope cassette, VSV-G, can enter a large variety of targets due to the omnipresence of the VSV-G phospholipic receptor, improving its ability to infect. The other function of the envelope protein is stabilization of vector particles from shear stress during concentration. The separation of the envelope-encoding sequence from the package and the vector improves safety issues in the use of lentivirus to infect cells [224; 225].

In the HSC field, the use of the shRNA library in integrating viral vectors for RNAi screens is a promising mechanism for the analysis of the negative regulation of engraftment. However, a low rate of false-positive results can still be observed [226]. This is an extremely powerful method, but it cannot be considered as an absolute indicator, but rather as a guide about the relative potency of transduced populations. For functional validations, the experiments with individual shRNAs are obligatory. Nevertheless, several large studies have demonstrated the power of using RNAi screens to identify novel regulators of normal and malignant stem cell regulation in hematopoiesis [222; 227-229].

Treatment

HSC transplantations

HSCs transplantation is the only routinely performed stem cell therapy and has been clinically used for more than five decades to treat hematopoietic malignancies like leukemia, lymphoma and genetic blood disorders. In a similar manner in which mice studies are conducted, patients need to be conditioned with radio- or/and chemotherapy prior to transplantations to eradicate the malignant cells and to ensure space for the new coming cells in order to rebuild the patient's blood system with healthy cells [202].

Although the first successful human BM transplantations were achieved in 1959 [11], it was not until our understanding of the importance of the human leukocyte antigen (HLA) that transplantations became a safe and viable option [230; 231]. HLA, as the name indicates, is expressed on white blood cells, specifically T cells. HLA matching between the recipient and the donor is crucial for successful transplantations as they are specific for each individual and immune system which uses them to determinate self-cells. This is why the graft-versus-host-disease (GVHD) is one of the major limitations in allogenic transplantations [202].

There are three sources for getting HSCs for transplantations in humans. The most classical practice is the aspiration of the BM; the most common nowadays is the mobilization of HSCs to enter peripheral blood (PB) and harvest HSCs from there; and lastly, the third and the newest and the most potent source is UCB. Unfortunately, due to the lack of HLA-matched BM donors or due to the low yield of HSCs – especially in UCB – not all patients that would benefit from this therapy can be treated. A long-standing goal in the field has been to establish protocols that would allow for the expansion of HSC in culture. Efforts to expand self-renewing human HSC that can engraft efficiently have so far been unsuccessful due to our limited understanding of their self-renewal mechanisms. Nevertheless, several seminal studies have identified crucial TFs such as RUNX1, MLL1, TFIIS, GATA2, NOTCH1, MEIS1, ERG, cMYB and cMYC (reviewed by Wilkinson and Gottgends, 2013 [232]), that regulate HSC fate options.

Medical use of HSCs

The most common reasons for hematopoietic cell transplantations are diseases of immunodeficiencies and malignancies. One of the approaches is the autologous transplantation of HSCs from the ill patient's BM. Own HSCs are first isolated and then highly enriched and transplanted back to the patient for reconstituting hematopoiesis. Unlike autologous graft, allogeneic transplantations use cells obtained from an HLA-matched donor, which is effective for cancer treatment [233].

Both types of HSC transplantations are in clinical use and, unfortunately, both still have some limitations. Many patients cannot benefit from the treatment due to a deficiency in stem cells for autologous transplantations or due to a lack of appropriate HLA-compatible donors for allogeneic transfers [234; 235].

An alternative method to treat hematopoietic diseases is the UCB transplantation. HSCs isolated from UCB represent a less mature phenotype than those in the BM and PB [236]. UCB has a high degree of haplotype diversity, but the number of harvested cells is insufficient for adults [237]. Rocha and colleagues has shown that, for hematopoiesis transplantations in children, UCB is a sufficient source of HSCs. Even if the reconstitution after UCB transplantation is slower than HLA-matches in the marrow from a sibling or an unrelated donor, the risk of the incidence of the acute and chronic GVHD is lower [238]. The safety of transplantation with the use of HLA-mismatched UCB from unrelated donors was tested and showed that the incidence and severity of GVHD was low regardless of HLA mismatching [235]. The biggest disadvantage in the use of UCB is the low number of harvested HSCs available to reconstitute the blood system of adult patients [237]. As a consequence, this type of transplantation remains significantly more successful for children. To increase the efficacy of UCB *in vitro*, expansion strategies are currently being developed [102]; they focus on generating a sufficient number of HSCs. Alternatively, modulation of adhesion molecules can improve the homing of HSC to their microenvironment in the BM. Combining the two strategies should ensure the best engraftment efficiency.

Gene therapy

The notion of gene therapy encompasses the correction of the disease process by restoring or modifying cellular functions; it is an especially promising alternative of treatment for monogenic disorders [239]. First, stem cells are isolated from a patient and then transduced with viral vectors carrying the

corrected gene. After the gene has been successfully corrected, the cells are re-injected into the patient [240].

The first clinical gene therapy trial was performed almost 18 years ago on patients with severe combined immunodeficiency, by transducing the patient HSCs with a gamma-retroviral vector [241]. The gamma-retroviral vector successfully incorporated the transgene into the host genome. However, 3 out of 11 patients developed leukemia due to insertional mutagenesis of the initial vector integration [242]. In comparison to gamma-retroviral vectors, lentiviral vectors exhibit a significantly lower probability to insert into proto-oncogenes and are considered safer [242; 243]. So far, no adverse side effects regarding genotoxicity have been recorded using lentiviral vectors. Therefore, they have been successfully applied in the clinics [244; 245].

Recent pre-clinical studies using the mouse model for the Wiscott-Aldrich syndrome, Gaucher type I and Diamond-Blackfan anemia using third generation self-inactivating vectors with cellular promoters, proved to be safer and without compromising the efficacy [246-248]. However, the struggle of the field to develop safer vectors and methods for targeted gene delivery is still under way [249].

If you know you are on the right track,
if you have this inner knowledge,
then nobody can turn you off…
no matter what they say.

– Barbara McClintock

Background to the articles

Despite intense research and more than five decades of clinical use as treatment of leukemia and genetic blood disorders, we still lack information about the mechanisms regulating HSCs to further improve their therapeutic potential. Finding the critical elements regulating the HSC fate will therefore be important in order to successfully expand HSC *ex vivo*, but also for understanding the initiation and progression of leukemia, especially as the LSC known to drive the disease, share the HSCs' capacity for self-renewal. Therefore, to successfully develop novel therapeutic strategies specifically targeting LSC, we would need to identify specific differences. Interestingly, global transcriptional profiling of HSC as well as LSC has identified HLF as a potent regulator of somatic stem cell function [250-252].

Hepatic Leukemia Factor

HLF is a member of the PAR bZip protein family, together with – DBP and TEF [184]. These proteins contain an *N-terminal* transcriptional activation domain, a *C-terminal* basic DNA binding domain and a leucine zipper dimerization domain used to form homo-/hetero-dimers with other bZips before DNA binding [253-255]. HLF, as many other known HSC regulators, was initially identified in leukemia as part of a translocation with E2A (**Figure 7**) [256].

Initially, HLF was found to be expressed in the liver, kidney, lung and the nervous system but was believed to be absent in lymphoid cells and the BM [251; 257]. However, more recent data have shown that HLF has a unique expression pattern in hematopoiesis centered on the stem cells. Importantly, the amino acid sequence of the mouse HLF showed 98% identity to its human homologue, which indicates a highly conserved function [254].

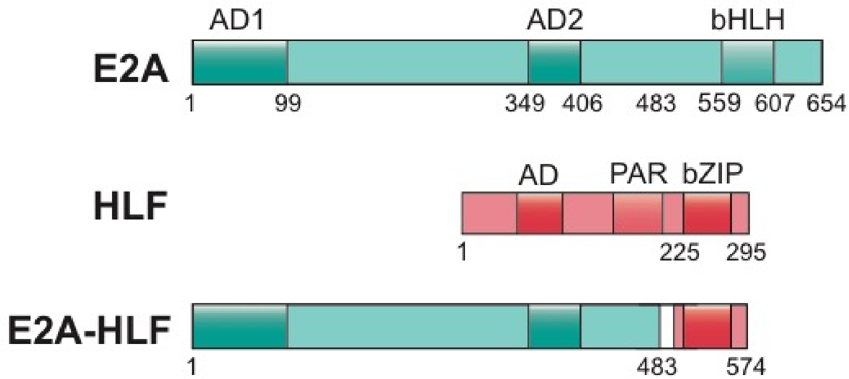


Figure 7. Domain structure of the HLF and its translocation with the E2A gene
The numbers indicate the first and last amino acid positions of the proteins or domains. AD (activation domain), bHLH (basic helix-loop-helix domain), PAR (proline acidic amino acid rich), bZIP (basic leucine zipper) (adapted from LeBrun, 2003 [258])

HLF is the mammalian homologue of the nematode protein *ces-2*, which has been described as regulating cell death of serotoninergic neurons during *Caenorhabditis elegans* development [256; 259] by inhibiting the expression of *ces-1*, a gene that normally inhibits programmed cell death by antagonizing the activity of the proapoptotic factor *egl-1* and, in doing so, induces apoptosis. In contrast to the proapoptotic role of *ces-2*, E2A-HLF blocks apoptosis by inducing the expression of SLUG, a *ces-1* homologue, normally responsible for protecting hematopoietic progenitors from DNA-damage-induced apoptosis [260-262]. Expression of a dominant-negative form of E2A-HLF in t(17;19)-carrying cell lines, blocks E2A-HLF function and results in apoptosis [256]. In addition, the expression of HLF is also aberrant in several cancer forms, and it was shown that the ectopic expression of HLF in epidermal cells and keratinocytes promotes resistance to cell death [263]. Those findings argue that the function of HLF is conserved between cell types and species. Taken together, those data suggest that the inhibition of cell death may be mediated through HLF.

The PARbZIP family

The PARbZIP family has been firmly implicated as circadian-dependant TFs [184], with the expression of HLF cycling with the circadian rhythm [184; 264; 265]. Recent findings have showed the importance of understanding the control of circadian oscillations on both HSCs and LSCs [87; 185] as, clinically, the timing of HSCs harvest or infusion may impact the yield or engraftment and affect the response to chemotherapy [266-268]. As regards circadian-dependent TFs [269], Gachon and colleagues demonstrated that the expression of HLF clearly cycles

with circadian rhythms in the liver and is involved in the regulation of this process [269; 270].

Furthermore, the depletion of all PARbZIP family members resulted in a disrupted neurotransmitter metabolism, and mice became prone to epileptic seizures [184; 271], which correlates with the high expression of these genes in the brain [251]. Also, it was shown that TEF, DBP and HLF have an important role in detoxification of chemotherapeutic agents in the liver and possibly play an important role in cancer treatment regulating the pharmacokinetics of the drugs [264], which may be very important for HSCs as well. However, Article I of this thesis shows that only low levels of *Dbp* and *Tef* mRNA were detected in purified HSCs [272]. Additionally, preliminary findings from Article III identified no phenotypic changes in DBP and TEF KO HSCs in comparison to wild type controls, indicating that DBP and TEF are dispensable for HSC regulation in contrast to HLF.

E2A-HLF fusion protein in leukemia

Leukemia occurs when hematopoietic progenitor cells are unable to respond to differentiation cures, leading to an accumulation of undifferentiated cells that interfere with normal hematopoiesis. Acute leukemia is the most common form of childhood cancer. Childhood leukemia can be divided into two main subgroups based on the affected blood cell type – acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL), with ALL being the most frequent. Normally, this subtype exhibits a high survival rate, but in contrast the E2A-HLF translocations display an extremely poor prognosis. Although relatively rare – approximately 1% of childhood B-precursor ALL [273; 274] – it was reported that, of 14 patients treated with chemotherapy, 13 relapsed or died within 2 years of diagnosis [275]. In addition, this leukemia is refractory to intensive therapy and frequently exhibits hypercalcemia and coagulation abnormalities [275-277]. Chromosomal translocations are a recurrent feature of human hematopoietic malignancies [278], often between TFs in ALL [279]. The t(17;19)-ALL translocation was identified as a fusion between E2A gene, encoding a basic helix loop helix TF on chromosome 19 and HLF on chromosome 17 [254; 280]. The E2A-HLF fusion gene generated by this translocation encodes a chimeric TF in which the *trans*-activation domains of E2A are fused to the DNA-binding domain of HLF [254; 280]. Although HLF and E2A-HLF share the same consensus binding site, studies have shown that they possess different transcriptional activities [281]. The altered transcriptional properties of this chimeric TF have been suggested as perturbing a normal lymphocyte development and rendering lymphocytes susceptible to malignant transformation and the development of ALL [282; 283].

More recently, it has been shown that the E2A-HLF fusion protein acts through BCL2 to immortalize hematopoietic progenitors. Excitingly, Fischer and colleagues demonstrated the clinical application of these findings by successfully targeting the chemoresistant primary E2A-HLF leukemic cells with the selective BCL2 inhibitor Venetoclax [284].

HLF in hematopoiesis

In hematopoiesis, HLF is expressed in HSCs and downregulated upon differentiation in both humans and mice [252; 285]. Global methylome analysis showed that HLF becomes progressively hypermethylated and transcriptionally silenced upon differentiation, which suggests that HLF plays a role in the maintenance of the HSC function and needs to be turned off to allow normal linage commitment [286]. Bryder's group further showed that the failure to downregulate HLF caused a block in lymphopoiesis, which may be a pre-stage to leukemia [285]. The mechanism was partly explained by HLF directly regulating the expression of NFIC.

Interestingly, HLF was also found to be rapidly downregulated upon *in vitro* culture, which may explain the immediate loss of HSC activity in culture [252]. Furthermore, enforced expression of HLF in isolated mouse or human HSCs prolonged their self-renewal capacity *in vitro* [250; 287]. Similar findings were shown *in vivo* by overexpression of HLF in human $CD34^+$ UCB cells, which resulted in an improved engraftment potential of the transduced cells in NOD-SCID mice without altered lineage distribution or indications of leukemia [288]. The improved engraftment was explained by HLF inhibiting apoptosis in human cells. In addition, Gazit and colleagues showed that Lin^-CD150^- cells isolated from the mouse BM became $CD150^+$ upon ectopic expression of HLF and gained multipotent colony potential *in vitro*, indicating that HLF plays a key role in preserving the HSC regulatory program *in vitro* [250]. Those findings are further supported by the crucial contribution of HLF together with 5 other TFs for successful *in vivo* reprogramming of committed lymphoid cells to HSC-like cells [151].

The expression of HLF has also been reported to be upregulated in LSCs, especially in AML with translocations containing HOX genes, indicating that these genes may directly regulate HLF expression [289-291]. Two separate, parallel studies reported on increased levels of HLF upon overexpression of HOXA cluster genes that successfully expanded HSCs *in vitro* [123; 291]. Intriguingly, HOXA10 was shown to directly bind and activate the promotor region of HLF in a luciferase assay [123]. The role of increased HLF levels in LSCs is not clear, but Roychoudhury and colleagues showed that HLF is a key mediator of MEIS1 in maintaining MLL-AF9 induced leukemia [290].

Interestingly, the role of MEIS1 was partly explained by the restriction of oxidative stress that was promoted by HLF. Still, our preliminary data (not reported in this thesis) show that transplanted HLF KO BM cells transduced with MLL-AF9 induced leukemia within the same time frame and frequency as wild type BM cells, demonstrating that HLF is dispensable for the development of MLL-AF9 induced leukemia. Instead, HLF may be an important factor for inducing drug resistance to LSCs by directly regulating the multidrug-resistance gene MDR1 shown in intestinal cells [292], inhibiting apoptosis as shown in E2A-HLF driven ALL [284; 293] or by maintaining the LSC quiescence, as shown in Article I of this thesis, with normal HSCs [272].

Even though HLF expression has been targeted in LSCs using natural compounds such as Cantharidin [289], further studies are needed to decipher HLF mechanisms in LSCs as well as in normal HSCs, in order to evaluate its true therapeutic potential for leukemia and stem cell expansion. Therefore, the main aim of this thesis was to investigate the physiological role of HLF in hematopoiesis and HSC regulation using a conventional HLF KO mouse model.

There is no such thing as a single-issue struggle
because we do not live single-issue lives.
– Audre Lorde

Investigation of this thesis

Hematopoietic stem cell transplantation is a life-saving treatment for many serious diseases because HSCs have the ability to self-renew (generate two identical copies of themselves) and to generate all mature blood lineages. However, the life-saving HSC transplantation in the clinical use of tissue regeneration still has some limitations. Thus, many patients cannot benefit from this treatment due to an insufficient number of stem cells for autologous transfer or due to a lack of HLA-compatible donors for allogeneic transplantations [234; 235]. UCB is a very promising source of HSCs because of its easy accessibility and haplotype diversity. However, the disadvantage is that there is too low a number of HSCs harvested from UCB to reconstitute the blood system of adult patients [237]. One possibility for improving the transplantation setting is to expand HSCs *in vitro* prior to transplantation by activating positive regulators of self-renewal or by improving the HSC capacity to engraft *in vivo.* Therefore, in this thesis, we have focused on both of these aspects. We successfully identified HLF as a new HSC regulator as well as a new molecule that can improve adhesion of transplanted cells into the niche. Both approaches are summarized in the published articles attached to this thesis.

Article I

Hepatic Leukemia Factor Maintains Quiescence of Hematopoietic Stem Cells and Protects the Stem Cell Pool during Regeneration.

Komorowska K, Doyle A, Wahlestedt M, Subramaniam A, Debnath S, Chen J, Soneji S, Van Handel B, Mikkola HKA, Miharada K, Bryder D, Larsson J, Magnusson M.

Cell Rep. 2017 Dec 19;21(12):3514-3523. doi: 10.1016/j.celrep.2017.11.084.

Article II

Hepatic Leukemia Factor is dispensable for hematopoiesis during aging.

Komorowska K, Miharada K, Larsson J and Magnusson M.

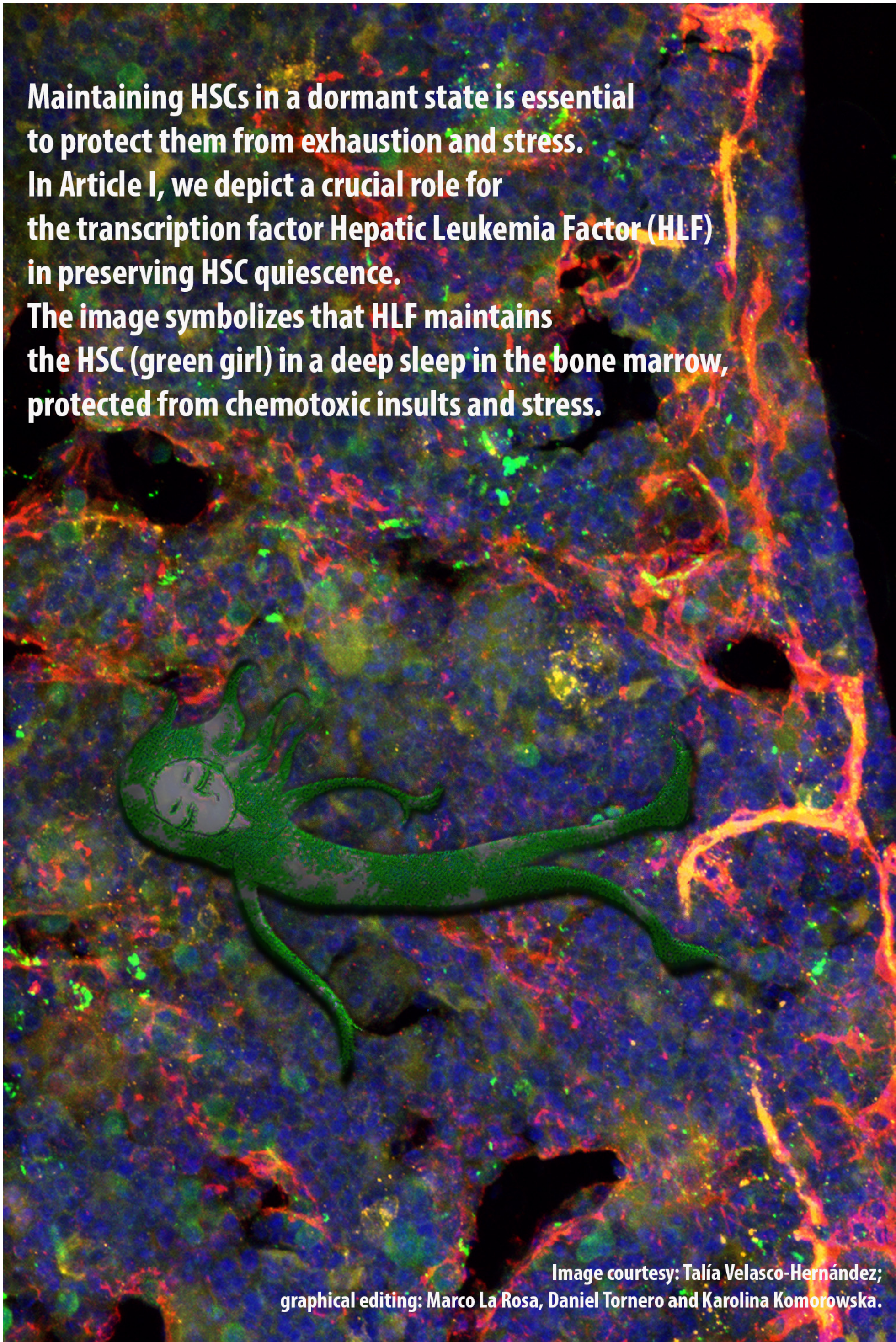
Manuscript

Article III

Cytohesin 1 regulates homing and engraftment of human hematopoietic stem and progenitor cells.

Rak J, Foster K, Potrzebowska K, Talkhoncheh MS, Miharada N, **Komorowska K**, Torngren T, Kvist A, Borg Å, Svensson L, Bonnet D, Larsson J.

Blood. 2017 Feb 23;129(8):950-958. doi: 10.1182/blood-2016-06-720649. Epub 2016 Nov 29.


Maintaining HSCs in a dormant state is essential
to protect them from exhaustion and stress.
In Article I, we depict a crucial role for
the transcription factor Hepatic Leukemia Factor (HLF)
in preserving HSC quiescence.
The image symbolizes that HLF maintains
the HSC (green girl) in a deep sleep in the bone marrow,
protected from chemotoxic insults and stress.
Image courtesy: Talía Velasco-Hernández;
graphical editing: Marco La Rosa, Daniel Tornero and Karolina Komorowska.

PL: Utrzymanie komórek macierzystych w fazie spoczynkowej jest niezwykle ważne dla ich ochrony przed wyczerpaniem i stresem, W Artykule I została przedstawiona bardzo istotna rola genu HLF w utrzymaniu stanu uśpienia HSC. Zielona dziewczyna (na zdjęciu przedstawionym na poprzedniej stronie) symbolizuje śpiącą komórkę macierzysta.

Conclusions from the published data

Article I

To study the physiological role of *Hlf*, we obtained a conventional $Hlf^{-/-}$ mouse model [184]. These mice were viable, displaying normal blood parameters, lineage distribution as well as normal frequency of immunophenotypic HSCs (Lin^- $Sca1^+$ $ckit^+$ $CD34^-$ $Flt3^-$), but with reduced platelet counts.

Upon serial competitive transplantation assays using the whole BM, $Hlf^{-/-}$ cells displayed a significant reduction in reconstitution capacity in primary recipients. This was strikingly enhanced in secondary recipients and no engraftment was detected from the $Hlf^{-/-}$ cells in tertiary recipients, demonstrating a reduced reconstitution and self-renewal capacity *in vivo*. This was further confirmed upon a competitive transplantation assay with purified HSCs ($Lin^-Sca1^+ckit^+CD34^-$ $Flt3^-$), showing a 2.4-fold reduced engraftment capacity of cells isolated from $Hlf^{-/-}$ donors. This data indicates that HLF is an important regulator of HSC self-renewal. Additionally, the reduced reconstitution ability was not due to a reduced HSC number, as enumerated by limiting dilution transplantation assay demonstrated on an intact HSC pool.

To delineate the mechanisms by which HLF regulates HSCs, we have performed next-generation sequencing on isolated HSCs at steady-state hematopoiesis from both $Hlf^{+/+}$ and $Hlf^{-/-}$ mice. The gene expression analysis identified several candidate genes directly regulated by HLF (verified by ChIP-Seq). A significant amount of the upregulate genes in the $Hlf^{-/-}$ were linked to proliferation.

In line, cell cycle analysis of $Hlf^{-/-}$ HSC showed a dramatic decrease in quiescence HSC in comparison to $Hlf^{+/+}$ HSC. Additionally, $Hlf^{-/-}$ mice displayed a striking inability of recovery following administration of the myeloablative agent 5-Fluorouracil (5-FU). 5-FU is a cytotoxic drug, a pyrimidine analog used in the treatment of cancer for over 40 years [294], whose administration induces cell death of rapidly dividing cells. None of the $Hlf^{-/-}$ mice survived past day 15 post 5-FU treatment, due to a depleted HSC and progenitor pool. These results strongly suggest a critical role of HLF in cell cycle regulation and HSC quiescence.

Taken together, our findings show that HLF is an important regulator of HSC activity and of maintaining HSC quiescence.

Article II

Stem cells must adapt their properties to follow the necessary demands for growth and tissue regeneration throughout the life of the organism. They divide rapidly during fetal development to support growth and, by adulthood, the majority of stem cells are quiescent [61], intermittently dividing to maintain tissue homeostasis.

We have previously shown that HLF is highly expressed in HSCs during human fetal hematopoiesis [252], and in Article I we confirmed that HLF is critical for maintaining the HSC pool during hematopoietic regeneration [272]. Since the hematopoietic system is continuously replenishing though out life, HLF may be critical for preserving the HSC pool during aging as such, contributing to the onset of aging and age-related diseases. The constant change of hematopoiesis over time is likely caused by changes in stem cell function [295].

Therefore, in Article II, we studied hematopoiesis and HSCs in aged HLF deficient mice (>36 months old), as well as in the FL from *Hlf*$^{-/-}$ embryos (E14.5). We observed a similar pattern to the phenotype in adult mice reported in Article I. The competitive transplantation using the whole BM from aged HLF deficient mice or HLF deficient FL cells demonstrated both impaired reconstitution ability, which is in line with the phenotype seen in young mice in Article I. Intriguingly, the phenotype in FL transplantation was stronger than in the young and aged *Hlf*$^{-/-}$ HSC, indicating that HLF may be particularly important during the expansion phase of HSCs during embryonic development.

According to the long-standing dogma, it is believed that HSCs are the main contributors to lifelong blood production [33; 35]. Indeed, HSCs are crucial during development to establish the hematopoietic system, which explains why in fetal and early postnatal life HSCs are actively cycling. However, more recent studies proved that once post-natal hematopoiesis has been established, LT-HSC are mostly quiescent and do not contribute to adult steady-state hematopoiesis, but are required and necessary after transplantation or any other cellular stress [37]. As HLF is upregulated on LT-HSCs and downregulated towards differentiation, these could potentially explain why HLF-deficient mice display a normal life span, with no changes to the HSC pool even after 18 months of age. Specific expression on immature cells could also explain why HLF is necessary upon a complete reconstitution of the hematopoietic system after transplantation.

Thus, our findings confirm an important role for HLF in maintaining the HSC function during hematopoietic stress, such as BM transplantation, throughout ontogeny. Our transplantation experiments demonstrate that the expression of HLF is crucial for HSCs to maintain their self-renewal potential during hematopoietic reconstitution. As the effect of the lack of HLF is only visual during regeneration

after stress, these results also support the notion that HSCs do not contribute to adult steady-state hematopoiesis.

Taken together, recent as well as our studies show that even if there is a defect on LT-HSC levels, it might be unnoticed if there is no extra stress put on the hematopoietic system, whereas any defect on downstream ST-HSCs or MPP would cause a rapid BM failure. In Article II, we showed that HLF is dispensable for preserving hematopoiesis during normal aging.

Article III

To identify the mechanisms regulating the homing of HSCs to their niche in the BM, we developed a functional RNA) screen for regulators of human HSC adhesion. For a precise study of the molecular mechanisms of adhesion between human HSCs and their cellular microenvironment, it was essential to establish a surrogate niche model derived from human cells.

MSCs are stromal components of the BM niche closely associated with HSCs. They provide crucial maintenance factors for HSCs *in vivo* [92]. As those factors are missing *in vitro*, we hypothesized that MSCs could constitute a suitable analogue of an *in vitro* niche model for studying molecular mechanisms of HSC adhesion towards the supportive cellular milieu of the niche [98]. Additionally, it was shown that co-culture of HSCs on a layer of MSCs resulted in an expansion of the HSCs [296; 297], indicating an important role of MSCs for attraction and maintenance of HSCs. As MSCs isolated from BM or UCB show a higher potential in the adhesiveness of HSCs compared to MSCs from the adipose tissue [298], we used the BM derived MSCs assuming that they would be the most suitable model for identifying molecules regulating HSC adhesion.

First, we performed an shRNAs screen, targeting 366 adhesion genes by transducing UCB $CD34^+$ cells with a pooled lentiviral shRNA library containing 5 shRNAs per gene. The transduced cells were then plated onto a MSC layer. Next, the plate was flipped upside down to let the force of gravity to remove the non-adherent cells. The unattached cells were collected and sequenced in order to identify the genes targeted by the shRNA. Using gravitational force permitted a high reproducibility of the experiments in standardized conditions. This also kept cells from being subjected to shear stress or vigorous pipetting/washing. Therefore, our adhesion assay provides a unique opportunity to study adhesive mechanisms of human HSCs *in vitro*.

The distribution of integrated shRNAs in the adherent vs non-adherent cell fractions was analysed by deep sequencing. One of the genes selected for further

validation was the GTPase Cytohesin1 (CYTH1). The knockdown of CYTH1 significantly increased the fraction of non-adherent cells in the stroma adhesion assay. The *in vivo* transplantation of CYTH1-knockdown cells to immunodeficient NSG mice significantly reduced both the short-term and the long-term engraftment of $CD34^+$ cells, indicating a reduced capacity of the CYTH1 transplanted cells to home and engraft in the BM. Homing assay using intravital microscopy showed that CYTH1 deficiency strongly affects HSC mobility and localization within the marrow space as visualized by a greater distance to the bone surface and endothelium compared to control cells. CYTH1 deficiency thereby impairs their proper lodgement into the niche. In summary, we identified CYTH1 as a novel regulator of cell adhesion and engraftment in human HSCs both *in vitro* and *in vivo*.

Knowledge is like water in a boundless sea...

The more you drink, the more thirsty of it you become.

– Stefan Żeromski (Polish novelist and dramatist)

Abbreviations

5-FU	5-Fluorouracil
AD	activation domain
AGM	aorta-gonads-mesonephros
ALL	acute lymphoblastic leukemia
AML	acute myeloid leukemia
ANG1	Angiopoietin 1
bHLH	basic helix-loop-helix domain
BM	bone marrow
bZIP	basic leucine zipper
c-kit	tyrosine kinase receptor
Ca^{2+}	ionic calcium
CD29	mature form of Integrin β1
CLP	common lymphoid progenitor
CRU	competitive repopulating unit
CYTH1	Cytohesin1
DBP	D box binding protein
E	embryonic day
ECM	extracellular matrix
EHT	endothelial-to-hematopoietic transition
ELDA	extremelimiting dilution analysis
ESC	embryonic stem cell
FACS	fluorescence-activated cell sorting
FL	fetal liver
FN1	fibronectin-1
GMLP	granulocyte-monocyte-lymphoid progenitor
GMP	granulocyte/macrophage progenitor
GVHD	graft-versus-host-disease
HIV-1	human immunodeficiency virus-1
HLA	human leukocyte antigen
HLF	Hepatic Leukemia Factor
HSC	Hematopoietic Stem Cells
IL2RG	IL-2 receptor common γ chain
iPS	human induced pluripotent stem cells
KO	knockout

Lin	lineage
LSC	leukemic stem cells
LSK	Lin^{-} $Sca1^{+}$ $c\text{-}kit^{+}$
LT-HSCs	long-term HSCs
MPP	multipotent progenitor
MSC	mesenchymal stroma cells
NK cell	natural killer cell
NOD	non-obese diabetic
OB	osteoblast
OC	osteoclast
PAR bZip	proline- and acidic amino acid- rich basic leucine zipper
PB	peripheral blood
pMegE	pre-megakaryocyte and erythroid
RNAi	RNA interference
ROS	reactive oxygen species
Sca-1	stem cell antigen-1
SCF	stem cell factor
SCID	severe combined immune-deficient
SDF-1	stromal derived factor 1 or CXCL12
shRNA	short hairpin RNA
siRNA	small interfering RNA
SLAM	signaling lymphocyte activation marker
SNS	sympathetic nervous system
ST-HSCs	short-term HSCs
TEF	thyrotroph embryonic factor
TF	transcription factors
UCB	umbilical cord blood
VCAM-1	vascular cell adhesion molecule-1
VLA-4	very late antigen-4
VSV-G	vesicular somatic virus glycoprotein G

When you talk, you are only repeating what you know.
But if you listen, you may learn something new.

– Dalai Lama

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Give a girl the right shoes, and she can conquer the world.

– Marilyn Monroe

Thank you Elante.pl

Cyprian Kamil Norwid "Fortepian Szopena"
VI
I — oto — pieśń skończyłeś — i już więcéj
Nie oglądam Cię — jedno — słyszę:
Coś?... jakby spór dziecięcy — —
— A to jeszcze kłócą się klawisze
O niedośpiewaną chęć:
I trącając się z cicha
Po ośm — po pięć —
Szemrzą: „Począłże grać? Czy nas odpycha??..."

Cyprian Kamil Norwid "Chopin's Grand Piano"
VI
And now — your hymn complete – your music mute
No more I'll see you – but what? is that there
I hear... as if a child's dispute — —
No more, but just the keys still chatter,
About the uncompleted rhyme
Shuffling final echoes spell
— Five a time — eight a time —
Rustling, "Did he begin? To play or to repel?"

Norwid C. K. - Polish poet, painter, philosopher and friend of
Fryderyk Chopin - Polish composer and pianist, often called the poet of the piano. His works were inspired by the patterns of Polish folk music.

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