Stem and progenitor cell involvement in acute lymphoblastic leukemia

Castor, Anders

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Anders Castor

Hematopoietic stem cell laboratory,
Lund strategic center for stem cell biology and cell therapy

and

Department of pediatric hematology/oncology,
Division of pediatrics.

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Professor Mel Greaves
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Abstract

Leukemic stem cells (LSCs) share the capacity of self renewal and extensive proliferation with normal hematopoietic stem cells (HSCs), and are therefore obvious targets for therapy. As such, they need to be identified and characterized in order to elucidate what drives them, and what separates them from their normal counterparts. The focus of this thesis is on pre B cell Acute Lymphoblastic Leukemia (ALL), the most common form of cancer in children. We have investigated 2 distinct subtypes of ALL, characterized by the gene fusions ETV6-RUNX1 (found mainly in pediatric ALL, conferring a favorable prognosis) and BCR-ABL1 (producing two different onco-proteins, designated P190 and P210, both of which are associated with a poor prognosis in both children and adults). We show that ETV6-RUNX1 ALL are propagated by B-cell committed LSCs expressing the lymphoid marker CD19, leaving the normal HSC compartment intact. In BCR-ABL1-positive ALL we show an unexpected difference between the two forms of the fusion protein, such that the LSC in P190 BCR-ABL1 ALL, similar to ETV6-RUNX1 ALL, are B-cell committed progenitors, whereas P210 BCR-ABL1 ALL originates in a multipotent HSC, expressing the same phenotypical markers as the normal HSC, and with a retained, albeit severely reduced, capacity to produce a clonal myeloid progeny. Interestingly, the LSC still displays the B cell commitment marker CD19, as only CD19+ cells propagates the disease in immunocompromised mice. We cannot, however, exclude very rare, and/or very quiscent CD19-ve P210 LSCs. This represents a hitherto unanticipated distinct biological difference between P190 and P210 ALLs, possibly indicating different requirements for eradication. In the second paper we describe a method to prospectively purify a large part of the leukemic cells from bone marrow or peripheral blood from patients with ALL, for relevant comparisons across samples. We compared ALL cells harvested from bone marrow and peripheral blood from the same patient by gene expression profiling, and found a striking similarity between cells from the two locations, indicating that bone marrow derived biological cues necessary for normal pre B cells not seem to segregate ALL cells in a blood and a bone marrow compartment, and that cells thus can be harvested from either compartment for further gene expression analyses. Finally, in the discussion part of the two papers, are preliminary data from follow up studies discussed, where we find indications for the existence of distinct sets of LSCs within the same patient with ALL or chronic myeloid leukemia in lymphoid blast crisis, contrary to the generally held view of a homogeneous LSC population.

Key words: hematopoietic stem cells, hematopoiesis, leukemic stem cells, acute lymphoblastic leukemia, philadelphia chromosome.

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"We choose to go to the moon… not because it is easy,
but because it is hard."

President John F. Kennedy
Address at Rice University on the Nation's Space Effort
Houston, Texas
September 12, 1962

"Pain is temporary.
Quitting lasts forever"

Lance Armstrong
7 times Tour de France-winner
and cancer survivor

"Sometimes I'm so far behind,
I think I'm first"

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Articles and manuscripts in this thesis

Distinct patterns of hematopoietic stem cell involvement in acute lymphoblastic leukemia.
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Nature Medicine, *11:* 630-637, 2005.

Minor differences in global gene expression between purified leukemic cells harvested from bone marrow and peripheral blood from pediatric ALLs
Castor, A., Hansson, F., Toporski, J., Månsson, R., Johansson, B., Norén-Nyström, U., Jacobsen, S.E, Wiebe, T., Larsson, M., and Sigvardsson, M.
Manuscript

Table of contents

List of abbreviations

AGM	aorta-gonads-mesonephros
ALL	acute lymphoblastic leukemia
AML	acute myeloid leukemia
BM	Bone marrow
CFU	Colony forming unit
CFU-S	Colony forming unit-spleen
CLP	common lymphoid progenitor
CML	chronic myeloid leukemia
CMP	common myeloid progenitor
CNS	central nervous system
CRU	competitive repopulating unit
CSC	cancer stem cell
ES cell	Embryonic stem cell
FACS	Fluorescence-activated cell sorting
G-CSF	granulocyte-colony stimulating factor
GMP	granulocyte-monocyte progenitor
HSC	Hematopoietic stem cell
IgH	immunoglobulin heavy chain
IgL	immunoglobulin light chain
LMPP	lymphoid-primed multipotent progenitor
LSC	leukemic stem cells
LTC-IC	long term culture-initiating cell
LT-HSC	long-term hematopoietic stem cell
MPP	multipotent progenitor
NOD	non-obese diabetic (*mouse strain*)
Ph	Philadelphia chromosome
Scid	severe combined immunodeficience (*mouse strain*)
SRC	*scid* repopulating cells
ST-HSC	short-term hematopoietic stem cell
YS	yolk sac

The “almighty” Stem Cell

The concept of stem cells has attracted huge interest recently, due to a number of break throughs’ in the last decade. These include the development of means to maintain and expand human **embryonic stem (ES) cells** [1]; several organs in the developed organism have been discovered to contain small populations of cells, **adult stem cells**, with the sustained capacity to regenerate all the different cells of that tissue[2-9]; several groups have reported an unexpected **plasticity** of stem cells to produce cells across tissue and even germ line boundaries[10,11]; and it has been shown in several cancers that only a small subpopulation of cells within the tumor has the capacity to infinitively divide and perpetuate the tumor, hence called **cancer stem cells**, and appearing to be the ultimate and necessary targets of curative anti-cancer therapy[12-16]. This has dramatically raised hopes for utility in regenerative medicine as well as increased understanding of normal and pathological development. As the tide of popular interest recedes, and some of the initial hopes are thwarted, the time for long and hard work is here, to realize at least some of the initial goals, and to discover and pursue new ones along the road. The concept of stem cells, in normal development and in disease, is for sure going to bear fruit, in expected or unexpected ways.

Stem cells are defined by their ability to **self-renew**, which means they are replenished by leaving at least one identical copy of themselves behind after each cell division, and to **differentiate** to all the mature cells of the organ or tissue to

which they belong[17]. Both characteristics need to be full-filled. Self-renewal without differentiation is what bacteria and other unicellular organisms do, and differentiation without self-renewal is the hallmark of cells in developmental transit between stem cells and mature cells. Thus, the ultimate test for a candidate stem cell is that it, as a single cell, should be able to completely recreate the tissue from which it is derived. This criterion, in its strictest sense, is to date only fulfilled by murine hematopoietic stem cells (HSCs)[18], and might not be applicable to all kinds of stem cells.

Stem cells are classified by the diversity of their progeny into **totipotent** (capable of creating an entire organism, i.e. both embryonal and extra-embryonal tissues, a capacity inherent only in the zygote and early cleavage-stage blastomeres in animals), **pluripotent** (able to form all tissues of the body, including germ cells, but with impaired ability to form extra-embryonic tissues, such as placenta, as seen in ES cells), **multipotent** (able to create multiple cell lineages constituting an entire organ or tissue, for example HSCs), **oligopotent** (progeny belonging to two or more lineages within a tissue, for example neural stem cells able to create subsets of neurons in the brain) and **unipotent** (contributes to only one lineage, for example spermatogonial stem cells)[19].

Embryonal carcinoma cells and embryonal stem cells

Stem cells can broadly be divided into ES cells and adult stem cells. Ethical issues surround research on ES cells, a feature attracting much attention in broad areas of society, and for political reasons skewing the possibilities of using this source of cells in different countries. This might be perceived as surprising in view of the 50 years of (more or less) active research on pluripotent cells[20]. The first pluripotent cells investigated were harvested from **teratocarcinomas**, a rare embryonal tumor histologically exhibiting an array of adult tissues and misshapen organs, where single cells from the tumor implanted intraperitoneally (within the abdominal

cavity) could recreate all the different cell types of the tumor[21], thus demonstrating a clonal ability of sustained growth and pluripotency as evidenced by differentiation into tissues of all three germ layers. By demonstrating that embryos grafted to extra-uterine sites in recipient animals formed retransplantable teratocarcinomas[22,23], the embryonic origin and nature of the tumor was established. The stem cells of the tumor could form cell lines, and because of their relationship with embryos were called **embryonal carcinoma cells**. When injected into mouse **blastocysts**, a very early embryonal structure composed only of embryonal stem cells and trophoblast cells (which will later form the placenta), these cells were shown to participate to varying degrees in producing chimeras (developing organisms with cells contributed by both recipient and donor, the later in this case teratocarcinomas cells). In some instances did the embryonal carcinoma cells even contribute to germline chimerism, thus showing retained embryonic features, but with occasional tumorigenic potential, questioning their normalcy. Although the use of embryonal carcinoma cells has diminished since the isolation of ES cells, they have been instrumental in the establishment of ES cell lines.

Efforts to obtain pluripotent cells directly from early mammalian embryos dates back several decades, and were crowned 1981 by the establishment of mouse ES cell lines[24,25], followed 17 years later by the development of human ES cell lines[1]. ES cells are derived from early, pre-implantation embryos at the blastocyst stage (5-6 days post conception)[1] or the even earlier morula-stage (4 days post conception)[26]. The fact that the establishment of human ES cell lines entails the destruction of an embryo with the potential of developing into a human being has led to significant, though geographically unevenly distributed, opposition on ethical and religious grounds. Arguments supporting the use of ES cells include the fact that the human embryos used are leftovers from in vitro fertilization procedures, otherwise destined for destruction, and the huge promise these immortal and versatile cells hold for regenerative medicine and as study objects for

normal and pathological development. Techniques are being developed, however, to alleviate the ethical concerns, for instance by using genetically altered embryos not capable of developing into babies[27], or removing single cells from the early morula, as is done today for pre-implantation genetic diagnosis, which leaves the embryo intact[28,29]. None of these techniques, however, seems to satisfy all critics[30,31].

There are still other hurdles to be overcome before the promise of ES cells can be realised in the clinic. The human ES cell lines existing worldwide today are established with the use of xenogenic (derived from animals) input from feeder-layers and serum, with a very small but realistic risk of contagious complications inhibiting their use on patients. Protocols for feeder-free and serum-free derivation and maintenance of ES cells need to be developed. Furthermore, there is a need to learn how to regulate differentiation of the ES cells prior to transplantation, since undifferentiated ES-cells will cause teratocarcinomas in vivo[32]. A further cause for concern is raised by recent reports on significant accumulation of genetic alterations in cultured ES cells[33,34]. Finally, since available ES cells virtually always will be of **allogeneic** origin (from another individual than the recipient), there is an immunological barrier that needs to be overcome by life-long immunosuppresion, with concomitant risks for morbidity and mortality.

Nuclear transfer

One way of getting around the immunological problems could be to use cells from the patient and revert them to an undifferentiated stage, from which they could be differentiated and expanded to the desired cell type. This is what is aimed for with **nuclear transfer** (previously called therapeutical cloning), a technique that dates back half a decade [35]. By transferring somatic nuclei from amphibians into unfertilized eggs, and observing them through embryonal development, researchers learned two important things: 1) that the genome was preserved through

differentiation, contrary to what was previously believed, and that the differentiation process is governed by genomic expression rather than content, and 2) the remarkable reprogramming potential of the egg cytoplasm[36]. Improvements in micromanipulation techniques allowed the successful nuclear transfer into much smaller mammalian eggs (usually <0,1% the size of amphibian eggs), and eventually to the transfer of a mammalian adult somatic cell nuclei (cultured adult mammary gland cells) into an egg, producing the cloned sheep "Dolly"[37]. Recently, in a "proof of principle"-paper, a genetic defect ($Rag^{-/-}$, leading to immune deficiency) in a mouse strain was corrected by first establishing an ES cell line, in which one Rag allele was repaired by homologous recombination and then *in vitro* differentiated into hematopoietic precursors prior to engraftment into mutated mice, restoring their immune function[38].

Although the concept holds promise, there is much to be learned and mastered before it will benefit patients. First and foremost, it has to be shown that human adult cells can be reprogrammed in this way. Recent reports claimed to have shown this, but was proven fraudulent[39,40]. The procedure is further very inefficient in producing viable totipotent cells, particularly correlating with maturity of the donor nucleus such that more immature cells are more efficient nuclear donors[41] (although this has recently been challenged[42]), and therefore puts great demands on numbers of eggs, which probably will be prohibiting for a widespread use. An alternative strategy employed for the reprogramming of the adult nucleus is by fusing it to an ES-cell, which has been shown to also produce reprogramming of somatic nuclei[43,44]. If the process could be understood in molecular terms, it might be possible to generate pluripotent cells directly from the patients' own cells *in vitro*. A step forward in this direction was recently reported, as it was shown that retroviral introduction of only four genes (*Oct3/4, Sox2, c-Myc* and *Klf4*) into adult mouse fibroblasts reprogrammed these differentiated cells into a pluripotent stage, which much like ES cells could be maintained on feeder layers for an extended

period of time[45]. These cells, however similar, are not identical with ES cells, and their true nature has to be revealed.

Finally, nuclear transfer is not devoid of potential ethical issues. If the reprogrammed cell would be viewed as a potential human being, it could be argued immoral to have produced it purely for the sake of providing spare parts for needing patients. However, the potential for a normal development will probably, at best, be very small, if at all existent, and some would argue that as long as the cell is not introduced into a womb whatever potential it possesses can never be realized.

Adult stem cells and plasticity

Partly because of the ethical issues surrounding research on ES cells, but also because of the profound impact on long-held views on developmental biology and implications for regenerative medicine, was recently a number of papers in well renowned journals reporting on an unexpected plasticity of adult tissue stem cells met with considerable interest and excitement by the scientific as well as the public community. According to these reports, blood could be turned into heart[46-48], pancreas[49,50], muscle[51-53], brain[54,55], liver[56,57], skeletal or more tissues[58,59], and stem cells from other tissues could be turned into blood[52,60,61]. Understandably, this caused as much expectations as it raised new questions. From the point of view of regenerative medicine this was extremely interesting, since this could potentially translate into easy access of stem cells for tissue repair or gene therapy and at the same time circumventing the potential immunological complications of using allogeneous stem cells and the ethical obstacles associated with ES cells. But what would be the possible mechanisms for this plasticity? Several models have been proposed[62-64], such as **transdifferentiation** of tissue specific stem cells into other tissue lineages; **de-differentiation** to a more primitive and multipotent stage with subsequent re-differentiation along a different lineage; the simultaneous

occurrence of many different tissue restricted stem cells residing in the same organ; the existence of rare pluripotent stem cells with the capacity to generate progeny of all germ layers; or fusion of a stem cell (or any cell) with a cell of a different lineage (so called **heterotypic fusion** – the most obvious normally occurring example being the fusion of a sperm cell with an egg).

However, the field has cooled substantially following difficulties in reproducing data[65-70], reports of alternative explanations for the observed plasticity[71-73], and the very low frequency of supposedly transdifferentiated cells (usually lower than a few percent). More rigorous experimental criteria has evolved as a requirement for true plasticity; for example the prospective purification of the cell population of interest, exclusion of cell fusion as a cause for the claimed transdifferentiation, and backing up of data with functional analyses and to not only rely on phenotype[62,63]. According to these more stringent criteria there has been no study convincingly demonstrating a true transdifferentiation event[62].

Will we recognize a stem cell when we see one?

The stem cell concept is in a sense simple and intuitively compelling: stem cells are the cells needed to supply the organism with all the different mature cells necessary for all vital biological functions over the life time of the individual. Mature cells and their immediate progenitors can not perform this feat - if they are placed in culture or transplanted into a recipient, they cannot uphold production of new cells for more than days or a few weeks at most. Something more long-lived, or self-renewing, is needed, such as stem cells. But as compelling as it may seem, this definition has inherent problems.

The most striking feature of stem cells is that they can self renew; i.e. produce exact copies of themselves. It is often stated that stem cells can perform three kinds of cell divisions: symmetrical divisions producing two new stem cells, or

asymmetrical divisions producing one stem cell and one daughter cell which commits to differentiation, or symmetrical divisions in which two committed daughter cells are produced. It is unclear what influences the HSC to choose either self renewal or differentiation, but most believe that the decision is largely stochastic, or random – which would imply that a "true" HSC inherently, at the same time, contain both choices, as yet unrealized. However, once the differentiation choice is realized in cell division, the HSC status of the preceding cell collapses, similar to Schrödinger's cat in the box: it might not be possible to prospectively identify a particular cell as a stem cell until it has divided, and thence is no more (or even to identify all stem cells even if they do divide)! Considering that we do not know to what extent a cell can differentiate without dividing suggests further complexity – how will one be able to show if a single stem cell can be forced to differentiate into a more mature cell without dividing, and still know that it was a stem cell in the first place? If stem cells can produce two daughter cells which are not stem cells, or even differentiate without division, then it will be impossible to know for sure if a candidate stem cell population is purified to homogeneity or not, since a fraction of this population in single cell assays will not produce any stem cells. These considerations also touches on the "uncertainty principle" of stem cell research, the inherent problem of simultaneous measurements of self-renewal and differentiative capacity in the same cell[74].

A related question is: how similar to the mother cell must the progeny of a cell division be, in order to satisfy the criteria for self-renewing divisions? Obviously, the progeny can never be an *exact* copy of the mother cell – random differences in type and number of expressed receptors, transcription factors, enzymes, or epigenetic state or in the number and activation status of sub-cellular organelles, such as mitochondriae, will inevitably lead to daughter cells that are subtly or significantly different from the mother. This could make the notion of a stem cell as a discrete state difficult to uphold, since the total sum of differences between mother and daughter cell (and possibly also subtle or significant differences in

their respective environments) dictates the state they are in and the probability for certain outcomes, such as self-renewal divisions. The non-random effects of both differently segregated intracellular components and environmental differences on stem cell fate have been shown in germ cells of the fruitfly (*drosophila*, see below). It has been suggested that, rather than modelling the stem cell hierarchy as consecutive, discrete compartments[75,76], a more proper and rewarding model would be to view it as a continuous variable allowing also all possible states in between[74,77,78].

Another approach towards defining and identifying stem cells is offered by the increasingly powerful microarray technology, by which genome wide up- or downregulation of genes in suitably enriched cell populations can be analysed. It has been proposed that the two main unifying themes among different stem cell populations, self-renewal and multipotency, could be mirrored by a shared set of genes, representing a conserved stem cell signature[79]. Recently a number of studies provided the first comparative genome wide transcription analyses of purified ES cells, HSCs and neuronal stem cells[80-82], which revealed a shared expression pattern of some 200-300 genes between the investigated populations, indicating a "stem cell signature". However, surprisingly, in a comparison between the three studies the shared "stem cell signature" boiled down to only one single gene: integrin α6[82]. Other studies have not been more successful in uncovering a stem cell specific fingerprint[83]. Plausible explanations for this failure could be differences in methodology, purification strategies, purity of enriched populations and computational algorithms used[79,84]. Other, more fundamental, reasons could be that stem cell specific genes are only transiently expressed, for example genes important for self-renewal only during cell cycle, which would make them difficult to detect within a largely quiescent population, or that they are not expressed exclusively on stem cells, but also on differentiated cells, making them hard to detect by differential expression analysis[79]. Or, in a sense an even more fundamental and simple reason: a specific stem cell signature does not exist. It has

been argued that even the two stem cell functions of multipotency and self-renewal by themselves are problematic[83], since some stem cells do not abide by them (germ cells, for example, are unipotent; and inner cell mass cells in the blastocyst perform a very limited number of self-renewing divisions before they differentiate to more specified stem cells) while some differentiated cells do (for example multipotent hematopoietic cells without self-renewing capacity, or differentiated B- and T-lymphoid cells in blood or β-cells in the pancreas which can self-renew), and therefore a unified gene expression pattern based on these characteristics cannot be found. Instead, another model has been proposed, in which a cell, moving along an imagined differentiation-line from ES cell to fully differentiated cell, is halted along its course, dividing and creating progeny endowed with the same features as the mother. In such a model, the resistance to progression/differentiation cannot be reduced to multipotency or self-renewal capacity, but might instead represent a fixed epigenetic state[83].

Whether stem cells are discrete states which can be only partly assessed retrospectively, or are part of a continuum were the individual cells are governed by continuous variables and defined by probability densities, or whether they are like any cell, but just halted, like a Peter Pan, on it's way towards fulfilling their final goals as mature cells – we still need to deal with them. The definitions we use today are operational, governed by the technologies we have at hand, and therefore the operational (and experimental) definitions differs between stem cells harvested from different contexts. A single test applicable for all stem cells is clearly not at hand – yet.

Hematopoietic stem cells and hematopoiesis

Hematopoietic stem cells

HSCs are the best characterised of all adult stem cells for several reasons. First, they represent a minor subpopulation of dissociated cells within a floating organ which is easily accessible in relatively large quantities. Second, they are vitally important providers of cells in an organ with a very high cell turn-over (exemplified by the rapid death of the organism in case the production stops, as for example after irradiation, which kills the HSCs), which may make both *in vitro* and *in vivo* assays faster and more yielding. A third reason is that the discovery of HSCs now dates almost half a century back, during which time instrumental methodological developments and insights have been made, which have aided in the enormous progress in the field that we witness today.

The first steps towards identifying HSCs was taken 1961 by Till and McCulloch, as they identified the **colony forming unit – spleen (CFU-S)**[85]. In their work on radiation sensitivity of tissues they stumbled upon the observation that bone marrow (BM) cells intravenously injected into lethally irradiated mice gave rise to macroscopically visible colonies of proliferating cells in the spleens of these animals. The colonies contained hematopoietic cells of all myeloid lineages at variable maturational stages, and were derived of recipient BM cells only, as irradiated but not transplanted mice never displayed such colonies. These colonies were termed colony forming units (CFUs). In order to prove that these colonies

were derived from single cells, recipient mice recieved a sublethal irradiation dose, followed by injection of donor BM cells and subsequent further irradiation of the recipient up to a lethal dose, which exposed the donor BM cells *in vivo* to a significant irradiation dose, enough to confer recognizable chromosomal abnormalities in a proportion of the cells without killing them, and at the same time ablating the host HSCs. In some of the resulting colonies abnormal karyotypes were found by cytogenetic analysis in all investigated cells[86], and as chromosome breakage by irradiation is a random process, the clonality of the colonies were proven. In a third landmark paper they defined a stem cell as a cell that should have the capacity to give rise to differentiated progeny and have an extensive proliferative potential, two features they already had been able to assay in the spleen colonies, but a stem cell should also posses the capacity for self-renewal, such that an organ like the BM throughout the life-time of an organism always has stem cells that can continuously give rise to both differentiated progeny and retain a sufficient stem cell pool. In order to test the CFU-S for self-renewal capacity they injected dissected and resuspended CFU-S into secondary hosts, and found that primary spleen colonies often, or possibly always, contained cells capable of forming more spleen colonies. Thereby they proved that their CFU-S assay detected cells with the capacities for differentiation, extensive proliferation and self-renewal, and cautiously suggested that they might be HSCs.

Although there is evidence that the CFU-S does not represent the true HSC, but a downstream immature progenitor population[87-90], the groundbreaking work of Till and McCulloch still laid the foundation of HSC research, not least by providing the definition of a HSC that is still used today.

Identification and characterization

Blood cells are born and develop in the BM, where they exist as a mixture of more or less differentiated cells on the way towards any of the mature blood cell types

found in the circulation, and interspersed among all these aspiring, proliferative young blood cells are a few quiet, morphologically insignificant HSCs. In spite of decades of efforts, the blood stem cells (and all other adult stem cells) have evaded final identification. No one can yet with certainty point out a specific cell as a stem cell, but due to continuous development of new methods, the circles around these enigmatic cells are drawing ever closer. Development of *in vitro* and *in vivo* assays for evaluation of lineage potential and self-renewal, and **fluorescence-activated cell sorting** (**FACS**) and monoclonal antibody technology for identification and purification of subsets of cells has been instrumental for the progress achieved in stem cell biology in general, and HSC biology in particular.

Purification

Cells, and particularly hematopoietic cells, can with increasing accuracy be subdivided into functionally distinct subfractions, based on the combined presence or absence of specific antigens on the cell surface. Antibodies raised against these antigens can either be attached to **fluorochromes** (particles emitting light with defined wavelengths when hit by a laser beam) which allows modern FACS instruments to simultaneously detect several antigens on single cells for analysis or sorting at a single cell level; or the antibodies can be attached to magnetic micro-beads, allowing positive or negative selection of cells by passing the cell suspension through a magnetic field. No single antigen specifically detects HSCs, but a high degree of enrichment can be achieved by combining several antigens. The relative ease and accuracy of assaying the homogeneity of purified candidate HSC populations in mice by transplanting the cells at limiting dilution or in a competitive manner into **congenic** (genetically identical except at a single genetic locus or region) recipients has lead to mouse HSCs being the most closely identified stem cells of all. This type of experiments cannot for natural reasons be done in humans, where researchers are confined to surrogate **xenogenic**

(transplants across species borders) or *in vitro* assays. The quest to identify human HSCs is seemingly not helped much by gleaning information from mouse HSCs, since they differ to a large degree in antigen expression from their human counterparts.

HSCs do not express any lineage specific markers on the cell surface, so using a combination of antibodies which detect mature blood cell types to purify a **lineage negative** (**lin**$^-$) population will enriche HSCs 20-500 fold, depending on the combination of markers used[91]. **CD34** is a cell-surface sialomucin adhesion molecule expressed on hematopoietic stem/progenitor cells and on small-vessel endothelial cells, which was the first antigen identified that enrich for primitive blood cells[92]. It has been suggested to play a role in adhesion to stroma, but seems to have no essential role in hematopoiesis[93,94]. CD34 is expressed on approximately 1-5% of all nucleated hematopoietic cells and includes virtually all *in vivo* and *in vitro* clonogenic potential [95-98]. Positive CD34$^+$ selection is used in clinical HSC transplantations to deplete the cell inoculum of alloreactive T cells, and possibly also solid tumor cells. This allows the collection and subsequent transplantation of megadoses of CD34$^+$ cells, which can overcome histocompatibility barriers such as in **haploidentical** HSC transplantations (in which the donor and recipient shares only half of the immunogenic major histocompatibility complex (MHC), as for example parents donating HSCs to their children (see further the section on adaptive immunity))[99,100]. Another commonly used marker for human HSC selection is **CD38**, which has a dual function as a promiscuous enzyme and receptor, regulating cell activation and proliferation, and is also involved in adhesion between lymphocytes and endothelium[101], and is up regulated on most cells carrying lineage specific markers on the cell surface. A commonly used combination of markers for purifying human HSCs (for experimental purposes, not clinical as of yet) is therefore **lin$^-$CD34$^+$CD38$^-$**, which identifies 1-10% of the CD34$^+$ cells, and which seems to purify for most of the long term repopulating activity[102-104]. The lineage depletion step is often omitted, since lin$^-$ cells to a large

degree overlap with $CD38^-$ cells, with perhaps some myeloid markers as important exceptions[105,106]. But even this population is not homogeneous, but seems rather to be mainly composed of short term repopulating cells with only few long term repopulating stem cells[107]. Other markers implicated in enriching for human HSCs are **CD90** (**Thy-1**)[108-110](which is the only marker except CD34 that has been used in clinical HSC transplantations[111,112]); **CD133**, which overlaps with, but are not identical to, CD34[113,114], and which incidentally have been used recently to identify several cancer stem cell populations (see below)[115-117]; **CD117** (**c-kit**)[118]; **Vascular endothelial growth factor receptor 2** (**VEGFR2**, also known as **KDR**)[119]; **VEGFR1**[120], **CD105** (**endoglin**)[121] and **CD201** (**endothelial protein C receptor, EPCR**)[122]

Most, if not all, mouse HSCs are identified by the phenotype **$lin^-Sca\text{-}1^+c\text{-}Kit^+$** (**LSK**)[123], but long-term HSCs are only ~1/30 of this population[124]. Additional markers used to enrich for HSC activity in mice are CD34[18], **Flt-3**[125,126], **Thy1.1**[127], **CD105**[128] and **CD150** (**Slamf1**)[129].

The expression of CD34 in mouse HSC is reversible and dependent on the age of the mice, such that HSCs from adult mice in steady state are $CD34^-$, whereas HSCs in young mice, or HSCs challenged by cytotoxic drugs, are $CD34^+$[18,130,131]. The expression of CD38 is reciprocally expressed[132], and therefore, human and adult mouse HSCs have opposite expression of both these markers. Several recent reports on $CD34^-$ human HSCs was therefore met with considerable interest[133-137], although the biological and clinical significance of this population is unclear, not least since it seems to be very small (the number of repopulating $CD34^-$ cells are two orders of magnitude less than $CD34^+$ cells) [138,139].

Stem cells can also be purified by other, more functional, means than expression of cell surface molecules. One of the oldest methods used for enriching HSCs is based on the fact that stem cells are mainly quiescent (non-dividing). The addition of cytotoxic drugs (e.g. **5-fluorouracil** (**5-FU**)**,** or **hydroxyurea**) to a suspension of hematopoietic cells (or *in vivo* to mice) will therefore selectively kill

dividing progenitors while sparing the non-dividing stem cells[140-142]. Another useful aspect of stem cells is their propensity to actively remove toxic substances from the cell interior by **efflux pumps** on the cell membrane, a feature not shared by more mature cells, whereby certain fluorescent dyes can separate dye-effluxing stem cells from dye-retaining progenitors. By staining cells with, for example, **Rhodamine-123** (**Rho**), a dye that binds intracellularly to activated mitochondria and is expelled by **p-glycoprotein** (also known as MDR-1, which is the product of the *ABCB1* gene)[143] has it been shown that both mouse and human HSCs are $Rho^{-/low}$[144-146]. Another widely used stain, **Hoechst 33342**, is expelled from cells by another efflux protein, breast cancer resistance protein-1 (BCRP1, which is the product of the *ABCG2* gene), which is mainly expressed on HSCs[147,148]. Expulsion of Hoechst from immature BM and other tissue cells identifies a **side population** (**SP**) - so called because of the characteristic FACS profile - which allows for purification of SP cells[149]. These cells are highly enriched for HSC in mice and several other species [150], with mouse LSK cells in the tip of the SP population ("SP-tip" $CD34^{-}$LSK cells) showing the highest enrichment of long term repopulating cells thus far achieved at 96%[151], with, however, about half the HSC population residing outside of the SP population[122,152]. Human repopulating fetal liver HSCs are enriched by a factor 10 and completely confined within the $lin^{-}CD34^{+}CD38^{-}$ SP population, but enrichment was impaired by the toxicity of the Hoechst dye to the HSCs[153]. The role of the SP phenotype in human cord blood or BM is unclear. Finally, the enzyme **aldehyde dehydrogenase** (**ALDH**) is selectively expressed in primitive hematopoietic cells, and $ALDH^{+}$ cells, detected and isolated with FACS following addition of fluorescent ALDH-substrates, have been shown to enrich for both mouse and human HSCs[154-156], and has been used in clinical autologous transplantations[157].

In vitro assays

The claim that a prospectively isolated population of cells is purified for candidate HSCs needs to be confirmed by assays for HSC function, since surface markers, and also some of the more functional means of enrichment, can be deceiving (i.e. some markers are differently expressed depending on activation status of the cells or maturational level of the investigated individual[130,132]); furthermore, several markers have no obvious functional connection with stem cells, and can therefore, at least in theory, fluctuate independently of stem cell function[158]. The need for functional assessment is further substantiated by the fact that most mouse, and certainly all human, purified HSC populations enriched so far are heterogeneous with regard to stem cell activity. *In vitro* assays investigating the properties of progenitor cells measure two cardinal parameters; cell proliferation and differentiation potential – and these two parameters are very difficult to measure simultaneously (the "uncertainty principle" of stem cell biology), since different requirements for the growth of different lineages generally leads to the favouring of one progenitor population at the cost of others.

The *in vitro* estimation of primitive hematopoietic cells needs a culture system which extends beyond 3-5 weeks, since that is the time primitive cells need to produce differentiated progeny, and since it effectively avoids the contribution of more short-lived precursors from the starting cell population to read out. This is usually accomplished by co-culturing the test cells together with feeder cells (mainly irradiated BM mesenchymal or stromal cells), which provide a BM-like microenvironment with necessary (but largely unknown) stromal substrates, and in some cases molecularly engineered to produce human growth factors. One immature cell type detected in long-term cultures is the **long-term colony-initiating cell** (**LTC-IC**), which is defined by their capacity to produce daughter colony forming cells (CFCs) after 5-12 weeks of co-culture on stroma (during which time more short-term colony forming cells (CFCs) in the initial inoculum disappear). Two immortalized mouse stromal cell lines are commonly used

simultaneously as feeder cells in this assay: $M_2 10B_4$, which is genetically engineered to produce human granulocyte-colony stimulating growth factor (G-CSF) and human interleukin-3 (IL-3), and sl/sl, which produces human kit-ligand[159]. Another long-term assay detects **cobblestone area-forming cells (CAFCs)**, which integrates with and are directly scored in the co-culture as "cobblestone"-like colonies. LTC-IC and CAFC are overlapping but not identical, with LTC-ICs representing a somewhat more immature cell[160,161].

Short-term colonies are usually grown in semisolid media (which has a lifespan not extending beyond 3 weeks, and therefore excluding long term cultures) which fixates single cells and allows read-out of their immobilized progeny as CFC. Different lineage restricted progenitors can be identified and quantified by using different standardized culture conditions. The full potential of immature progenitor cells with regard to both quiescence and multipotentiality would require the combination of long-term and short-term assays, which have successfully been achieved to some extent in mice with so called **switch-cultures**, but have been hampered in the human setting, mainly due to the difficulty to grow lymphoid cells[162]. The golden standard for proving that a cell population contains stem cells, by showing both self renewal and multipotentiality, is *in vivo* transplantation. However, *in vitro* cultures are necessary to evaluate differentiation and proliferative potential in cells without self renewal capacity, which do not reach sufficient numbers *in vivo* for evaluation.

In vivo assays

The golden standard for assaying stem cell properties are *in vivo* transplantations. This is readily done in mice, by transplanting test cells from a donor to a congenic recipient, which is genetically identical to the donor except for one trait – for example sex difference, or by the expression of different isotypes of a commonly expressed cell surface antigen such as the pan-hematopoietic marker **CD45** (where

the two isoforms CD45.1 and CD45.2 often are used). The **competitive repopulating unit** (**CRU**, which essentially represents the HSC content of the test cell population) can be calculated by co-transplanting decreasing numbers of test cells (with a sex- or congenic marker differing from the recipient) together with a fixed number of radioprotective recipient cells (necessary for the rapid short term reconstitution) into a lethally irradiated recipient[163].

Subfractionated human progenitor cell populations cannot for evident reasons be tested in this way by transplantation to other humans, and instead xenogenic transplantation models (transplantation between different species) are used. The first successful transplantations of human cells into mice were done in **severe combined immunodefiency** (***scid***) mice[164], which are rendered immunodeficient by a mutation in DNA-dependent protein kinase (DNA-PK_{CS}) leading to failure of immunoglobulin rearrangements (see further below) and the production of very few B- and T-lymphocytes[165], and the **beige/nude/xid** (**bnx**) mice, displaying defects in their B-, T-, and NK cells[166]. To further increase the immunodeficiency, and concomitantly the tolerance for injected human cells, SCID mice were back-crossed to **non-obese diabetic** (**NOD**) mice, which displays defects in NK-cell, antigen presenting cell and macrophage functions[167], and these **NOD-*scid*** mice have been the most widely used strain for xenogenic human cell transplantations. Injection of unmanipulated or purified subpopulations of human hematopoietic cells in decreasing numbers into sub-lethally NOD-*scid* mice allows the identification and enumeration of **scid repopulating cells** (**SRCs**), similar to CRUs in mice. Although this model represents the gold standard for assaying human HSCs today, it is important to recognize its limitations. First and foremost, it is important to acknowledge the fact that the human cells assayed for their capability of sustained engraftment (perhaps even into secondary recipients) and multilineage engraftment are also selected for their capacity to home to, engraft and function in a xenogenic environment. For example, homing efficiency of human cells to the mouse BM seems to be less (5%)[168] than in corresponding congenic mouse

transplants (towards 100%)[151,169]. To what degree human HSCs and SRCs overlap is therefore unclear. In addition, NOD-*scid* mice display a bias for human B cell development; usually do not support human T-cell development; have a certain "leakiness", especially when getting older, for a small production of lymphoid cells; and are relatively short-lived and can therefore not be monitored for longer than about 6 months. The remaining NK cell activity, even if deficient, seems to impact on engraftment. This has been shown in NOD-*scid* mice where NK cell function is further impaired by injection of **interleukin-2 receptor β** (**IL-2Rβ**) antibodies[170], or by gene manipulation, such as in NOD-*scid* **β2-microglobulin**$^{-/-}$[171] or NOD-*scid* **γc**$^{-/-}$ [172,173]mice, where engraftment is enhanced, particularly by short-term myeloid progenitors (for a comprehensive review of available mouse strains accepting human grafts, see ref[174]). It is interesting to note that the state of immunodeficiency of the mouse strain is not the sole determinant of human cell engraftment, but also other, poorly understood, traits are influential. For example, mice completely lacking an adaptive immune system (B- and T cells) due to deletion of the genes encoding the common gamma chain and the enzyme rag2, do not support intravenously injected human cells (but they do proliferate if injected in the liver of newborn mice)[175,176].

A further xenogenic model for assaying human HSCs is the pre-immune fetal sheep model, where test cells are injected intraperitoneally (the abdominal cavity) into sheep fetuses at a developmental stage where they are old enough to accept and maintain hematopoietic cells, but before they have developed an immune system capable of rejecting the transplant[177,178]. This model allows the differentiation of all human hematopoietic lineages, and monitoring of individual engraftment over a number of years by serial BM aspirations. The method is, however, resource demanding and challenging to set up, and therefore not particularly widespread.

Hematopoietic hierarchy

The development and refinement of tools for detection, purification and assaying discrete subpopulations of hematopoietic cells have allowed the delineation of progenitors down-stream of the HSC (***Figure 1***). This has been done to a greater detail in mice than in humans.The multipotent hematopoietic compartment (which includes all cells which at the single cell level can produce all the mature blood cell

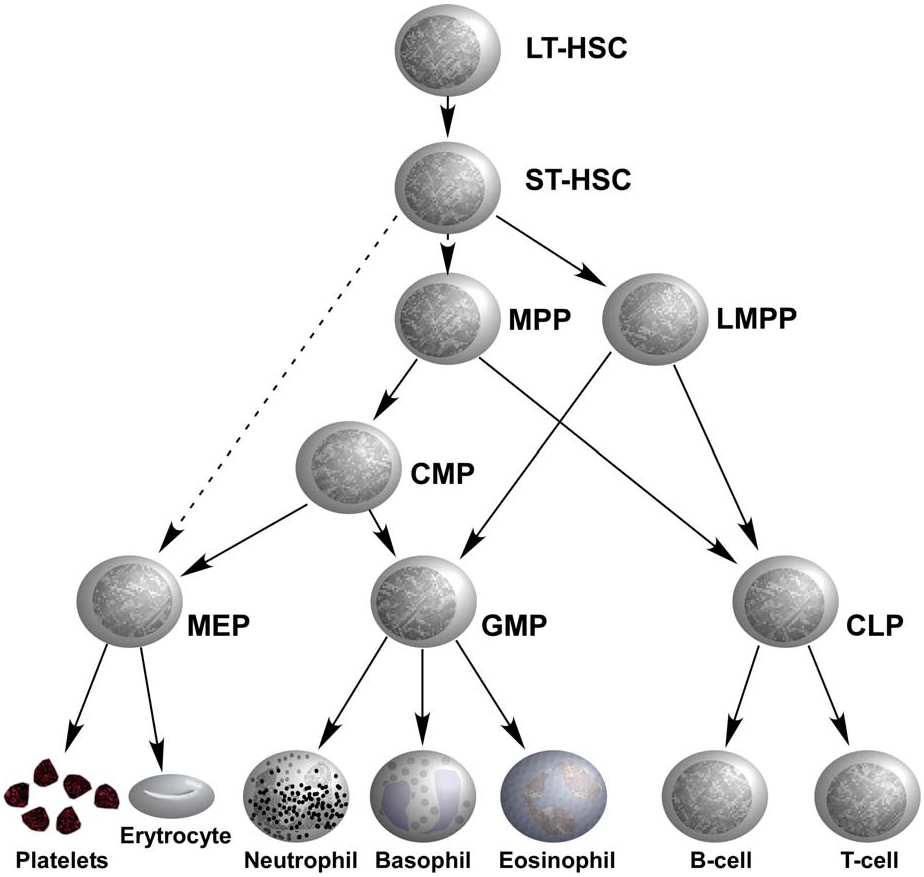

Figure 1. Cellular hierarchy of the hematopoietic system. HSCs produce intermediaries with restricted differential potential, which in turn produces the mature cells entering circulation. In the standard model is the first differential division between CMP and CLP. In an alternative modelis erythrocyte-megakaryocyte potential lost in the LMPP. The relationship between the LMPP and MPP is not clearly defined.

lineages) is heterogeneous and consists of cells with decreasing levels of self renewal capacity but increasing mitotic activity[13]. This compartment in mice can be phenotypically subdivided and prospectively purified into **long-term HSCs** (**LT-HSCs**, sustaining hematopoiesis for life), **short term HSCs** (**ST-HSCs**, sustaining hematopoiesis for up to 6 weeks) and **multipotent progenitors** (**MPPs**, without any self renewal capacity). A similar hierarchy is evident in humans, although it has not been phenotypically characterised[107,179]. For a long time, the first lineage branching point has been thought to be between myeloid and lymphoid cells. Common progenitors for these two cell populations (**common myeloid progenitor**, **CMP**, and **common lymphoid progenitor**, **CLP**) have been identified in both mice[180,181] and humans[105,182,183]. Recently, however, an alternative model has been proposed, where the first branching point is represented by a division between a common progenitor for red blood cells and megakaryocytes (the precursor for platelets) on the one hand and a progenitor with potential for all other lineages, called **lymphoid-primed multipotent progenitor** (**LMPP**), on the other[184]. An alternative way of interpreting the data has been offered though[185].

The CLP gives rise to committed precursors differentiating into the effector cells of the lymphoid system; B-, T- and NK cells, and the CMP differentiates into **granulocyte-monocyte progenitor** (**GMP**), the common precursor for granulocytes and monocytes/macrophages, and the **megakaryocyte-erytrocyte progenitor** (**MEP**)[105,180], although it is somewhat unclear if this last progenitor is a direct descendant from a MPP or from a CMP produced by a LMPP[184]). None of these oligoclonal progenitors have any measurable self-renewal capacity[13].

Fate and commitment

HSCs face, at any given moment, several possible fates. The choice most often taken at steady state is to remain **quiescent**, which seems to be a *sine qua non* for

stem cells in the adult organism. Other possible fates of a HSC are to **self renew**, **differentiate**, or to **die** (***figure 2***). The knowledge of how the fate of a HSC is regulated is very limited. It is even controversial whether fate decisions are **stochastic** (random)[186] or **deterministic** (governed by intrinsic or extrinsic cues)[187], although the prevailing view argues for a largely stochastic choice at the HSC level, while deterministic forces (which can be either **permissive** or **instructive** in their actions) gain importance during differentiation[188,189].

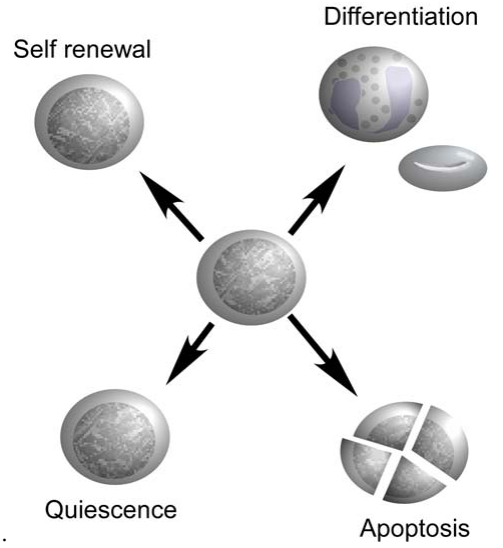

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Figure 2. HSC fates.

Quiescence

Most stem cells are quiescent, which might not be so surprising for stem cells residing in tissues with normally low cell turnover, like the central nervous system (CNS) and heart muscle, but perhaps more so for stem cells in tissues with very high cell turnover like blood and the intestinal mucosa. HSCs cycle asynchronously, with virtually all HSCs having cycled on average once every 2 months[190]. The fact that stem cells seldom cycle has led to their identification in several tissues by using different label retaining assays, or, in the case of HSCs, by

the use of cytotoxic drugs killing more proliferating cells, as explained above. One suggested reason for their quiescence could be to limit the chances of mutations to occur and to allow enough time to repair DNA damage[191].

A certain twist to the notion of quiescence as a fate choice comes from two competing models describing the dynamics of HSC usage, where the **clonal succession model** posits that quiescent HSCs are sequentially recruited to divide and differentiate, and when exhausted are replaced from a new set of HSCs activated from a quiescent state[192], while the other, and today preferred, **clonal maintenance** model postulates that, after an initial flux of clones following HSC transplantation, the engraftment is maintained by a few, stable clones for the life of the individual[179,193,194].

Self renewal

A stem cell can self renew by two profoundly different types of cell divisions; either by a **symmetrical division**, were both daughter cells are stem cells, and which therefore expands the stem cell pool, or by an **asymmetrical division**, were one daughter is a stem cell and the other enters differentiation, leading to a steady state with regard to the stem cell compartment. In a third, non-self renewing type of division is the stem cell symmetrically producing two differentiated daughters, which depletes the stem cell pool. Much has been made of the idea that asymmetric division is a characteristic trait of stem cells. However, it seems obvious that most, if not all, stem cells at times divides mainly (or perhaps even exclusively) symmetrically to expand themselves, as for example during embryonal development, or during wound healing or regeneration; and therefore this trait cannot be necessary for the stem cell identity [195]. Very little data exist on the mode of division of mammalian stem cells *in vivo*[195,196], and it is theoretically conceivable (even if unlikely) that stem cells upholds their activity mainly, or even exclusively, by symmetrical divisions, producing either more stem cells or more

differentiated progeny, depending on the needs of the organism. The scarcity and quiescence of most stem cells *in vivo* makes this question technically very challenging to approach.

Regardless of the mode of cell divisions, stem cells can perpetuate themselves and serve their host during a life time and beyond, and recent years have seen a dramatic progress in identifying signalling pathways influencing HSC self renewal, such as Wnt[197], Notch[198], sonic hedgehog (Shh)[199], Hoxb4[200], Bmi-1[201,202], Lnk[203], and thrombopoietin[204] (the loss of which is implicated in the BM failure syndrome congenital amegakaryocytic thrombocytopenia[205]) The use of extrinsic signalling to amplify HSCs, like cytokines and growth factors, has shown at best very limited expansion[206-210], with perhaps recent reports of purified Wnt3a protein[211], fibroblast growth factor-1 (FGF-1)[212] and angiopoietin-like proteins[213] expanding murine HSCs as notable exceptions. The quest for promoting HSC self renewal continues, however, since success could clinically benefit HSC transplantations were donor cells are limited in numbers (such as adult patients receiving cord blood transplants) or were large doses of HSCs are desired to overcome immunological barriers (such as for haploidentical transplantations). Mastering HSC self renewal can also enhance gene delivery to HSCs for gene therapy[214].

Differentiation

When considering that in man 10^{12} new blood cells are produced daily[215] (which roughly corresponds to the total hematopoietic cell content in the BM), an enormous expansion must obviously occur somewhere between the mainly quiescent HSC and the mature, non-dividing blood cell. The progenitors responsible for this are called transiently amplifying cells, and their regulation needs to be tightly calibrated to the needs of the organism. Our understanding of this regulation, and at which level in the hematopoietic hierarchy it occurs, is still incomplete. As mentioned in the previous section, it is believed that the HSC fate

decision of self renewal versus differentiation is mainly stochastic, and it has even been postulated that this randomness maintains the HSC contribution to all hematopoietic lineages in times when there is substantial needs for a particular lineage, such as during infection or severe blood loss[188].

The hematopoietic hierarchy, as outlined a few sections above, could in essence be created by the sequential addition of lineage specific transcription programs on the background of a *tabula rasa*, but a wealth of findings rather supports the opposite – a sequential restriction of available lineage choices as a HSC goes down the differentiation pathway[216]. In line with this, promiscuous expression of genes associated with lineage-committed hematopoietic cells (and also non-hematopoietic lineages) has been observed in purified HSCs[217-221], and expression of lineage specific genes prior to commitment has been called **priming**[218]. This expression promiscuity is thought to mirror a mainly open HSC **chromatin structure** (a complex of DNA and specific proteins regulating transcriptional accessibility of the DNA), enabling access to a wide variety of lineage affiliated programs[222]. When a HSC is fated for differentiation, it will sequentially up regulate lineage specific genes, but also, as importantly, abrogate the transcriptional program of the unselected lineages[223,224]. Primed and activated lineage programs fluctuate, interact and compete, and the ensuing lineage choice might be a threshold event which by its nature is probabilistic[223,225]. Cytokines and growth factors do not seem to influence the balance between lineage programs in uncommitted cells (since knocking them out in mouse models impede differentiation but not commitment) but rather support proliferation, development and survival of the committed cell, and can thus direct a probabilistic HSC output in certain lineage directions by clonal selection/suppression[188].

The “mainly open” chromatin of HSCs does not seem to involve lymphoid specific genes, which thus far have not been found expressed in HSCs[184,220,221,225]. Commitment to the B cell lineage is initiated by the expression of the B cell specific genes early B-cell factor (*EBF*) and *E2A*, which in turn cooperatively

induce the expression of several other B lymphoid specific genes. However, in the absence of a third transcription factor, *Pax5*, the B cell development is blocked at the pro B cell stage, and due to continuous co-expression of myeloid genes, this cell can instead differentiate into several other lineages, such as macrophages, dendritic cells, granulocytes and T cells. Upon restoration of *Pax5* expression the myeloid genes are down regulated and B cell associated genes such as CD19 and Igα are induced, and the cells loose their potential towards other lineages rather than the B cell lineage[226]. So it seems that the decision to differentiate might be partly independent of the lineage fate adopted[227], and the transition from uncommitted to lineage biased to lineage restricted might be floating and hard to pin down as a discrete developmental step[228]. After conditional inactivation of *Pax5* in early committed B cells, the capacity to differentiate into alternative lineages is regained together with the expression of myeloid genes[229], indicating that the commitment to differentiate, elicited by *E2A* and *EBF,* needs *Pax5* to downregulate myeloid associated genes and upregulate genes promoting B cell development in order to stabilize the lineage choice.

Apoptosis

The *ars moriendi* of unwanted cells by apoptosis, the clean and swift disappearance following intrinsic or extrinsic suicide signals, is vital to development and maintenance of all life. Apoptosis sculptures our bodies by carving out the empty space between our digits or the inner parts of tubes to create lumina; it moulds our central nervous system into a functional fabric of interconnecting cells by removing great numbers of excess neurons and oligodendrocytes; and it controls our immune system by eliminating B and T cells producing dangerous self-reactive receptors or failing to produce useful antigen-specific receptors[230]. Deregulation of apoptosis leads to pathological conditions as diverse as neurodegenerative diseases, autoimmunity and cancer[231,232].

Apoptosis has been suggested as an important alternative fate for HSCs[189,233]. The observation that overexpression of the anti-apoptotic regulator *Bcl-2* leads to an increase in size of the HSC pool, and enhance the competitiveness *in vivo* of these cells, indicates a role for apoptosis in regulating the HSC compartment[234]. However, BCL-2 does not seem to be the main regulator in HSCs, but rather its homologue BCL-X_L[235]. Fas, a surface receptor relaying death signals to the interior of cells, is not expressed on HSC in steady state, but can be induced by interferon γ, tumor necrosis factor α (TNFα) or culture[236-239]. Interestingly, it seems to exert dual roles on immature progenitors by promoting survival of human $CD34^+CD38^-$ candidate HSCs while inducing cell death in more mature $CD34^+CD38^+$ progenitor cells[240]. The extent of HSC demise or the signals regulating it under physiological conditions remains elusive, however.

At home – the niche

It has become evident that at least some stem cells are not solo artists starring on the stem cell biology stage, but are merely players of a team of cells and substances in a much larger and more complex system, deployed to maintain and repair tissues during an organism's life-time. The study of the integration of stem cells and their progeny into tissues has taken use of the ecological concept of "niche", which describes a living entity's specific interaction with its environment. A hallmark of a functional stem cell niche is then to balance activity and quiescence, or self-renewal and differentiation, in such a way that enough progenitors are produced to answer the need of the organism, while safeguarding against an excessive stem cell proliferation which would pose a risk for malignant transformation[241,242].

The concept of stem cell niches was first proposed by Schofield almost 30 years ago, in reference to mammalian hematopoiesis[87], but experimental evidence has only recently emerged, first in invertebrate models. The *Drosophila*

melanogaster (fruit fly) has a tightly regulated number of germ line stem cells enveloped in small pockets of cells constituting a stem cell niche (Cap cells in the ovary and Hub cells in the testis), that physically holds on to the stem cells, and delivers signals that block their differentiation. Under normal, steady state conditions, as the germ stem cell divides, one daughter cell remains under the constraints of the niche, whereas the other is displaced from the niche, and starts moving down the developmental pathway towards differentiated sperm or eggs[243,244]. The fate decision for the two daughter cells is partly taken by the orientation of the mitotic spindle, such that one daughter remains connected to the niche while the other "buds out" of it[245]. In case one or more germ stem cells are missing within the niche, the spindle orientation can cause both daughters to remain within the niche. Interestingly, an empty germ line stem cell niche can support proliferation of ectopic cells[246] and even revert progenitor cells to a stem cell like state[247]. Niches have subsequently been described for several other tissue stem cells, such as neuronal, hair follicle, intestinal and blood stem cells (for reviews see[241,244,248,249])

Although the niche concept was coined in conjunction with HSCs, a deeper understanding of the hematopoietic niche (or niches) has only recently emerged. Morphological and functional studies have indicated that HSCs and immature colony-forming progenitors reside in relation to the bone surface, away from the central cavity[250-252]. It has also been observed for some time that the bone tissue, and particularly the bone-forming **osteoblast**, by itself can support HSCs in co-cultures[253-255], and mouse models where osteoblastic differentiation has been blocked show a complete absence of hematopoietic activity within the BM[256,257], implicating the osteoblastic lining of the BM cavity as the anatomical location of the HSC niche. In two important papers, published back-to-back in the same issue of *Nature* 2003, genetically engineered mouse models where used to convincingly show the osteoblast dependency of HSCs[258,259]. In the first, Zhang *et al* [258]observed that in mice, in which the bone morphogenetic protein receptor type IA (BMPRIA)

were conditionally inactivated, an increase in HSCs correlated with an increase in trabecular bone lining within the BM. Histological analysis revealed that long-term HSCs attached only to immature, spindle-shaped osteoblasts expressing N-cadherin, and that N-cadherin adhered specifically to β-catenin on HSCs. The number of the spindle shaped osteoblasts correlated to a high degree with the increased number of HSCs, suggesting that the rise in HSC numbers where due to an expanded niche. In the second paper, Calvi *et al*[259] utilized the fact that osteoblasts are activated by parathyroid hormone (PTH), and constitutively expressed the PTH receptor under the control of a osteoblast-specific promoter (α1-collagen promoter) in mice. They observed, similar to Zhang *et al*, a correlation between increased osteoblasts and an increase in HSC numbers and function. In both papers, the increase in hematopoietic cells was restricted to HSCs. A third group reported the complementary finding, that induced osteoblastic deficiency was followed by reduction of HSC numbers, and that restoration of osteoblast function normalized hematopoiesis[260].

However, other cells than osteoblasts must also be able to support HSC function, since hematopoiesis continues in vivo in the absence of osteoblasts[260], and can be maintained over time outside of the bone in tissues like spleen and liver[261]. Recently it was suggested that **sinusoidal endothelial cells** (cells creating the vessels in BM and elsewhere) might constitute a second major niche for HSCs[129,262,263]. The cells in contact with HSCs are discontinuously dispersed along the vessels, and express high levels of chemokine ligand 12 (CXCL12, also known as stromal cell-derived factor 1 (SDF-1), a chemokine known to be important for homing of HSCs to the BM (and which also happens to function as the entry receptor for HIV-1[264]) and E-selectin[262,263]. Although far more HSCs reside attached to these endothelial cells than to osteoblasts, very sparse evidence for any specific function of this niche exist. It might be just a way-station for HSCs mobilizing from the osteoblastic lining, or it might regulate stem-cell function in specific ways. Megakaryocyte maturation and thrombopoiesis have been reported

to be dependent on BM endothelial cells[265], but whether similar dependence of other hematopoietic cell types on the endothelial niche exist is not known. It has been suggested that HSC at this vascular niche might be more cycling, and therefore a more readily accessible pool for maintaining cellular output, than the more quiescent HSCs residing attached to the endosteal lining[266-269]. The suggestion that the low oxygen tension within BM is a means for the HSC niche to keep potentially dangerous radical oxygen species (ROS) low to protect against negative effects on HSCs[241,270] would be in line with the suggestion that the more cycling (and therefore more vulnerable) HSCs should reside in the vicinity of vessels, benefiting from a better supply of oxygen, nutrients and circulating growth factors. Their cycling status would also explain why they are not readily detected at sites outside of the endosteal lining by label retaining assays[258,271].

Although the composition of the niche in terms of cells, matrix and fluid seems comprehensible, the connections and interactions between these players and their modes of signalling are complex and still poorly understood. Whether asymmetric stem cell divisions in BM is regulated by differences in location of the two daughter cells relative to the niche, as has been described for the *drosophila* germ stem cells, is unknown (it is, in fact, not even known whether HSCs divide symmetrically or asymmetrically in steady state). But cues other than orientation which has been implicated in the maintenance and function of HSCs within the niche are for example signalling molecules like Jagged/NOTCH[259], membrane bound stem cell factor (SCF, kit ligand)/c-kit[272], Tie2/Angiopoietin-1[271,273], chemokines like CXCL12/CXCR4[263,274], and adhesion molecules like osteopontin/β_1 integrin[275,276] and N-cadherin/β-catenin[258,269,272,273].

The role of the niche is thus to maintain the HSC compartment, but a further function seems to be to control the traffic of HSCs in and out of the niche. HSCs are known to briefly exit the BM into the circulation and home back to BM[277] (although it has been argued that the exiting might often not be followed by homing again[278]). This reflects the physiological basis for therapeutical stem cell

transplantation, in which HSCs from a donor is injected into a recipient, whose blood forming system is ablated by prior irradiation and/or chemotherapy, and were they eventually home to the BM and reconstitutes the hematopoietic system. From a developmental point of view (as will be discussed in a later section) it is not so surprising that HSC travel through the circulation to take residence at other sites, since the entire hematopoietic system moves from the yolk sac to the liver and finally to the BM during embryonal development - but it is less obvious why that should happen in the adult organism. Perhaps it is just a secondary consequence of permanent bone remodelling[269], or it is a means for the hematopoietic system to replete niches reduced in HSC content due to trauma, disease, random non-self renewing HSC divisions, or other reasons. A few ways for the organism to physiologically regulate this process has recently been elucidated. For example, the increased calcium ion concentration in BM (due to the normal bone turnover) regulates the lodging (but not the homing) of HSCs within the niche by affecting the seven-transmembrane Ca-sensing receptor on HSCs[279]. Nervous signalling in the form of noradrenergic release within the BM has been described to, somewhat unexpectedly, impact HSC release by negatively regulating osteoblasts and CXCL12 expression[280], and thus integrating tissue signalling with niche function. The ability of circulating HSCs to home back to the niche depends on the chemoattractant activity of CXCL12/CXCR4, and has been used for decades in HSC transplantation - but also the ability to migrate out of the niche is increasingly used clinically. By pre-treating the HSC donor with G-CSF and/or certain cytotoxic drugs (i.e. cyclophosphamide) HSCs briefly mobilizes into the circulation following degradation of the CXCL12/CXCR4 axis[281,282], increasing the circulating pool of $CD34^+$ cells from <0,1% to >1%, which allows harvest of sufficient numbers of HSCs through leukapheresis directly from the circulation[283]. This is today the preferred way of acquiring HSCs for transplantation, because of the relative ease for the donor as compared to BM aspiration, and since mobilized

HSCs generally engrafts faster, and thus shortens the dangerous neutropenic phase following myeloablation of the recipient[283,284].

If the niche is fundamental for HSCs, could the same be true for leukemic stem cells (LSCs), or any cancer stem cells (CSCs)? It is not known whether LSCs depends on a niche for self-renewal, but they share at least some features with HSCs, such as responding to CXCL12 by co-localizing with HSCs in their niche[262,263,285,286]. It is intuitively appealing to believe that CSCs, which seem to resemble normal stem cells in so many ways, would be also dependent on a niche, and this notion is supported by the observation of BM derived cells creating a pre-metastatic niche for subsequently incoming tumor initiating cells[287], and by the dependence of chronic myeloid leukemia (CML) and acute myeloid leukemia (AML) stem cells (but, interestingly, not normal HSCs) on the actions of CD44, a cell-surface molecule important for cell-cell adhesion[288,289]. A differential dependence on the niche between normal and malignant stem cells (such as for CD44 and PTH) could implicate new, highly interesting targets for treatment of cancer[290,291].

Hematopoietic development

In all vertebrates, hematopoietic development is occurring in two successive waves, primitive and definitive hematopoiesis, the first from the extra-embryonic **yolk sac** (**YS**) and the second from the developing vessel wall in a structure called the **aorta-gonads-mesonephros** (**AGM**) region within the embryo proper. In mice, the first blood cells are primitive nucleated erythroid and macrophage precursors, detected in the YS at embryonic day 7.5 (E7.5). They enter circulation between E8,5 and E9, after which time the hematopoietic potential of the YS disappears. The second wave is detected in the AGM region at E8.5-E9 and disappears by E13. HSCs circulates and seeds the fetal liver (which is not believed to form any hematopoietic progenitors by itself) at around E10, and thereafter develops as the

major hematopoietic organ until birth, after which time the BM takes over this role throughout adult life[292,293].

It seems that the two waves of blood forming elements arise separately[294], but their respective contribution to definitive hematopoiesis has been controversial for decades. YS blood cells do not long-term reconstitute irradiated adult recipients, while AGM derived hematopoietic cells do. It seems, however, that YS cells can acquire a long-term reconstituting potential if let to mature in culture with AGM-derived stromal cells, or even when passaged through the fetal liver in transplant experiments with newborn recipients. It is, however, still unclear if both cell types in the end effectively colonizes the fetal liver[295].

Several molecular cues important for the early hematopoiesis have been identified by targeted disruptions of various transcription factors. A hierarchical importance through the early phases of blood formation is evident by the absolute requirement of SCL/tal, Lmo-2, GATA-1 and GATA-2 for the initiation of both primitive and definitive hematopoiesis[295], while RUNX1 (also known as AML-1) is necessary for definitive but not primitive hematopoiesis[296], and ETV6/Tel for the establishment of hematopoiesis within the BM, but not for YS, AGM or fetal liver hematopoiesis[297].

The knowledge of human hematopoietic development is less detailed due to scarcity of very early embryos and limitations of functional assays for human hematopoietic cells, but available data suggests that the human program closely parallels the mouse program in both sequence and anatomy[298]. Blood formation is initiated in the YS at E16, and disappears before E60, while AGM hematopoiesis occurs between E27 and E40. Importantly, hematopoietic LTC-ICs can be detected as early as E19 in the splanchnopleura, a structure preceding the AGM in the embryo proper, 3 days before onset of circulation, indicating that YS and AGM hematopoiesis arises separately in the two locations also in man[299]. Hematopoiesis is transferred to the liver at E30, which is the major hematopoietic organ from E42 to 20 weeks of gestation, after which time hematopoiesis occurs in the BM[298].

HSCs change phenotype and function through ontogeny. The aging HSC pool increases in size and self renewal capacity but suffers a measurable functional decline, such that stem cells from a younger source always has a competitive repopulation advantage over stem cells from an older donor[300]. Older HSCs are also skewed in myeloid direction with a diminished lymphoid potential and a loss in immune function as a result[301]. Recently a steady increase with age of the tumor suppressor $p16^{INK4a}$ was suggested to cause most of this ageing phenotype in HSCs[302].

Hematopoiesis

The cells

The ultimate importance of stem cells reside in their progeny, the mature, non-dividing effector cells, which leave the BM and enters circulation to perform their diverse, life sustaining activities. The cellular content of blood is sometimes seen as a floating organ, but should perhaps be seen as several distinct organs, sharing only their ancestry and the capacity to perform their functions as single cells dispersed throughout the body, but otherwise as different in their purposes and actions as, say, the heart and the pancreas. Three distinct cellular compartments sustain the life of the organism by performing three distinct and vitally important functions. First, the **erythrocytes**, or the red blood cells, which as mature cells have lost their nucleus, and therefore have no means to answer to cues from the environment. They are delivered to the blood stream armed with haemoglobin, a molecule that colors blood red, and which transport oxygen to all tissues. Oxygen is absolutely necessary for all cells as a source of energy, and therefore there are few living cells in the body (with corneal cells as a notable exception) who survives at a distance greater than 0,2 μm from a capillary. Second, the platelets, the smallest of all blood cells, also without a nucleus, which patch vascular leaks, and without which we would be at constant risk for fatal bleedings. Third, the

white blood cells, which constitute the immune defence. The immune system in higher organisms is quite complex, and consists of several specialized subsystems accumulated during evolutionary time, with diverse functions performed by specialized cells.

Cancer in the hematopoietic system arises almost exclusively from cells in the third group, the white blood cells making up the immune system, with **erythroblastic leukemia** (affecting the lineage leading to red blood cells) and **megakaryocytic leukemia** (the platelet lineage) as very rare exceptions. Therefore my focus below will be on the diverse white blood cells in general, and on a specific subset of white blood cells, the lymphoid cells, in particular, as the leukemic counterpart of these are the main subjects of study in this thesis.

The immune system

(As general references on the sections on the immune system, see[303,304]). Immunity is conferred by a complex system of barriers, cells and chemical compounds, which interact to protect the host from invading microbes, but also detects and destroys tumor cells and inactivates certain toxins. The immune system is commonly considered to consist of two components, which differs in immunologic specificity and time to respond. The **innate immunity** represents the phylogenetically oldest component and the first line of defence against invasion. Its functions are based on a broad reactivity against common themes displayed by invaders, and are not specific against any particular pathogens. The very first line of defence is made up of physical and physiological barriers, such as the integrity of the skin, entrapment and anti-microbial activity of mucus on mucus membranes, fever (which confers an inhospitable condition to some pathogens), acid secretions (such as gastric acid to the stomach content and sebum produced by sebaceous glands on the skin), and various soluble or cell-associated molecules with the property of **pattern recognition** (which is the ability to recognize and interact with

classes of molecules usually not found in multicellular organisms, but are commonly displayed by microbes) like the **complement system** or **Toll-like receptors** on macrophages[305]. The cellular part of the innate immunity consists of **phagocytes**, a group of cells with the capacity to ingest and digest exogenous cellular or particulate antigens, and endogenous matter such as dead cells or cell debris. The **monocytes** are phagocytic cells produced in the BM and resides only briefly (~8 hours) in the circulation before they enter into tissues and differentiates into tissue **macrophages** or **dendritic cells**. The macrophages can either remain motile and migrate through the tissues, or be fixed in certain tissues, and are then given different names depending on the tissue in which they take residence, for example histiocytes found in connective tissues, Kuppfer cells in the liver, mesangial cells in the kidneys, microglial cells in the brain and osteoclasts in bone. Myeloid cells, or **granulocytes**, are divided into **neutrophils**, **eosinophils** and **basophils**, of which only the neutrophils and eosinophils are phagocytic. The neutrophils are the most important cells in the immediate cellular defence against bacteria and fungi, partly because of their numbers, and partly because they are the phagocytic cells most likely to kill the microbes they have ingested. It is a fact in clinical practice that an extended neutropenic phase after for example cytotoxic therapy inevitably leads to infection by bacteria and/or fungi. All of these players are important in their own right, but together they also contribute to activate an even more powerful and versatile part of the immune system – the **adaptive immunity** of lymphoid cells

Adaptive immunity

Adaptive immunity is from an evolutionary point of view a more recent component of the immune system, abruptly appearing in jawed fish (were sharks are the phylogenetically oldest member) and then in all higher vertebrates, while even plants can mount some form of innate immunity. Adaptive immunity offers, in

contrast to the innate system, a highly specific targeting of virtually every possible antigen, and a subsequent memory of the assailant, allowing an augmented response when reencountered. The main actors are the two kinds of lymphoid cells; **B lymphocytes** (**B cells**) and **T lymphocytes** (**T cells**). They differ in what antigens they recognize, such that B cells are activated by antigens located outside of the cells in the body, while T cells reacts against intracellular antigens, such as for example viruses. They also differs with regard to what kind of responses they evoke, with B cells producing **antibodies** (**immunoglobulins, Ig**), antigen specific molecules which either binds to and inactivates antigen (such as bacterial toxins or virus) or coats pathogens and thus mark them for destruction by macrophages or complement by a process called **opsonisation**; while T cells display a more diverse array of responses, including direct killing of antigen presenting cells or activation of B cells or macrophages.

The great antigen diversity and specificity of lymphocytes rests on their antigen receptor molecules; in B cells called the **B cell receptor**, which in its membrane-bound form acts as a recognition and activation molecule, and which is secreted as antibodies from the activated B cell; and the **T cell receptor**, a molecule closely related to immunoglobulin but always membrane-bound and never secreted, which acts by recognizing antigens and activating the T-cell. The mechanism by which the diversity of antigen specificities is created for these molecules is unique, since the genes describing the antigen receptors are not laid down as such in the germline, but uniquely produced by every single B- and T cell in an active process of randomly rearranging gene-segments to a unique sequence. This means that a single B or T cell has specificity towards only a single antigen, and the great diversity within the system is the result of billions of different lymphocytes displaying different antigen receptors.

The B cell receptor can recognize antigen directly, but the T cell receptor can only recognize antigens degraded to small peptide molecules inside other cells and presented on the surface embedded in a large molecule called **the major**

histocompatibility complex (**MHC**, referred to as the **HLA complex** in humans, or the **H-2 complex** in mice). There are two classes of MHC molecules; **class I MHC** (**HLA-A, B** and **C**) molecules are expressed on virtually all cells and activates cytotoxic T cells, whereas **class II MHC** (**HLA-DP, DQ** and **DR**) molecules are expressed by antigen-presenting cells and activates mainly T helper cells (There is also a class III MHC, which includes molecules critical for immune function, but do not share structural similarity or functions with class I or II molecules). The MHC molecules are polymorphic (have different amino-acid content between individuals) and since they are central in activating T cells constitutes the major immunological barrier for transplantation of organs or tissues between individuals.

Recognition of an antigen by a B or T cell receptor is not enough to activate the lymphocyte to become an effector cell, but a second **co-stimulatory signal** is needed. B cells encountering antigens internalizes the antigen-B cell receptor complex, processes the antigen and presents it on the surface to a helper T cell, which in turn activates the B cell by the interaction of CD40 (on the B cell) and CD40L (on the T cell). T cells, on the other hand, are activated by **antigen-presenting cells**, particularly mature dendritic cells, but also macrophages and B cells, displaying the antigen together with a MHC class II molecule and a co-stimulatory signal (CD80 or CD86, interacting with CD28 on the T cell) . The innate and the adaptive immune systems are thus intimately interconnected.

After a lymphoid cell has been activated by encountering an appropriate antigen and has received the necessary co-stimulatory signal, it will proliferate and thus cause a clonal expansion of lymphocytes with the necessary antigen specificity. Activated B cells can differentiate into **plasma cells**, which produce and secrete antibodies with the same antigen specificity as the B cell receptor, and T cells differentiate into either **cytotoxic T cells**, recognizable by their cell surface expression of CD8, and which detects and kills cells containing pathogens, or into **helper T cells**, expressing CD4 on the surface, which activates other

immunoactive cells by secreting cytokines or by direct cell-cell signalling. Both B- and T cells can also differentiate into **memory cells**, which confer immunological memory by reactivation when encountering their specific antigens again.

Immunoglobulin gene rearrangement

Antibodies and T cell receptors are similar in structure and in the way they are rearranged. Both are made of two different polypeptides (a duplication of a **light chain (IgL)** and a **heavy chain (IgH)** in antibodies, which corresponds to a **α**- and **β chain**, or rarely a **γ**- and **δ chain**, in the T-cell receptor), each with a **variable region**, which confers antigen diversity and recognition function, and a **constant region**, which defines the **isotype** of the antibody (and therefore its specific function), but has a negligible role in the T cell receptor (***Figure 3***). The rearrangement of the immunoglobulin genes will be discussed below, but the principles are the same for the T cell receptor.A complete antibody molecule consists of two identical IgH and a pair of IgL, of which there are two types,

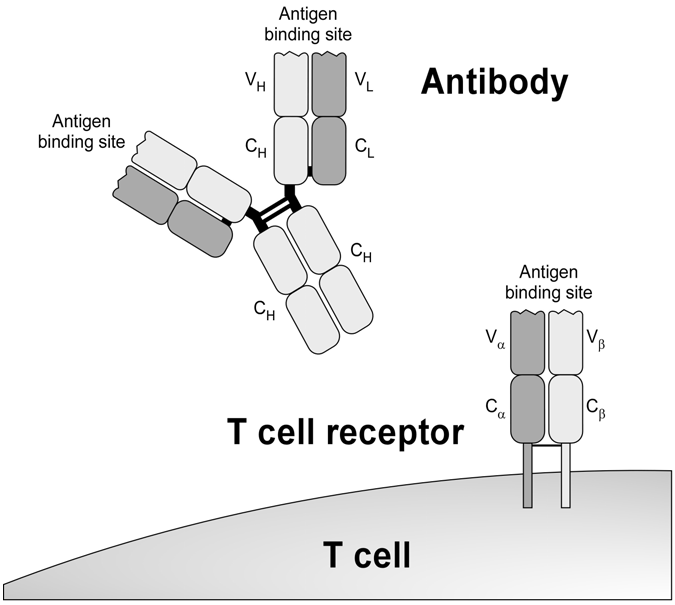

Figure 3. Structure of an antibody and a T cell receptor.

termed **kappa** (**κ**) and **lambda** (**λ**), which are mutually exclusive on single mature B cells. The genes for these molecules are located on different chromosomes (IgH on chromosome 14, Igλ on 22 and Igκ on 2), and exist as loci containing many alternative gene segments which can be randomly joined to produce a specific antibody. Three different gene segments are needed to produce an IgL molecule: one for the constant region, called the **C_L gene segment** (where the L indicates that it is the light chain), and two for the variable region, called **variable** or **V_L gene segment** and **joining** or **J_L gene segment**. The IgH gene also contain a C_H gene segment (with H denoting Heavy), but has 3 variable segments, with a **diversity** or **D_H gene segment** in between the V_H and J_H segments. There are multiple copies of the gene segments describing the variable region, which are randomly selected by **somatic recombination** of separate gene segments (***Figure 4***). Only one gene segment is rearranged at a time, in a fixed order; first IgH is rearranged, followed by IgL. During this process, all other segments are deleted, leaving the B cell with

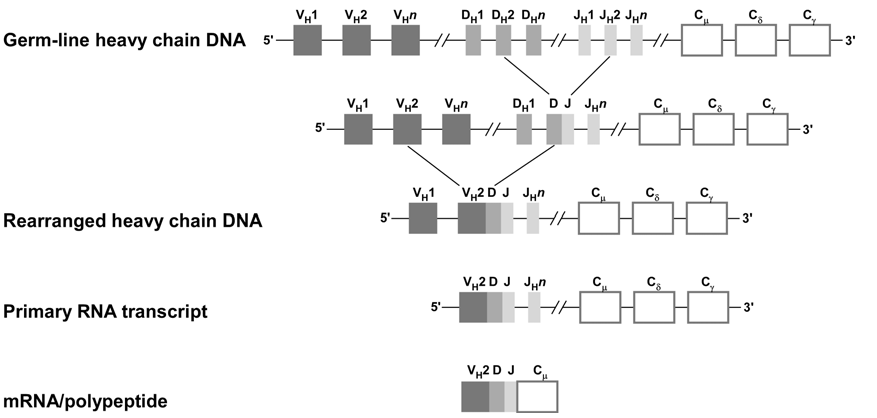

Figure 4. A simplified view of the rearrangement process of the heavy chain (IgH). There are multiple gene segments to choose from – in humans there are 40 V_H, 25 D_H and 6 J_H genes – numbers which can vary slightly between individuals. The recombination process is ordered, such that first is a D_H segment joined to a J_H segment, and then is a V_H segment joined to the D_HJ_H sequence. The process is similar for the light chains (IgL), but the V_L segment is joined directly to a J_L segment.

the gene sequence for only one, unique antibody. The C_H segment is also available as several copies, but these are not somatically recombined and do not contribute to antigen detection, but rather specifies the isotype of the immunoglobulin molecule. B cells can change isotype of secreted immunoglobulins by alternative splicing at the RNA level, so that translation of C_μ, for example, results in an IgM molecule and C_γ translates into an IgG molecule. These different isotypes have different functions, and are secreted at different anatomical localisations, but they all have the same antigen specificity.

The diversity of the immunoglobulin molecules is created by the random combinations of V_H, D_H, J_H and V_L and J_L segments, which theoretically results in $1.9x10^6$ different antibody molecules. Diversity also increases by the random combination of IgH and IgL pairs. The random addition of up to 20 nucleotides at the junction between IgH segments by **terminal deoxynucleotidyl transferase** (**TdT**) increases the total number of possible antibodies to as many as 10^{11}. The actual number of different antibody specificities created by these combinatorial diversity mechanisms in an individual at any given time is, however, substantially less. Finally, a further increase in diversity is caused by **somatic hypermutation**, which introduces point mutations at a high rate into the V regions of IgH and IgL in activated B cells – a process leading to the selection of some B cells expressing antibodies with an increased affinity for their antigens, and therefore called **affinity maturation**, which is one reason adaptive immunity against a particular antigen increases in strength with time[306].

The specificity and diversity of the adaptive immune system adds power to the overall protection against pathogens. But considering that invertebrates, lacking an adaptive system, thrive and remain healthy in the face of the same microbial challenges as the more developed vertebrates invokes the question: what is the evolutionary need for an adaptive system, were the risk of autoimmunity is a price to be paid? One function, only partly covered by the innate system, is to recognize and destroy malignant cells displaying aberrant antigens on the surface. Another

recently proposed function could be to recognize and manage complex communities of beneficial microbes residing in or on all mucosal linings in vertebrates[307].

Lymphoid cell development

Both B- and T cells are descendants from the HSC within the BM, and seems to share a common progenitor for a short while, the CLP[308]. B cells continue their differentiation within the BM after commitment, while T cell progenitors mature in the thymus. B cell development will be described at some detail, since their leukemic counterparts are the focus of this thesis, while the T cell development, although more complicated, will be briefly discussed.

The first precursor cell restricted to the lymphoid lineages is the CLP, which has been defined in mouse as cells displaying the phenotype $Lin^{-}Sca\text{-}1^{lo}c\text{-}kit^{lo}Thy\text{-}1^{-}IL\text{-}7R\alpha^{+}$[181], but which is poorly defined in humans due to limitations of clonal lymphoid assays and differences in antibodies/fluorochromes giving contradictory results. A proposed composite phenotype would be $Lin^{-}CD34^{+}CD38^{+}CD45RA^{+}CD7^{+}CD10^{+}$, where $CD7^{+}CD10^{-}$ cells are skewed towards T/NK lineages, $CD7^{-}CD10^{+}$ cells towards B lineage and the small $CD7^{+}CD10^{+}$ fraction possibly represents the precursor, although this awaits prospective purification and clonal analysis[182,183,308-310]. The further progression towards mature B cells is defined by the rearrangement of the immunoglobulin genes and the sequential expression of transcription factors and surface molecules. The fact that immunoglobulin gene rearrangements and expression of surface markers are not fully synchronized leads to a sometimes confusing terminology, depending on if the stage ascribed to a B cell population is defined by surface markers or the progression of immunoglobulin gene rearrangement. B cell development in mice is elucidated in much greater detail than in humans, and the terminology and

discrete steps does not necessarily overlap in all instances. The description below refers to human B cell development.

Early B cells are cells in transit between the CLP and B cell commitment, which does not seem to constitute a discrete step, but rather a progressive restriction of potentials other than the B lineage. These cells are $CD10^-$ and $CD19^-$ and have often initiated DJ_H rearrangement and express B-lineage specific proteins such as VpreB, Igα (CD79a), EBF, E2A, TdT and Rag-1 but do not express Pax5[311,312]. Cells with this phenotype illustrate a stage of lineage bias before full lineage restriction since they can be forced to differentiate into macrophages, NK cells and T cells, while retaining the DJ_H rearrangement[312]. How often, or if at all, a B cell biased progenitor actually changes fate into another lineage in vivo is not known. The next stage, represented by **Pro B cells,** is defined by the expression of CD19 and CD34, but lacking cytoplasmatic or cell-surface μHC (the heavy chain protein resulting from a completed IgH rearrangement). IgH is actively rearranging during this stage, although a few pro B cells have IgH in germline[313] while 5-10% of purified CD34+CD19+ pro B cells display cytoplasmatic μHC[311]. As Pax5 is locking mouse pro B cells into the B lineage fate and concomitantly induces the expression of CD19, it seems that CD19 would be the definitive marker including all "true" B cells[226,229]. This notion is supported by the finding that CD19 is never turned on, even transiently or promiscuously, in any other cell than B cells *in vivo*[225]. A pro B cell must successfully rearrange its IgH gene to proceed to the next stage; otherwise it will die by apoptosis. The **pre B cell** looses expression of CD34, and displays the VDJ_H rearrangement product as μHC in the cell interior or on the cell surface, where it forms together with a surrogate light chain (φLC, composed of the IgL-like proteins VpreB and λ5) the pre B cell receptor, which signals several rounds of division prior to the rearrangement of the IgL genes[313]. Upon completion of a functional IgL rearrangement (either Igκ or Igλ) a fully formed immunoglobulin molecule (IgM) is displayed on the cell surface, which signifies the transition to **immature B cell**[308,311]. This cell is not yet

immuncompetent, since it is not reactivated upon antigen challenge, but rather dies after crosslinkage of its surface IgM molecules, which effectively removes self-reacting developing B cells. Next it develops into a **naïve mature B cell,** which displays both IgM and IgD on the cell surface. When encountering an appropriate antigen it is activated and can become either an immunoglobulin-secreting plasma cell or a long-lived memory cell.

T cells need the stroma of the thymus for proper development. The nature of the BM derived progenitor cell which seeds the thymus and becomes T cells is still obscure[314]. Post-commitment T cell development parallels B cell development in its dependency of sequentially completing the different stages of gene rearrangement in order to progress to the next stage, with concomitant characteristic changes in cell surface phenotype. There are, however, more choices for the developing T cell as it can turn into CD4 helper T cells or CD8 cytotoxic T cells, and possibly also suppressor T cells, and can rearrange the T cell receptor genes in two distinct ways, producing the more common αβ T cells, or a small, non-MHC restricted γδ T cell subset. Upon activation are T cells turned into effector T or memory T cells (similar to plasma cells and memory cells in the B lineage.).

Immunity is in many cases life-long, and depends upon clonal B and T cells. This is accomplished by the memory lymphocytes, which can self-renew or differentiate into at least two distinct populations of cells (new memory cells or effector cells), and therefore formally fulfils the criteria of being stem cells[315-318]. All other subsets of B and T cells lack self renewal capacity and are not long lived (in fact, more than 90% of all maturing B and T cells die within the BM or thymus by apoptosis due to non-productive antigen receptor rearrangements or by selection processes deleting self reactive cells or cells displaying antigen receptors without any affinity), with plasma cells, which can survive in the BM for several years, as the only exception[319,320].

Leukemia

The word leukemia means white blood (Gr. *leucos* white + *haima* blood) and refers to the white layer formed by increased numbers of leukocytes (white blood cells) on leukemic blood samples that has been left standing for a while. The term encompasses very different diseases with the common denominator that they are cancers of the hematopoietic system. Leukemias can be subdivided into acute and chronic leukemia, which historically reflects the natural history of the disease when no treatment was given, in which case acute leukemias usually leads to death within a few months, whereas a patient with a chronic leukemia can live many years. These two subtypes can be further subdivided into lymphoid and myeloid leukemia, depending on the morphology and phenotype of the leukemic cells; resembling either immature lymphoid or myeloid cells, and for long time thought to reflect the lineage of the initiating cell. The two chronic diseases, chronic lymphoid leukemia (CLL) and chronic myeloid leukemia (CML), are mainly diseases of the elderly patient (CML accounts for a few percent of all leukemias in children; CLL does not afflict children at all). Acute myeloid leukemia (AML) can be classified according to the French-American-British (FAB) system into 8 groups (FAB 0-7), based on lineage characteristics (or lack thereof) of the blast cells, while acute lymphoblastic leukemia (ALL) is subdivided into B- and T-cell ALL.

ALL is the most common cancer in children, and accounts for 80% of all leukemia in this age group. ALL can, based on phenotype, be classified as B-cell

lineage (80% of ALL) or T ALL (20%). In adults AML dominates, followed by CML, CLL and ALL.

The focus in the following sections is on acute leukemias in general and ALL in particular.

Pathogenesis

The ultimate cause for acute leukemia in individual patients is very rarely known. Several genetic syndromes are associated with an increased risk for developing acute leukemias (either lymphoblastic or myeloid), such as for example Down syndrome, Neurofibromatosis type I, ataxia telangiectasia, Fanconi anemia, Wiscott-Aldrich syndrome and several immune deficiency syndromes[321]; however, they account together for less than 5% of cases. Population based studies show no excessive risk for first degree relatives to children with cancer that cannot be explained by known hereditary syndromes[321], indicating that other, as yet unknown, inheritable predispositions probably exert no major role in the pathogenesis.

Exposure to ionizing radiation is another established risk factor for leukemia, but its impact on the incidence is presumably small[322,323]. Chemical exposure to, for example, benzene, or to certain cytotoxic drugs (e.g. alkylating agents or epipodophylotoxins, used for the treatment of cancer) are known precipitating factors for AML[324], but no firmly established link between exposure to chemicals and ALL have been established.

Pediatric ALLs have further been proposed to be a developmental disorder, similar to osteosarcomas of the bones and germ cell tumors, which all have their peak incidences at ages corresponding to the maximum growth rate of the tissues from which the tumors develop. The intense proliferation would then simply increase the probability of mutational and potentially oncogenic events.

A strong case has been made for infections as an etiological factor. Viruses are directly implicated in the pathogenesis of certain lymphoid malignancies, such as Burkitt‘s lymphoma (which also occurs in a disseminated form as B-ALL), which has a very high incidence in tropical Africa and Papua Guinea (where it is the most common cancer among children) and has a strong association with EBV virus, and possibly also with malaria[325,326]. EBV is also thought to be a contributing factor for Hodgkin’s lymphomas[326,327]. However, albeit no specific virus has been linked to common pediatric ALL and no viral genomic inclusions have been found within leukemic cells, there is evidence for space-time clustering, excess incidence with higher population mixing, and seasonal variation suggesting infection as contributing causal factor[328]. The marked incidence peak of pre B-ALL around 2-5 years of age typical for socio-economically developed countries, is suggestive of important environmental factors[329]. Greaves[330] has proposed a model for leukemogenesis similar to Knudsons two-hit model for retinoblastoma[331], with at least two independent events leading to leukemic transformation. The first event seems to occur most commonly in utero[332,333], creating a pre-leukemic clone of cells, which are the basis for the second hit, occurring several years later. The delayed exposure in more affluent societies of the immune system to common microbes could invoke a hyper reactive or unbalanced immune response, acting as the second event on the pre-leukemic clone and precipitating overt leukemia[322].

Pathophysiology, clinical features and diagnosis

Leukemia is a clonal disorder[334,335] arising from a blood forming progenitor cell in the BM, which due to genetic perturbations has lost the normal restraints to proliferation and therefore gradually replaces the normal hematopoietic cells with its progeny. In accordance with its blood heritage leukemia is a floating cancer, which probably very early mobilizes out of the BM and enters the circulation, from where it seeds the BM in all other bones and can be found more or less in any

tissue or organ within the body. The infiltration of BM with leukemic blast cells compromises normal hematopoiesis, and the ensuing anemia, thrombocytopenia and neutropenia causes most of the initial clinical symptoms and findings, such as pallor, fatigue, petechiae or purpura (small punctuate or larger bleedings into the skin) or overt bleedings, and fever. White blood cell counts (WBC) are abnormally high in approximately half of patients, due to circulating blasts, but are within or below normal reference values ($<10x10^9$/L) in the remaining half, were differential counts usually reveals reduced numbers of several or all of the normal blood cell lineages. Circulating leukemic cells causes clinically apparent enlargement of liver, spleen and/or lymph nodes (localized or generalized) in between half and two thirds of cases. Bone pain, presumably caused by infiltration of periosteum (a specialized connective tissue covering all bones) and the bone itself, is a common complaint, as is anorexia and other unspecific symptoms.

The diagnostic procedure is usually a BM aspiration followed by analysis of the cells with regard to morphology, phenotype and genotype. Morphological examination of BM smears is fast and has a long track record in diagnosis of haematological diseases, and is still often necessary in order to evaluate possible differential diagnoses. Establishment of the phenotype is also fairly fast, and informs of the type of leukemia (AML or ALL, which often, but not always, can be distinguished by morphological examination; and B- or T ALL, which can not be distinguished by morphology). Information about genotype can sometimes aid in difficult cases to establish an exact diagnosis, but is more often important for prognostic purposes. In comparison with some solid tumors, a diagnosis of leukemia is usually arrived at very fast, and a child presenting at an emergency department in the morning can often have the diagnosis of acute leukemia established and treatment started the same evening.

Leukemic biology and genotype

Cancers are considered to be caused by disruptions of the genome, leading to the loss or amplification of gene expression, or to the creation of hybrid genes through fusions, which results in an altered cell function. The disruptions might target the DNA directly, as in point mutations, structural alterations in the genome sequence (translocations, deletions, inversions and so on), and changes in **ploidy** (chromosome numbers), or it might alter gene transcription by epigenetical changes. Several cellular alterations, and therefore also several concurrent genomic disruptions, have been proposed to be critical for malignant transformation, such as increased proliferative potential, reduced apoptosis, reduced dependency on survival signals and reduced responsiveness to inhibitory signals, increased angiogenesis and genomic instability[232]. However, despite a dramatic increase in our knowledge of the molecular machinery of normal and malignant cells is the link between the observed genomic alterations in human cancers and the actual malignant phenotype poorly defined in most cancers in general, and in ALL in particular.

Among the hematopoietic malignancies, CML and AML are the best characterized from a molecular and mechanistic point of view (reviews on the molecular wiring of AML and CML cells are legio), while the same is not true for ALL - and still ALL has met with the greater success in terms of treatment results. This difference might very well be due to biological differences, such that AML and CML are simply more difficult to cure, but seems to have led to different research approaches towards improving the treatment of patients. With regard to AML and CML, the gain in molecular knowledge has translated into drug developments, such as for example **all-*trans*-retinoic acid** (**ATRA**), which specifically targets the chimeric protein encoded by *PML-RARα* in promyelocytic leukemia, and **imatinib**, which targets the fusion tyrosin kinase protein BCR-ABL1 caused by the t(9;22) in CML. The amazing progress in the care of patients

with ALL, on the other hand, is mainly due to the incremental gain of empirical knowledge through large, multicenter (and often international) trials, which have informed on the details of combination chemotherapy, risk group stratification and supportive care. The impact of the molecular progress has been mainly in risk classification, but not in the development of new treatment modalities (all drugs used in the treatment of pediatric ALL today were at hand already in the end of the 1960s, *before* the disease was even thought of as curable![336]). Since two thirds of ALL patients are children (in whom it is the most common cancer) the disease is mainly attracting the interest of pediatric oncologists, who follows the line of research which has been so successful thus far, i.e. identifying risk factors and treatment variables influencing outcome. Perhaps both camps, adult hematologists seeing the majority of AML patients, and pediatric oncologists with their ALL patients, have things to learn from each others´ approaches. Considering that the survival curve of patients with ALL, which rose so impressively during the 1970s and 1980s, is flattening out, and that most contemporary treatment protocols reach virtually the same results using somewhat different approaches[337], suggests that new, molecularly informed, treatment modalities might be needed in order to cure the last 10-20% of patients, who might otherwise not be cured, whatever combination of known drugs are given. Chromosomal aberrations can be identified in the leukemic cells of virtually all patients diagnosed with ALL (***Figure 5***). Most aberrations are recurrent and non-random, and are due to **translocations**, which mean breaks and exchange of genetic material between at least two chromosomes, or an abnormal chromosome number (**aneuploidy)**. Some are important due to their impact on prognosis, and some predict for response to specific therapies[338]. Although most of the non-random aberrations have been well characterized at the molecular level, their relevance for the leukemic phenotype is largely unknown. Two of the translocations, **t(12;21)** causing the *RUNX1-ETV6* fusion and t(9;22) resulting in the ***BCR-ABL1*** fusion, due to their importance in this thesis are described in detail in the following sections.

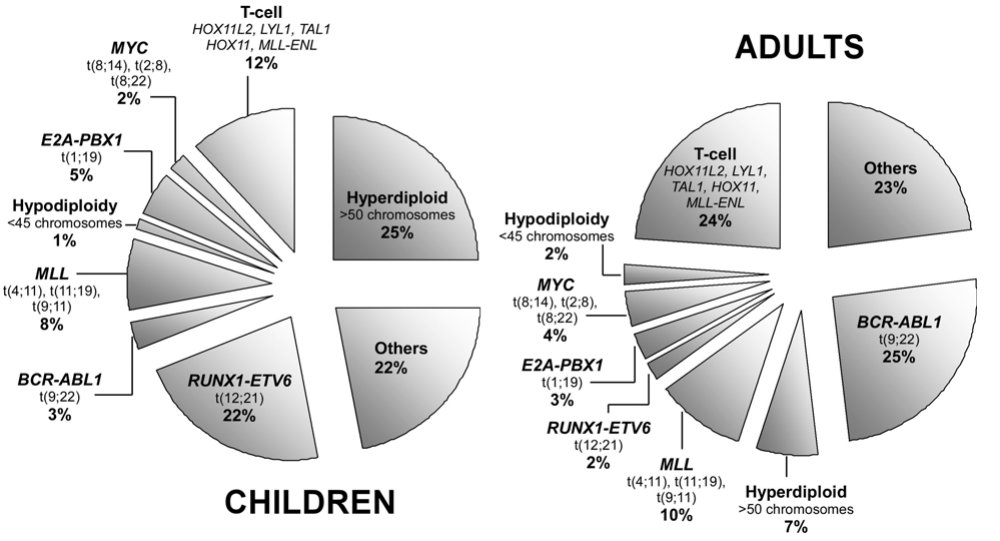

Figure 5. The relative occurence of genetic aberrations in ALL in children and adults. T-cell ALL have characteristic translocations usually involving a TCR gene as one partner, fused to several other genes as indicated in the chart. The translocations involving MYC and one of the Ig chains are typical of mature B ALL, while the other aberrations are found mainly in pre B ALL.

ETV6-RUNX1 (TEL-AML1)

The 12;21 translocation is a cryptic fusion usually not detected with conventional cytogenetic analysis, and its relevance as the most common specific genetic lesion in pediatric ALL, found in 20-25% of the cases, is therefore a relative recent finding[339-342]. The translocation fuses two genes with important functions in hematopoiesis, *RUNX1* (also known as *AML1*, *CBFA2* and *PEBP2αβ*) and *ETV6* (also known as *TEL*), to create the t(12;21)(p13;q22). Both genes are known as fusion partners with many other genes, and with each other, in hematopoietic malignancies.

RUNX1 is one of three DNA-binding heterodimeric transcription factors (*RUNX1-3*), which together with the non-DNA-binding partner, *CBFβ*, forms a core-binding factor (CBF), which mainly acts as an activator (in mammalians) on

target genes. *CBFβ* increases affinity of RUNX1 protein to DNA, and protects it from proteolysis, and is essential for RUNX1 function, since loss of *CBFβ* function phenocopies *RUNX1* mutations[343]. *RUNX1* is important for the specification of definitive HSCs in early embryonal development, as evidenced by the profound block in definitive hematopoiesis when both alleles of *RUNX1* (or *CBFβ*) are disrupted[296], but is largely dispensable for HSC maintenance[344,345]. Inducible disruption of *RUNX1* in adult mice further revealed impaired megakaryocyte maturation and defective B and T cell development[344]. CBFs are also implicated in AML through the fusion between *RUNX1* and *ETO*, creating the t(8;21)(q22;q22), and the inversion including *CBFβ* in inv(16)(p13;q22), together representing 20-25% of all AML[343]. How the same transcription factor can generate two such disparate diseases as ALL and AML is unclear, but important differences in the involvement of *RUNX1* could possibly explain the lineage bias, such as the relative contribution of the gene in the translocations (almost the whole *RUNX1* in 12;21 but a somewhat truncated *RUNX1* in 8;12), the difference in promoter (12;21 is regulated by the *ETV6* promoter whereas 8;21 is expressed under the *RUNX1* promoter), and the choice of fusion partners which can add specific functions to the fusion protein[346]. The specific role of the *RUNX1* in leukemic development is also dual as it seems to be able to act as both oncogene and tumor suppressor[346]; for example is amplification of *RUNX1* in 2-5% of pediatric ALLs associated with a poor prognosis[347-349], while its role in the *ETV6-RUNX1* fusion seems to be to dominantly inhibit the transcriptional activity of the remaining *RUNX1* allele through recruitment of nuclear co-repressor complex (N-CoR)[350], suggesting a tumor suppressor function.

The fusion partner, *ETV6*, is an ETS-domain transcription factor expressed in most tissues and acting mainly as a transcriptional repressor[351,352]. It is, like RUNX1, vitally important for early hematopoietic development (the transition from fetal liver to BM hematopoiesis)[297], but unlike *RUNX1* it is essential for survival and maintenance of HSCs in adult life[353].

The acquisition of the *ETV6-RUNX1* fusion is an early event in the leukemogenic process, as it is found on retrospective analysis of Guthrie cards (blood samples taken on all newborns for screening of several metabolic disorders) from children later developing ALL; but seems not to be sufficient for transformation, since the concordance rate between homozygous twins is rather low (<10%), latency is long, and only one out of a hundred children with the translocation present at birth will ever develop leukemia[332,354-356]. Mouse models with transduced expression of *ETV6-RUNX1* supports the notion that the translocation is not sufficient for transformation, but rather show a deficit in B cell development with an accumulation of pro-B cells[357-359]. However, another study observed instead enhancement of pre B cell self renewal and promotion of B cell development[360], which perhaps might be explained by the transduction of hematopoietic fetal liver cells instead of adult BM cells, which might more truthfully model the fetal origin of the human disease. Thus, the acquisition of t(12;21) might be a first hit, creating a "pre-leukemic" clone, which can act as a substrate for subsequent genetic lesions eventually leading to full leukemic transformation[361]. For example, the expression of *ETV6-RUNX1* in mice lacking the gene segment encoding the tumor suppressors $p16^{INK4a}$ and $p19^{ARF}$ caused an enhanced leukemogenecity, as compared to mice with functional $p16^{INK4a}$ and $p19^{ARF}$ [362]. Patients with t(12;21) often lack the second *ETV6* allele[363,364], and this loss seems to be secondary to the translocation, since newborns with the *ETV6-RUNX1*fusion retains the other *ETV6*; twins developing $12;21^{+}$ ALL have lost different parts of their *ETV6*[365], and relapses often display the exact same fusion, but different breakpoints for the lost *ETV6*, possibly indicating that the relapse arises from a pre-leukemic clone[366-368] and suggesting that loss of the other ETV6 allele could represent a second, and possibly transforming, hit[368-370].

It is not known at what level in the hematopoietic hierarchy the *ETV6-RUNX1*fusion is created, or at what level the subsequent transformation occurs. Available data indicates that only $CD19^{+}$ cells carries the translocation and not

$CD19^-$ cells[356,371], and it has been generally believed that a committed B cell progenitor is the target cell[369]. However, a recent study on t(12;21) modelled in zebrafish showed that only when the *ETV6-RUNX1*fusion was ubiquitously expressed in all hematopoietic lineages (including the HSCs) did leukemia ensue, but not when only lymphoid restricted cells were targeted[370].

The *ETV6-RUNX1* fusion is restricted to pre-B ALLs affecting children 1-10 years of age, and is seen very rarely in infants or adults, and never in T-ALL or myeloid leukemias[372]. The impact on prognosis is somewhat controversial, but the translocation is generally regarded as carrying a favourable prognosis [343,373,374]. The age incidence pattern is intriguing - why does this translocation occur only in utero, resulting in leukemia in only small children and almost never in adults? Either the cause is restricted to the environment of the developing fetus, or the cause, whatever it is, acts on a cell specific for fetal hematopoiesis. A recent report indicates that a developmentally transient hematopoietic cell actually can be the target of distinct pathology: children with Downs syndrome often displays a transient myeloproliferative disorder (TMD) around or shortly after birth, caused by a mutation of the transcription factor GATA1, and which in some cases can progress to AML. In mice embryos, the hyperproliferative effect of mutated *GATA1* is restricted to a unique yolk sac and fetal liver megakaryocyte progenitor, which is not present after birth. The authors suggests that this explains the restriction of TMD to infants, and proposes that target cells in childhood leukemias in general could be distinct from those in adult leukemias[375].

BCR-ABL1 (The Philadelphia chromosome)

The reciprocal translocation of the long arms of chromosomes 9 and 22, resulting in a truncated chromosome 22 commonly called the Philadelphia (Ph) chromosome, was the first specific karyotypical abnormality consistently associated with a human neoplasm[376-378]. The t(9;22)(q34;q11) translocation gives

rise to a fusion between the Breakpoint cluster region (*BCR*) and tyrosine kinase Abelson murine leukemia viral oncogene homolog 1 (*ABL1*) genes, resulting in production of the constitutively activated tyrosine kinase BCR-ABL1. The oncogenic effect of this fusion protein results in deregulated cellular proliferation, decreased adherence of leukemic cells to stroma, and a reduced apoptotic response, and these effects are due to both compartmentalization of the fusion protein and the effect on different molecular pathways through its many downstream effectors[379]. The normal ABL1 tyrosine kinase shuttles between the cytoplasm and nucleus, where it exerts apoptotic effects in the latter[380,381], whereas the BCR-ABL1 is restricted to the cytoplasm, due to it being constitutively phosphorylated[382]. In fact, when CML cells are treated with the BCR-ABL1 tyrosine kinase inhibitor Imatinib the nuclear entry of BCR-ABL1 is stimulated. If the export is then blocked by leptomycin B, and Imatinib treatment is discontinued, the now nuclear BCR-ABL1 reactivates and induces, rather than prevents, cell death. The tyrosine kinase activity of BCR-ABL1 moreover phosporylates a host of downstream targets[379], resulting in dysregulation of multiple cellular pathways[383]. However, the complexity of possible interactions between the signalling pathways has made it difficult to identify any key targets or pathways[384].

It is unclear if t(9;22) is the first and also sufficient event for the transformation of normal hematopoietic cells into CML[385]. Although most cancers are generally believed to be the result of a multistep process, there is still very little evidence of genetic abnormalities preceding the t(9;22)[331,386]. It has been speculated that BCR-ABL1$^-$ clonal populations sometimes observed in patients with BCR-ABL1$^+$ CML in remission after treatment with the BCR-ABL1 tyrosine kinase inhibitor Imatinib could represent a pre-leukemic clone preceding the acquisition of the *BCR-ABL1* fusion[387], but this clone could also be treatment related[388,389]. The proposition that the acquisition of *BCR-ABL1* is sufficient to cause leukemia is supported by murine models, in which transduction of the fusion gene into HSCs alone results in myeloproliferative disease[390] or lymphoid

leukemia, which remits upon suppression of *BCR-ABL1* expression[391]. However, *BCR-ABL1* is found in white blood cells in 30-70% of healthy individuals, the majority of whom will never develop leukemia[392,393]. Although it is unclear whether the Ph chromosome is a first event in the development of CML, it is fair to say that it is probably a very early and necessary, but not sufficient, event with regard to the clinically more dramatic entities of CML blast crisis and Ph^+ ALL.

The tyrosine kinase activity of BCR-ABL1 can be inhibited by the tyrosine kinase inhibitor Imatinib mesylate[394]. This drug represents the breakthrough of designer drugs, since it was developed as a specific tyrosine kinase inhibitor with activity restricted to ABL1, C-KIT, platelet-derived growth factor (PDGF) and ARG. It has shown dramatic effects particularly on Philadelphia positive malignancies (see below under CML), but also on other cancers, such as gastrointestinal stromal tumor (GIST) with activated C-KIT, and dermatofibrosarcoma protuberans and hypereosinophilic syndrome, both involving activation of PDGF[395].

The location of the breakpoint on BCR can vary, which together with different splicing options of the primary RNA results in three different fusion proteins, designated according to their molecular weight as P190 BCR-ABL1, P210 BCR-ABL1, and P230 BCR-ABL1, respectively. P210 BCR-ABL1 is found in virtually all cases of CML, but also in approximately 20-50% of adult Ph^+ ALL[396-398], and 10-20% of pediatric Ph^+ ALL[399,400]. The remaining Ph^+ ALLs therefore have the shorter P190 BCR-ABL1 fusion gene, which is found as single variant only in ALL (but in a low frequency together with P210 BCR-ABL1 in CML[401]). The much rarer P230 BCR-ABL1 causes a distinct, indolent chronic neutrophilic leukemia[402,403]. There is a biological difference between the splice variants in that the tyrosine kinase activity is highest for P190 BCR-ABL1 and lowest for P230 BCR-ABL1, with P210 BCR-ABL1 falling in between[404,405], translating into a shorter latency for leukemic development in mice transduced with P190 BCR-ABL1 as compared with P210 BCR-ABL1 [405].

Chronic myeloid leukemia

The Philadelphia chromosome was discovered in the context of CML, a disease shown to harbour the genetic lesion in more than 95% of the cases. The cell of origin in CML has for long been considered to be a multipotent hematopoietic stem cell. An accumulation of predominantly granulocytes, but also to varying degrees red blood cells and platelets, has been observed for a long time[406]. This observation has subsequently been extended by the use of different techniques to show the presence of clonal Ph^+ cells in virtually all blood lineages[407-409], indicating an origin in a multipotent cell. A recent report even claimed an origin in the even earlier common hematopoietic-endothelial precursor, the so-called hemangioblast[410], although this finding awaits confirmation by other investigators. The long-term repopulating CML stem cells also display the same surface markers ($Lineage^-CD34^+CD38^-$) as normal HSCs[411]. Interestingly, these CML stem cells, which initially coexist with normal HSCs, have a selective disadvantage in reconstituting NOD-*scid* mice transplanted with BM or PB from patients with early phase CML[412-414]. The mechanisms used by the CML stem cells to gain clonal dominance is not clear, but might be caused by a diminished sensitivity to the effects of the increased levels of neutrophil elastase often seen in CML patients[415], or by inducing apoptosis in neighbouring Ph^- cells by secreting lipocalin[416]. Whatever mechanism is at play, it evidently leads to mobilization of normal HSCs into the circulation[412,413]. The CML stem cell pool seems to remain relatively small, and the massive expansion of myeloid cells apparently occurs downstream of the CML stem cell[417].

CML is a disease of the elderly, with a median age at diagnosis of 60 years, with only very rare cases diagnosed in children, and with unknown etiology. It runs a biphasic course, with an initial chronic phase, during which the patient might be relatively symptom free, and which is characterised by an expansion of a functionally normal myeloid compartment. Symptoms and findings are related to the massive accumulation of mainly granulocytes, but also basophiles, eosinophiles

and platelets, which leads to hepatosplenomegaly (enlargement of the liver and spleen) and impaired blood flow due to hyperviscosity, which in turn might cause difficulties breathing, mental alterations, visual- and hearing problems and cyanosis. The increased metabolism caused by the excessive proliferation can lead to fatigue, fever, sweating and weight loss. This phase lasts from days to years, until it eventually transforms into a blastic phase (sometimes preceded by an intermediate phase called accelerated phase), which essentially equals an acute leukemia with differentiation block and blast cell accumulation in BM and peripheral blood. The chronic phase transforms into AML in approximately two thirds of cases, and in the remaining third into ALL. A fully developed blast crisis usually causes death within 3-6 months.

The only proven curative treatment for CML is allogeneic hematopoietic stem cell transplantation (HSCT), but this treatment is available to only a fraction of patients due to either advanced age or limited donor availability[379]. Historically hydroxyurea and Interferon-α have been used for cytoreduction and prolongation of life, but with small or no expectations for cure[418]. Imatinib mesylate was made available in 1998, and showed dramatic response rates, including complete cytogenetic response in up to 65% of patients, significantly better than previous therapies[394,419,420]. It is however clear that some patients will not benefit from this treatment, but instead progress in their disease due to failure of the drug to inhibit proliferation of the BCR-ABL1^{+} clone. Furthermore, Imatinib may not offer a lasting cure, since discontinuation of therapy can lead to relapse[421], even in patients with long-term complete molecular remissions during treatment[422]. One obvious reason for some of these failures is inherent or acquired resistance to Imatinib, due to mutations in the ABL-kinase domain[423], amplification of *BCR-ABL1*[424,425], or enhanced drug efflux[426]. Another intriguing reason is that the CML stem cell might evade the anti-leukemic effect of the drug. The eradication of differentiated leukemic cells concomitant with insensitivity of a select CML stem cell population has recently been mathematically modelled on observed treatment responses

among Imatinib treated CML patients[427]. Although CML stem cells generally are spending more time in cell cycle than their normal HSC counterparts, a small population of Ph^+ deeply quiescent cells has been identified[428], which exhibit insensitivity to Imatinib[429] and other cytotoxic drugs, possibly through resistance to apoptosis[430]. The role of quiescence *per se* is controversial, since a recent report suggest it to be directly responsible for the insensitivity to treatment[431], whilst other studies show that by pushing these cells into cycle by administering G-CSF or GM-CSF, the effect of Imatinib is increased [432,433]. Other reasons for these cells to escape therapeutic effects might be due to the effects of the drug efflux pump p-glycoprotein[426], normally highly expressed on HSCs; or because of reduced activity of the influx protein OCT-1[434]; or because little or no BCR-ABL1 mRNA or protein is produced in this cell compartment[435,436]. The latter assumption is, however, contradicted by observations that quiescent CML cells do express *BCR-ABL1*[437], and might even do it to such a high degree that pharmacological doses of Imatinib does not suffice to block it[438].

Philadelphia chromosome-positive ALL

Patients presenting with the features of an ALL carrying the *BCR-ABL1* fusion gene but without a preceding chronic phase are diagnosed as having a *de novo* Ph^+ ALL. For those carrying the P210 splice variant there are no signs or findings that definitely differentiates between a *de novo* Ph^+ ALL or a CML in lymphoid blastic phase where the chronic phase has passed unnoticed. The shorter P190 fusion gene, however, indicates a *de novo* ALL, since this variant is exceedingly rare as the dominant fusion in CML[439].

There is a difference in tyrosine kinase activity between P210 and P190 BCR-ABL1[404], with a 7-fold increase of P190 and 5,4-fold increase of P210 relative to wild type c-abl[405]. A biological difference in leukemogenic potential has also been observed, with a greater potency of P190 as compared to P210 to induce B-

lymphoid leukemia in vitro[440], and in vivo[405,441]. The two splice variants seem to activate the same molecular targets, with the notable exception of preferential activation of GATA6 by P190[442]. Given these biological differences, it is interesting to note that reports on differences in clinical behaviour and outcome between ALLs with P190 *BCR-ABL1* and P210 *BCR-ABL1* have failed to provide consistent results[396,443-445].

P190 BCR-ABL1 is more potent to induce B-lymphoid disease than P210, but it also induces myeloproliferative disease with the same frequency in mouse models as P210. So why is the P190 fusion not found more often in CML? Proposed reasons are the higher tyrosine kinase activity, preferential activation of GATA6, but also that P190 might selectively target B-cell committed progenitors. Mouse cells treated with 5-FU (a cytotoxic drug which preferentially kills dividing cells, and therefore enriches for quiescent HSCs) and then transduced with either P210 or P190 and injected into recipient mice give rise to a myeloproliferative disease with many similarities to human CML (with the important difference that the induced mouse disease has a very short latency and is fatal within weeks). However, when non-5 FU-treated cells are transduced and then injected into recipients, half of the recipients develop either a B-cell leukemia or a macrophage tumor (or a mix thereof)[405]. In this mouse model, therefore, it seems that both P190 and P210 can give rise to either a CML-like disease or B-cell leukemia, depending on target cells. However, several attempts, using different techniques, to identify the initially targeted cell as a multipotent HSC or a committed pre B-cell progenitor in patients with P190 or P210 ALL have yielded inconsistent results[446-449]

Regardless of splice variant, Philadelphia chromosome positive ALL carries a poor prognosis in both adults and children, with long-term survival rates ranging towards 40% in pediatric patients to less than 20% in adults[450-453]. *BCR-ABL1* occurs in ALL with a frequency that increases with age, from 3-5% in children to 25-40% in adult ALLs[453-455]. It has been proposed that the higher frequency of

BCR-ABL1 in adult ALL relative to pediatric ALL patients could be a contributing factor to the worse prognosis of ALL in general among adults. However, even among Ph^+ ALLs, the prognosis is better in children, partly because children tolerate chemotherapy better than adults, but probably also because of inherent differences in the disease[456]. Ph^+ ALL is a heterogeneous disease, displaying very different outcomes depending on prognostic features in the patient[399,400]. For example, children below the age of 10 and with a white blood cell count below $50x10^9$/l have a relatively favourable outcome with a disease-free survival at five years of 50%, whereas children presenting with white blood cells in excess of $100x10^9$/l fare worse, with only 15% experiencing long-term survival[450]. Further supporting the heterogeneity is the weak signature of Ph^+ ALL on gene expression profiling studies as compared to other distinct subclasses of ALL[457,458].

Allogeneic stem cell transplantation has proven its greatest benefit relative to conventional chemotherapy in patients with Ph+ ALL (both children and adults) as compared to all other subgroups of acute leukemias. Treatment of pediatric Ph+ ALL with transplantation of stem cells from a matched related donor leads to an overall long-term survival of 72%, as compared with 42% after conventional intensive chemotherapy[450], and the trend is the same for adult patients[459]. However, only 30% of patients can expect to have a matched related donor, which leaves the remainder with the option of a matched unrelated donor, in which case the outcome is significantly worse, mainly due to transplant related mortality (the relapse frequency, however, is the same as for related donors)[459]. This might change as the matching techniques and the supportive care gets better. Older patients and patients with concurrent diseases experience a dramatically increased transplant-related mortality, and the best treatment for them is debated. Imatinib, alone or in combination with other treatment modalities, have been used with good short term results, but with very short remission durations[453]. New and more efficient treatments are eagerly sought for these patients.

Treatment and outcome

The development of treatment for pediatric ALL is one of the most dramatic success stories in medicine in the last century. Before 1960, acute leukemia was a uniformly fatal disease, with death from infections or hemorrhage occurring usually within three months after diagnosis[336]. In the late 1940s, the first pharmacological drug with activity against leukemic cells was developed, aminopterin, a forerunner to methotrexate, which still is a critically important chemotherapeutical in treatment protocols for ALL. The decades until the late 1960s, saw the development of combination chemotherapy, the recognition of the different treatment phases (induction, consolidation and maintenance treatment), CNS directed therapy necessary to avoid CNS relapses, and the introduction of *all* drugs used in ALL protocols to this day (corticosteroids, vincristine, the anthracyklines, asparaginase, cyclophosphamide, cytarabine, methotrexate and 6-mercaptopurine). But it was not until 1972 that ALL, in a landmark paper, was declared a curable disease[460].

Treatment is usually divided into an **induction phase**, which aims to induce **remission** (in leukemia defined as the absence on BM smears of lymphoblasts, and a full recovery of normal hematopoiesis) by eradicating as many leukemic cells as possible during a four to six week period of cytotoxic treatment, which is immediately followed by **consolidation** of the remission by the use of different cytotoxic drugs as was used during induction (albeit often also incorporating reinduction therapy, which essentially repeats the initial treatment). Treatment ends with a prolonged **maintenance phase**, which lasts for a total of two years of treatment or more, and consists of orally taken methotrexate and 6-mercaptopurine.

Modern treatment is based on **risk assessment**, which assign patients to three or four risk groups; usually standard risk, intermediate risk and high risk groups, and sometimes an extra high risk group. Adults are generally divided into only two risk groups (standard and high risk). The risk factors most often used today are **age** (with 2-10 year old having the best prognosis, while the youngest, particularly

those <1 year, and the oldest having the worst prognosis); **WBC count** (the higher the worse prognosis); certain **cytogenetic aberrations**, such as Ph^+ ALL and hypodiploidy (<45 chromosomes in the leukemic cells) conferring a poor prognosis, and *RUNX1-ETV6*$^+$ and hyperdiploidy (>50 chromosomes) confering a favourable prognosis; and **response to treatment**, such that attaining a complete remission within four weeks of induction treatment is necessary for a good prognosis[337]. The assessment of treatment responses is refined by the use of flow cytometry or molecular methods for the sequential measurements of cytoreduction (minimal residual disease, **MRD**), which reflects the combined effects of the biology of the leukemic cells, host pharmacogenetics and treatment pharmacokinetics, and thereby predicts prognosis[461]. Many other risk factors have been identified through the years, but most have lost their predictive value due to increased effectiveness of the therapy, such as for example T-ALL or mature B ALL in children. In fact, the most predictive factor of all for prognosis is the treatment itself.

Today cure rates of around 80% are achieved for pediatric ALL[462-466]. Adults still face a worse prognosis, with cure rates rarely exceeding 40%[337]. Suggested reasons for this difference in outcome are a higher incidence of high risk leukemias in adults, poorer tolerance of treatment, and less effective treatment regiments[467]. The last point has been substantiated by several reports on young adults treated according to either pediatric or adult protocols, showing a better outcome when treated on pediatric protocols[468,469], and suggestions that a greater adherence to treatment protocols by pediatric oncologists might influence outcome: "Pediatricians administer these treatments with a military precision on the basis of a near religious conviction about the necessity of maintaining prescribed dose and schedule come hell, high water, birthdays, Bastille Day, or Christmas"[470].

The history of pediatric ALL may be glorious, but should also instil a certain admiration for the first pioneers (and their patients with families) who, during the early days when all children treated with the first cytotoxic drugs died, met with

collegial disapproval and antagonism caused by misguided protectionism of the children and a prevailing pessimism regarding the disease[336]. In spite of this, and aided by the willingness (and sometimes even eagerness) of patients and their parents to participate in research studies, they continued their efforts until cure was within reach, and beyond[471].

Leukemic stem cells

Cancer stem cells (CSCs) have attracted considerable interest recently, although this concept also dates a long way back. A huge body of conceptual work has been published in recent years regarding the impact of the "stem cell view" on cancer biology and treatment, and some have wryly observed that the number of reviews seems to outnumber original papers[472].

It has been noted for long that cancers are built up of morphologically and functionally heterogeneous cells[473-475], where only a subset of the cells seem capable of creating colonies *in vitro*[476,477], or recreating new tumor *in vivo*[478-481]. For example, autotransplantation of cells from partially resected tumors (adenocarcinoma, epidermoid and sarcomas) into the anterior thigh or forearm of patients resulted in local growth in only 5 out of 27 individuals, and only from inoculums containing greater than 10^6 cells[478]. These observations have been explained by the existence within the tumor of a minor subpopulation of CSCs[476,477,480,482], endowed with the capacity of both differentiating into mitotically less active or inactive tumor cells, and self-renewal, allowing them to perpetuate the tumor, and to seed metastases to distant sites. Another formal explanation of the observed heterogeneity in proliferative capacity of tumor cells is proposed in the **stochastic model**, which states that the low clonogenicity could be due to a low but more or less evenly distributed probability of all tumor cells to enter cell

cycle and proliferate[13,478]. For a comprehensive review on the historical development of the cancer stem cell concept see ref[483,484].

With seminal work done in the mid 1990s on AML, the stem cell model gained strong support. Primary AML cell populations were purified based on their differential cell surface expression of CD34 and CD38, and only the minor $CD34^{+}CD38^{-}$ population was found to repopulate the leukaemia when injected into NOD-*scid* mice, whereas the other populations did not[12,485]. In similar ways, minor cancer stem cell-containing populations have been separated from significantly larger, non-tumorigenic cancer cell populations in brain tumors[115], breast cancer[486], colon cancer[116,117], head and neck squamous cell carcinomas[487], pancreas cancer[488], and prostate cancer[489], and it has been suggested that most, if not all, cancers are driven by CSCs[13]. It is important, however, to acknowledge the fact that although the above mentioned cancer stem cell populations have been enriched for CSCs, the purity of self renewing cells, and therefore their actual frequency, is unknown. It is also highly conceivable that the NOD-*scid* model underestimates the number of cells with the capacity to perpetuate the cancer in patients, since the assay likely selects for only those self renewing cells which can survive in a xenogenic environment. For example, 50% of AML samples do not engraft NOD-*scid* mice at all, although they obviously maintain the disease in the patients and presumably contain leukemic stem cells (LSCs)[490], and in a mouse AML model with an induced myeloid oncogene (MLL-AF9), 25-30% of all leukemic cells were found to be self-renewing[491].

Inherent in the cancer stem cell theory is the notion of a hierarchy of cells, with CSCs at the top and mature cells at the bottom, and most in vivo studies mentioned above report on a more mature, and often diversified, progeny, mimicking the original tumor. It has even been reported on a hierarchy within the leukemic stem cell population, similar to the normal HSC compartment, with varying degrees of self renewal [492].

"Cell of origin"

The observation of cellular hierarchies in tumors with self renewing cells at the top suggests an analogy between normal stem cells in tissue formation and CSCs in tumor formation. The observed similarities in stem cell function, together with the candidate stem cell phenotype ($CD34^+CD38^-$) of AML[12] and Ph^+ ALL[493] LSC, and clonal involvement of $CD34^+CD38^-$ cells in common pediatric ALL[494], could suggest that a normal HSC is the target for malignant transformation[13,495]. Two theoretical considerations further support this notion: first, a long-lived HSC might be a more probable target for the multiple genetic events believed necessary to subvert a normal cell into a cancer cell than short lived progenitors; and second, it might be simpler to transform an already self-renewing stem cell, than to turn the self renewal machinery on again in a progenitor [13]. An origin in a multipotent HSC has indeed been shown for chronic phase CML[407-409] and subtypes of the pre-leukemic condition myelodysplastic syndrome (MDS) [496,497], by identifying disease specific genetic markers in several or all different blood lineages. However, both these diseases are characterized by a global hematopoietic affliction – how would one reconcile the usually narrow lineage restriction of an AML or ALL with a transformed multipotent cell? It has been suggested that the transforming event itself might block differentiation into all other lineages[13,495], or it could conceivably also support a specific lineage, which would out compete rivalling lineages. A third proposed option is that transformation occurs in a lineage biased HSC (which also would suggest an explanation for the higher incidence of AML in elderly people)[187,498-500]. Another way of reconciling both a HSC origin and a uni-lineage disease is if the first initiating event occurs in a normal HSC, creating a "pre-leukemic" clone, but the transforming event targets a committed progenitor of the affected lineage. Data suggested to support this model has been reported on CML in myeloid blast crisis, were the clonal counterpart of a normally non-self renewing progenitor, the GMP, was endowed with self renewal capacity[501]. Here it is

important to make a distinction between the **"cell of origin"**, the target of the first hit, and the **leukemic stem cell**, which has the capacity to repopulate the leukemia and thus act as a stem cell, but which is derived from a committed progenitor[19].

Several recent and conceptually important reports on leukemic mouse models indicate that it is possible to create LSCs from committed progenitors. The transduction of oncogenic fusion genes like MLL-ENL, MOZ-TIF2 or MLL-AF9 (all described in the context of human AMLs) into prospectively purified HSCs or committed progenitor fractions (CMP, GMP and MEP) have shown that all of these progenitor fractions except MEP can be transformed into transplantable myeloid leukemias, with similar morphology and phenotype regardless of which progenitor or HSC population was transduced initially[502-504]. In leukemias derived from MLL-AF9 transduced GMPs, the leukemic stem cells was found to be enriched in a fraction of cells with a GMP-like phenotype. These cells were found by comparative gene expression analysis to be highly similar to normal GMPs, except for a few hundred genes whose expression pattern was shared exclusively with HSCs, and which therefore was hypothesized to represent a leukemia self-renewal-associated signature[504].

A final hypothesis for the origin of malignant clones is the fusion of a HSC and a progenitor cell, where one of them is normal and the other partially transformed, and were the fusion contributes to the full malignant potential. There are, however, hardly any data supporting this sequence of events[505,506].

In conclusion, the hierarchical level at which the "cell of origin" or the cancer stem cell are recruited in human cancers is still mostly elusive. All we know is that the "cell of origin" in CML and MDS is a HSC (allthough clonal T cells have rarely been demonstrated, and therefore a HSC origin is strictly not proven in most of these cases), and that most pediatric ALLs are initiated in (and therefore also driven by) lymphoid committed progenitors (article I in this thesis); the answer to the origin of all other cancers lies ahead of us.

Clinical relevance

The major foreseeable impact the cancer stem cell concept will have on the clinical care of cancer patients is probably the realisation that from a treatment perspective only a minority of the tumor cells have a consequence for cure (while without treatment it is the sheer number of all the other tumor cells which will kill the patient). For the ultimate cure CSCs must be eradicated – all other tumor cells, with limited long term capacity to proliferate, will eventually disappear if their source is removed[507]. Today, in clinical trials evaluating new drugs, the principle is rather to look at the effect on the bulk tumor, which might shrink and even disappear if the patient enters remission, but is often followed by recurrence of the tumor sooner or later.

By applying a stem cell perspective on cancer, several similarities between normal stem cells and CSCs have been revealed, as described in the previous section. Further characteristics of normal stem cells found in their malignant counterparts are relative quiescence, expression of efflux proteins on the cell membrane and resistance to apoptosis[507-510]. Since many currently used cytotoxic drugs are both cell cycle-phase specific and substrates for any of the several efflux proteins expressed by LSCs they are expected to have an inferior effect on the primitive tumor population as compared to the bulk of the disease [511,512]. In pediatric ALL, a hierarchy of sensitive cells is obvious, with a dramatic reduction of leukemic blasts during the first few weeks of treatment, but with often minimal clones (not even detectable by high sensitivity PCR) needing several additional cytotoxic drugs for a very extended period of time (years) to be eradicated. The least sensitive cells are the ones giving rise to relapse if treatment is insufficient, which can occur several years later and thus possibly indicates a certain self renewal capability.

The many similarities between normal and malignant stem cells (regardless of the origin of the later) could raise concerns of how to design treatments that kills

only the latter and not the former (which brings the matter to a more relevant level as compared to most contemporary treatment modalities which unspecifically aims at killing as many malignant cells as possible while keeping collateral damage to normal tissues within tolerable limits). As important as the similarities with normal stem cells might be, the major impact for patients might arise from the differences. AML stem cells share the $CD34^{+}CD38^{-}$ phenotype with normal stem cells, but differs with respect to other markers, such as c-kit (expressed on HSCs but not on LSCs)[513], IL-3Rα (expressed on LSCs but not on HSCs)[514] and SP phenotype (were $SP^{+}CD34^{+}CD38^{-}$ cells from AML samples are enriched for normal stem- and progenitors)[515]. These differentially expressed markers might be useful for graft selection in an autologous stem cell transplantation setting, but their relevance for leukemic stem cell function is uncertain, and they will probably not constitute good targets for drug intervention. The search for pharmacological targets has directed much focus on molecular pathways involved in normal stem cell functions, particularly self renewal. Several of these pathways are known to be relevant for cancer, such as the wnt- and sonic hedgehog pathways[516], but no mutations within these pathways have been reported in leukemias[517]. The wnt/β-catenin pathway might still be important for acute leukemias through other mechanisms [518,519], such as a constitutively expressed wnt-16 induced by the t(1;19) E2A-PBX1 fusion in pre-B ALL (although the role, if any, of wnt-16 in this disease awaits clarification), and epigenetical downregulation of wnt-inhibitors in ALL[520]. Bmi-1 and several Hox genes are also implicated in self renewal of both normal and malignant stem cells[202,504]. Another subverted stem cell function in cancer, perhaps most relevant for epithelial tumors, was revealed in *drosophila*, where induced mutations of several genes responsible for asymmetric divisions led to tumor development, presumably by disrupting the control of self renewal[521]. The corollary of this finding, the loss of cell polarity, is a common feature of all epithelial cancers[522].

Experimental proof-of-principle that LSCs can be selectively eradicated while sparing the normal HSCs is shown by the selective up-regulation and dependence of nuclear factor κB (NF-κB) in LSCs. By blocking NF-κB with a proteasome inhibitor, and concomitantly administering a cytotoxic anthracykline (idarubicin), which normally induces expression of NF-κB as a survival mechanism, LSCs were killed while normal HSCs were mainly unaffected[523]. The phosphatase and tensin homologue (PTEN), which negatively regulates proliferation and survival through regulation of the PI(3)K pathway, is commonly inactivated in cancers, including leukemias, and seems to contribute to the malignant phenotype. When blocked by rapamycin, a selective effect on LSCs is seen[524]. Finally, Parthenolide, a naturally occurring small molecule, has also been reported to act specifically on LSCs, presumably by triggering LSC-specific apoptotis[525,526].

The possibility to selectively kill CSCs is also evident from the clinic. A majority of children with ALL are cured by chemotherapy, which obviously spares their normal HSCs; and a significant fraction of patients with testicular germ cell cancer cured by chemotherapy has enough remaining spermatogonial stem cells to remain fertile[527].

The analogy between normal and CSCs might be very instructive, but should perhaps not be drawn too far. Proof of CSCs is still limited to a relatively small number of cancers, and the strength of the evidence is further limited by the surrogate assays available for human stem cells. As already mentioned, the “golden standard” stem cell assay, the long term engraftment in immunocompromized mice, detects cells with long term repopulation capability, but also inevitably selects for only those cells capable of surviving without human-specific cues, which likely underestimates the true frequencies of the cells of interest. Given these caveats, the field of cancer stem cell biology might still offer surprises, and constitutes an exciting research area for anyone with an open mind.

Summary and discussion of articles

Article I

The recent breakthroughs in the identification of CSCs in general[115,486], and LSCs in particular[12], have inspired a conceptual framework for cancer stem cell biology [13,507,528,529]. The theory suggests that LSCs, like their normal counterparts, could be more resistant to therapy as compared to the major bulk of tumor cells, which could explain clonotypic relapses from a state of attained remission. The phenotype of AML stem cells is suggesting a close relationship with normal HSC[12], and the fact that myeloid progenitors are short lived and therefore are unlikely candidates for the multiple genetic hits necessary for transformation further suggests a stem cell origin of AML[13]. In ALL, the picture is less clear. It has been proposed that ALL derives from committed B cell progenitors[361], but recent reports of clonal involvement of $CD34^{+}CD38^{-}$ ALL cells instead implicates a HSC origin[493,494].

We reasoned, as others have done before us[530], that if there are leukemias arising from cells at different levels of maturation, the more immature origin might confer a greater resistance to treatment and thus assignment to a higher risk group than would be the case if a more mature cell is targeted for transformation. We therefore focused on two distinct subgroups of ALL with markedly different prognosis, pediatric ALLs characterized by the *RUNX1-ETV6* fusion which generally is associated with a favourable outcome, and *BCR-ABL1*$^{+}$ ALL, which

carries a very poor prognosis in both children and adults. We hypothesized that *RUNX1-ETV6*$^+$ ALL might arise from a committed B cell progenitor, while *BCR-ABL1*$^+$ ALL (similar to CML) might arise from the HSC compartment.

In patients with the *RUNX1-ETV6* fusion we found an enlarged CD34$^+$CD38$^-$ candidate HSC pool as compared to healthy individuals, containing approximately 90% t(12;21)$^+$ cells, in line with previous reports[494]. However, this population could be subdivided into a greater (~90% of the cells) CD19$^+$ and a smaller (~10%) CD19$^-$ fraction. The former population was found in all patients to be virtually enriched in leukemic cells, which was not so surprising given the aberrant phenotype (CD38 is expressed on all CD34$^+$CD19$^+$ cells in normal BM), whereas the latter, strikingly, represented a purified normal CD34$^+$CD38$^-$CD19$^-$ *RUNX1-ETV6*$^-$ HSC compartment in most patients. LTC-IC activity in the CD34$^+$CD38$^-$CD19$^-$ population was found to be comparable to CD34$^+$CD38$^-$ cells from healthy volunteers, whereas it was absent in CD34$^+$CD38$^-$CD19$^+$ and CD34$^+$CD38$^+$CD19$^+$ ALL cells, as would be expected of lymphoid committed cells in this myeloid biased assay. To test if the self renewing t(12;21) LSCs were uncommitted, HSC-like cells or committed towards the B cell lineage, we sorted CD19$^+$ and CD19$^-$ cells from diagnostic BM samples (based on data implicating this marker to identify cells locked unto the B cell fate[225,226,229]) and injected them into NOD-*scid* mice. All mice injected with CD19$^+$ cells engrafted with ALL, whereas CD19$^-$ cells only engrafted with normal, multilineage, *RUNX1-ETV6*$^-$ cells. These findings indicates that the normal HSC compartment in t(12;21) ALLs remains unaffected as to size, phenotype, clonal involvement and function, and that the leukemic clone, including the LSCs, is restricted to the B cell committed CD19$^+$ fraction.

When we subsequently turned our attention to *BCR-ABL1*$^+$ ALLs, we had to acknowledge the fact that this disease is characterized by two different splicing variants of the fusion protein; P190 BCR-ABL1 which is found in ALL, and P210 BCR-ABL1 which is found in almost all cases of CML, and in 10-50% (depending

on age) of ALLs. Regarding the latter variant there is an unavoidable issue of whether the disease is truly a *de novo* ALL, or rather a CML in lymphoid blast crisis, where the chronic phase has passed unnoticed. No clinical, biological or genotypical data can with certainty, to our knowledge, distinguish the two, and we therefore relied on the clinical diagnosis of *de novo* P210 BCR-ABL1 ALL in those cases. When we investigated diagnostic samples from these two entities in similar ways as we had for t(12;21) ALLs, we found a striking difference between the two. P190 BCR-ABL1 ALLs unexpectedly turned out almost identical to t(12;21) ALLs, whereas P210 BCR-ABL1 ALLs displayed a (often massive) clonal involvement of the $CD34^{+}CD38^{-}CD19^{-}$ population, with undetectable LTC-IC activity (although the significance of this is hampered by the increased size of the population, making the relative contribution of any remaining HSC smaller), suggesting competition within the uncommitted stem cell compartment of clonal cells. To further investigate whether the LSCs in P210 BCR-ABL1 ALL originate from an uncommitted, multipotent progenitor we analyzed the BM samples for multilineage involvement by sorting $CD34^{+}CD33^{+}$ myeloid progenitors and analysing them by FISH for clonality. In all P210 BCR-ABL1 ALLs the myeloid compartment was involved, whereas in no *RUNX1-ETV6* or P190 BCR-ABL1 ALL samples were translocation-positive myeloid cells (above the cut off level for FISH) detected. Together these findings are strongly indicating a multipotent HSC origin for P210 BCR-ABL1, but a committed progenitor origin for P190 BCR-ABL1 and *RUNX1-ETV6* ALLs. With regard to our initial hypothesis, that a more immature origin would implicate a worse prognosis, the difference between P190 and P210 was surprising, given the similarity in clinical outcome between the two entities.

Transplantation of $CD19^{+}$ and $CD19^{-}$ cells from P190 BCR-ABL1 ALLs into NOD-*scid* mice revealed the same engraftment pattern as t(12;21) ALLs, with leukemic engraftment from $CD19^{+}$ cells and normal, *BCR-ABL1*$^{-}$ engraftment from $CD19^{-}$ cells. Unexpectedly P210 BCR-ABL1 $CD19^{-}$ cells also gave normal

engraftment in all investigated cases, even after serial transplantations in order to assay any deeply quiescent LSCs. Clearly, the dominating LSC activity is confined to the $CD19^+$ fraction, but rare LSCs, also in the $CD19^-$ fraction can not be excluded. They might escape detection due to extreme rarity, paracrine dependency of similar immature cells which become too diluted in the mice, competitive disadvantage *vis-à-vis* the co-injected normal HSCs, or deficient homing capability. The finding of clonal involvement of both phenotypical $CD19^-$ cells and myeloid progeny in P210 ALLs together with the seemingly committed nature of the LSC (always $CD19^+$) could indicate the co-existence of a pre-leukemic, non-committed and thus multipotent (and possibly poorly engraftable) clone and a $CD19^+$ B cell committed transformed clone. This would in theory be in accordance with a recent report of CML in myeloid blast crisis, were purified committed myeloid cells had acquired self-renewal capacity[501], and could thus suggest that our patients diagnosed as *de novo* ALLs in fact were CMLs in lymphoid blast crisis, with myeloid Ph^+ cells produced from a remaining chronic phase LSC. It is, however, interesting to note that in three individual samples of CML in lymphoid blast crisis both $CD19^+$ and $CD19^-$ cells gave rise to ALL in the recipient mice (unpublished observation, AC, SEJ, and Anna Lübking), indicating a significant biological difference between CML in lymphoid blast crisis and *de novo* P210 ALL.

Whith regard to ALLs with the *RUNX1-ETV6* or P190 BCR-ABL1 fusions, we conclude that the LSC seems to be a committed progenitor, and not a multipotent HSC-like cell. We base this assumption on the expression of CD19 on the LSCs, the lack of myeloid potential of the clonal cells, and the unaffected normal HSC compartment. CD19 is, in our view, a particularly suitable marker for detecting committed B-cells, including the earliest progenitors (but excluding the plasma cells), for reasons explained above. However, it needs to be acknowledged that CD19 under certain conditions can be expressed on cells not belonging to the B-cell lineage, such as on a few percent of AML cells[531,532], and the formal

possibility therefore remains, that within the CD19+ ALL population uncommitted clonal cells, with aberrant CD19 expression, might reside. It should also be noted, that it is practically impossible to exclude the possibility of extremely rare clonal CD19− cells, below the detection of FACS purification and FISH cut-off levels, unless a huge number of leukemic cells are investigated.

The question of the identity of "the cell of origin" for the pediatric leukemias can not be definitely answered from our work, and might be very difficult to answer at all. It is, as stated above, possible that the t(12;21) or P190 BCR-ABL1 clones might contain very rare $CD19^{-}$ cells, perhaps representing pre-leukemic cells, or that such cells are only transient, due to the need for further genetic "hits" in order to be sustained, or because they are outcompeted by their transformed progeny. It has been observed that in otherwise healthy babies with $t(12;21)^{+}$ pre-leukemic cells detected shortly after birth, these cells have a $CD19^{+}$ phenotype[356]; but again, the existence of very rare $CD19^{-}$ cells cannot be excluded. Another interesting question in this context is; why are *RUNX1-ETV6* ALL (and other ALL subtypes more or less restricted to childhood) hardly ever seen in adults? One explanation could be that the postulated cause for the secondary hits, the mismatch between immunological development and postnatal infections, is age specific[322]. But the question remains if the genetic event causing the pre-leukemic clone also is restricted to childhood, and if so, why? Perhaps the initiating event targets only developmentally transient cell populations, as has been suggested in the case of transient myeloproliferative disease in children with Down syndrome[375]. One potential candidate cell population in the lymphoid lineage, which seems to have a largely prenatal origin, could be the so called B-1 cells, a rare population of B-lymphocytes with restricted immunoglobulin repertoire and mainly T-cell independent responses as compared to conventional (B-2) B lymphocytes[533]. The existence and characteristics of this cell population in humans, however, awaits further clarification.

For P210 BCR-ABL1-positive ALLs, on the other hand, it seems reasonable to assume that the "cell of origin" is a multipotent stem cell, given the finding of myelo-lymphoid potential whithin the leukemic population, and assuming that this multipotent cell is downstream of the originating clone.

We conclude that:

- ALLs with *RUNX1-ETV6* and P190 BCR-ABL1 fusions are maintained by $CD19^+$ B cell-committed LSCs, leaving the remaining normal HSC pool intact with regard to size, phenotype, genotype and function.
- ALL with P210 BCR-ABL1 fusion originates in a multipotent progenitor/stem cell, but might be maintained by a committed progenitor.

Future directions:

We have in our paper speculated that the small $CD34^+CD38^-CD19^+$ population found in ALLs with *RUNX1-ETV6* or P190 BCR-ABL1 fusions might represent the LSCs, due to phenotypical similarity to normal HSC and its small size. It is, however, important to acknowledge that we have no data supporting that hypothesis, or any data separating LSCs from the bulk of leukemic cells.

In subsequent attempts to identify the LSC in ALL, guided by our own hypothesis and the results of others indicating that the LSC might be restricted to cells with the candidate HSC phenotype CD34+CD38−[493], we have unexpectedly found that the expression of CD38 do not aid in demarcating the LSCs in most samples, since LSCs can be found in both $CD38^+$ and $CD38^-$ populations, with a dominating LSC activity in the $CD38^+$ fraction in most cases.

By comparing the global gene expression profiles of the $CD34^+CD38^+CD19^+$ and $CD34^+CD38^-CD19^+$ ALL fractions, a small but significant difference are

noted. The data suggests that the $CD34^+CD38^-CD19^+$ fraction might be the more immature of the two, since it displays a reduced expression of cell cycle regulating genes as compared to the $CD38^+$ fraction. Moreover, using principal components analysis (PCA), where similarities between normal and malignant cells can be investigated, we observe that the $CD34^+CD38^-CD19^+$ population displays an expression pattern consistent with its pre B cell phenotype, but still slightly closer to the HSC cluster than the $CD34^+CD38^+CD19^+$ fraction. An obvious limitation of these analyses is that the true frequency of LSCs in the two fractions is not known, and might be so small that the differences in gene expression levels rather reflects the differences between non-tumorigenic blast populations contained within the $CD34^+CD38^+CD19^+$ and $CD34^+CD38^-CD19^+$ fractions. However, there are *in vitro* data indicating a high frequency of LSCs in ALL[534]. We have further found another stem cell marker, CD90 (Thy-1), incapable of discriminating between LSCs and non-tumorigenic blasts (unpublished observation, AC and SEJ), which together with the same findings for CD38 could suggest that ALL LSCs might be rather prevalent within the blast population, and not possible to confine by any particular marker expression.

We also have preliminary data indicating the existence of distinct clones, based on specific immunoglobulin rearrangemt patterns, within the $CD34^+CD38^+CD19^+$ and $CD34^+CD38^-CD19^+$ ALL fractions, indicating distinct clonal LSCs. We are in the process of investigating if these distinct clones remain restricted to the population of origin upon reconstitution in NOD/*scid* mice, which formally would prove the existence of distinct LSCs, possibly hierachically organized within the leukemic population.

We have further turned our attention to CML in lymphoid blast crisis, as a model disease with a known origin in a multipotent HSC, to investigate at which level the transformation to blast crisis occurs, and thereby the identity of the LSC in this disease. As mentioned above, we have transplanted $CD19^+$ and $CD19^-$ CML/blast crisis cells into NOD/*scid* mice, and found that both fractions

reconstitute the same ALL-like diseae in mice (in striking contrast to all investigated ALL samples, including all P210s, where only $CD19^+$ cells reconstitute leukemia). Interestingly, when investigating the expression of several myeloid and lymphoid genes in purified $CD34^+CD38^-CD19^+$ and $CD34^+CD38^-CD19^-$ CML cells, we find that the $CD19^-$ fraction lacks expression of lymphoid genes but do express myeloid genes (as also normal HSCs do), while the $CD19^+$ cells, as expected, do express lymphoid genes, and none to low levels of myeloid genes. Together, these data indicates that whitin the same individual (at least) two quite distinct populations of cells, one B cell-committed and one uncommitted, can give rise to phenotypically identical disease in the mice. Possibly there is a spectrum of cells, traversing from uncommitted to committed, whith the capacity to self renew and thus act as LSCs. This would add to the recently proposed model of acquired self renewal capacity within a committed cell population in CML in blast crisis[501], the possibility that self renewal is not acquired again in the progenitor, but never lost in the first place, as the most immature, uncommitted, HSC-like LSC differentiates into a more mature, committed cell type.

We want to further corroborate the maturational difference between the $CD19^+$ and $CD19^-$ fractions by investigating in more detail any differences in lineage potential (we have, for example, observed a small myeloid, clonal population in mice reconstituted by $CD19^-$ cells, but not from $CD19^+$), and possible differences in sensitivity towards Imatinib.

Article II

Modern treatment of pediatric ALL leads to cure rates around 80%, and slightly higher is predicted for the near future[337]. These cure rates are achieved by more or less different international protocols, and it is generally assumed that cure rates might not increase much more only from intensifications or modifications of existing therapies, but new modalities, based on knowledge of the biology and molecular wiring of leukemic cells, needs to be developed. All children treated on contemporary protocols receive at least 2 years of continuous multidrug chemotherapy[461], although as many as two thirds may be cured with only 12 months of treatment[535], and a few percent with perhaps only a few weeks of single drug treatment[471] - the problem being that this group can not reliably be identified prospectively. Global gene expression analysis has emerged in recent years as a potentially very powerful tool to address these issues. The technology has already been applied to identify known genetic and phenotypic subtypes of ALL[457,536-539], and hopes are that new subtypes might be identified, allowing for a more specific and accurate risk group assessment, which will prospectively identify the group of children with excellent prognosis needing less intense treatment, as well as the group of patients who relapses in spite of often displaying classical low risk characteristics, such as age between 1 and 10 years and a low white blood cell count. Furthermore, microarray technology has been deployed to elucidate the molecular basis of drug responsiveness, in an effort to understand the cause of the 20% treatment failures. Hopes are that increased molecular understanding will generate new therapeutic targets for these unfortunate children.

However, to realize this, it seems pertinent that the cells to be studied should be the leukemic blasts, as uncontaminated by residual normal cells as possible. In order to achieve this a FACS strategy was developed, which identifies $CD19^+$ pre-B cells lacking immunoglobulin (detected by antibodies against the IgL) on the

cells surface, and which purifies pre-B ALL cells to a high degree (mean 99 ± 1,3%), as assessed by FISH on sorted populations.

ALL is generally thought to have its origin in a hematopoietic cell within the BM, but can often, to varying degrees, be found in the circulation or in other organs, like lymph nodes, spleen, liver and kidneys. Since blood is a more accessible tissue than BM, we wanted to compare the leukemic blasts from either source in order to evaluate if blood-derived leukemic blasts can substitute for their BM-derived counterparts in gene expression analysis experiments. We thus sorted phenotypically identical $CD19^{+}IgL^{-}$ pre-B ALL cells from blood and BM samples from patients (n=3) and performed microarray analysis, which revealed a striking similarity between blood and BM derived ALL cells (although the differences between the patients were significant). The only notable exception was VEGF, which consistently was expressed higher in BM than in blood. The most probable reason for this might be that the relative hypoxia in the BM induces VEGF transcription through hypoxia-inducible factor 1 (HIF-1)[540], but alternative explanations, such as a greater dependence of BM derived pre B ALL cells of VEGF, can not be excluded.

We conclude that:

- The majority of pre-B ALL cells (including the LSCs according to article I) can be prospectively purified by the phenotype $CD19^{+}IgL^{-}$.
- Pre-B ALL cells harvested from BM and blood are very similar, indicating that either source should yield valid gene expression data, whether for MRD analyses or biological studies.

Future directions:

One obvious experiment we are planning to do is to compare VEGF expression in normal $CD19^{+}IgL^{-}$ pre-B cells harvested from BM and blood in healthy donors, to

see if the differential expression of this gene in the two compartments found in ALL patients is reflected in healthy individuals. Since we feel that the most relevant comparison would be with age matched controls, the experiment is awaiting the accruement of samples from children, where BM aspiration is done but found not to be diagnostic for malignancy.

Further, we unexpectedly found a significant proportion of clonal $CD19^{+}IgL^{+}$ immature B cells from two patients. This finding was unexpected, since the disease overall has a pre B cell phenotype, and the reasons for a subset of leukemic cells to mature beyond that stage is not obvious. Can all leukemic cells reach this stage, but few do because of apoptosis, or can only a few do this, representing a certain "leakiness" of the differentiation block? We have preliminary data indicating that some leukemic cells from one of the above mentioned patients can mature even further into $CD19^{+}IgL^{+}IgD^{+}$ mature B-cells, suggesting a complete release from the pre B cell block (or escape from apoptotic signals).

One interesting experiment would be to sort $CD19^{+}IgL^{-}$, $CD19^{+}IgL^{+}IgD^{-}$, and $CD19^{+}IgL^{+}IgD^{+}$ cells from the same patient and inject into NOD/*scid* mice, to investigate the possibility of LSC activity in several of these distinct maturational stages. In case self renewal capacity would be found to span two or all three stages, it would reinforce our hypothesis that ALL is not driven by one homogeneous population of LSCs, but several.

Articles and manuscripts not included in the thesis

Pharmacokinetics of doxorubicin in children with acute lymphoblastic leukemia: multi-institutional collaborative study.
B. M. Frost, S. Eksborg, O. Bjork, J. Abrahamsson, M. Behrendtz, A. Castor, E. Forestier, G. Lonnerholm
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B. M. Frost, G. Lonnerholm, P. Koopmans, J. Abrahamsson, M. Behrendtz, A. Castor, E. Forestier, D. R. Uges, S. S. de Graaf
Acta Paediatr, 2003. **92**(5): p. 551-7.

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M. Buitenhuis, H. W. M. van Deutekom, L. P. Verhagen, A. Castor, S. E. W. Jacobsen, J-W. J. Lammers, L. Koenderman, P. J.Coffer
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Protein kinase B (C-AKT) regulates hematopoietic lineage choice decisions
M. Buitenhuis, L. P. Verhagen, H. W. M. van Deutekom, A. Castor, S. Verploegen, L. Koenderman, S. E. W. Jacobsen, P. J.Coffer
Submitted for publication.

Enforced expression of GATA-2 confers quiescence on human haematopoietic stem and progenitor cells *in vivo* and *in vitro*
A.J. Tipping, C. Pina, A. Castor, A. Atzberger, D. Hong, L. Lazzari, G. May, S.E. Jacobsen, T. Enver.
Manuscript

Sammanfattning på svenska (Swedish summary)

Våra kroppar består av ett mycket stort antal celler, varav en del sällan eller aldrig behöver bytas ut efter att en gång ha bildats (till exempel nervceller, hjärtmuskelceller och äggen i kvinnans äggstockar), medan andra har en mycket kort livstid och ständigt måste ersättas (till exempel cellerna i tarmslemhinnan som byts ut var 5-7 dag, och flertalet blodceller, varav en del existerar bara några timmar). För vävnader med snabb celldelning måste det finnas celler – stamceller - som kan dela sig men inte mogna ut och försvinna. Två egenskaper karakteriserar en stamcell; 1) den kan självförnya sig, dvs vid celldelning producera minst en dottercell som också är en stamcell och som inte mognar ut till en kortlivad, mogen cell, och 2) den kan bilda ett flertal mogna celltyper, så som till exempel blodstamcellen kan bilda alla olika typer av blodkroppar. Med denna definition kan man identifiera flera olika sorters stamceller, som till exempel embryonala stamceller, vilka är mycket omogna celler som existerar bara en kort tid under tidig embryonal fas och som har förmågan att bilda alla celler som finns i en kropp; ett annat exempel är blodstamceller, som finns under hela vår levnad och som producerar våra blodceller.

Stamceller har, trots att de varit kända sedan åtminstone 50 år, rönt mycket publicitet under de senaste åren, på grund av ett flertal spektakulära framsteg. Till exempel har man lärt sig att odla embryonala stamceller från mänskliga foster, vilket i sin förlängning kan innebära ett outtömligt förråd av celler som kan

användas för att ersätta förlorade eller sjuka celler hos patienter med till exempel diabetes, parkinsons sjukdom eller hjärtsvikt efter infarkt. Man har också insett att stamceller finns inte bara i vävnader med livlig cellomsättning, som blod och tarmslemhinna, utan också i organ som man trott var färdigbildade och utan cellnybildning vid eller strax efter födelsen, som till exempel nervsystemet och hjärtmuskeln. Man har dessutom sett att flera tumörer (till exempel vissa typer av leukemier, hjärntumörer, bröstcancer och tjocktarmscancer) drivs av ett fåtal cancerstamceller, som likt normala stamceller bildar ett stort antal mer mogna celler som inte kan självförnya sig och därför så småningom försvinner. Dessa cancerstamceller är därför i grunden det som driver en tumörsjukdom, och som måste behandlas bort. Det finns flera anledningar till att cancerstamceller kan vara svårare att behandla bort med cellgifter än mer ”mogna” tumörceller, men det är inte möjligt att bedöma behandlingseffekten på dem så länge vi inte vet hur de ser ut och kan studera dem. Vi vet inte heller i vilken mån en cancerstamcell liknar (och kanske rentav är bildade från) sin normala motsvarighet – vilket är viktig kunskap då vi med nya läkemedel inte vill riskera att skada eller förlora de normala stamcellerna.

Arbetena i den här boken bygger på studier av Akut Lymfatisk Leukemi (ALL, blodcancer som drabbar celler i det lymfatiska immunsystemet), vilket utgör ca 25% av all cancer hos barn, och är därmed den enskilt vanligaste cancerdiagnosen hos individer under 18 år. Hos vuxna utgör ALL däremot mindre än 1% av all cancer, och överskuggas framförallt av solida tumörer, men också av andra, framförallt kroniska, leukemiformer. I det första arbetet har vi försökt utröna i vilken typ av cell ALL uppstår – en normal blodstamcell eller en mer mogen cell som förlorat möjligheten att bilda några andra typer av celler än just lymfatiska – och därmed vad typ av cell som driver sjukdomen. Vi har undersökt två olika typer av ALL; en form som ses i princip bara hos barn och som har en generellt god prognos med modern behandling; och en svårare form, karakteriserad av förekomsten av en så kallad Philadephia-kromosom (Ph^+; en sammanslagning

av två kromosomer, där själva brottpunkten mellan dem bildar ett nytt protein, sk fusionsprotein, som antas vara viktigt för sjukdomen) i de sjuka cellerna, och som ses hos barn men framförallt hos vuxna. Vid den första, lindrigare varianten fann vi att ursprungscellen (och därmed också cancerstamcellen) *inte* var en normal stamcell, utan en mer mogen lymfatisk cell. Vid Ph^+ ALL däremot fann vi överraskande att sjukdomen kunde starta i *antingen* en stamcell *eller* en lymfatisk cell, beroende på hur fusionsproteinet såg ut. Det är känt sedan länge att det finns två varianter av detta fusionsprotein, men man har hittils inte kunnat påvisa någon signifikant skillnad med avseende på sjukdomsförlopp, behandlingssvar eller prognos mellan dem. Vi fann nu att den form som är vanligast hos vuxna bidrar till en leukemi som startar i en normal stamcell, medan den form som är vanligast hos barn startar i en lymfatisk cell. Denna skillnad är stor ur biologisk synvinkel, och kan också ha klinisk konsekvens, då en mer ”stamcellslik” ursprungscell av teoretiska skäl kan antas vara mer svårbehandlad (eller i varje fall kräva annan behandling) än en mogen cell. Vi fann också att den normala blodstamcellspopulationen var helt bevarad vid ”barnformerna” av ALL, men verkade reducerad vid ”vuxenformerna” (även om det senare var svårare att fastställa med säkerhet).

I det andra arbetet ville vi undersöka om leukemiska celler i benmärgen och i cirkulationen utgör samma typ av celler. Leukemi drabbar ursprungligen en omogen cell i benmärgen, som delar sig och efterhand fyller ut det begränsade utrymmet och därmed tränger undan de normala blodbildande cellerna. Den tilltagande förlusten av de olika blodkropparna orsakar i sin tur de symtom patienten får i form av blodbrist, blödningar och infektioner. Hos många patienter hittar man också varierande mängder leukemiska celler i blodet. Det är inte givet att dessa celler är desamma som dem man hittar i benmärgen – i den mån leukemiska celler fortfarande har något gemensamt med normala lymfatiska celler skulle man kunna tänka sig att de cirkulerande är mer mogna, eller i varje fall inte beroende av benmärgsmiljön för sin utveckling och överlevnad. För att kunna göra

denna jämförelse så precis som möjligt utvecklade vi först ett sätt att anrika de leukemiska cellerna, för att bli av med de varierande mängder av normala celler som finns i både benmärg och framförallt i blod, och som möjligen skulle kunna påverka vår analys. Efter anrikning jämförde vi så de båda populationerna med en teknik som kallas *microarray*, varvid det relativa uttrycket av ett mycket stort antal gener i cellerna undersöks, och där resultatet ger en mycket detaljerad bild av cellens funktionella identitet. Vi fann en mycket hög grad av likhet mellan de leukemiska celler som tagits från benmärg och de som tagits genom vanligt blodprov. Detta arbete ger således både en metodbeskrivning för hur man kan göra denna typ av jämförelser, samt indikerar att man lika gärna kan undersöka de betydligt mer lättåtkomliga leukemiska cellerna som cirkulerar i blodet, snarare än de som finns i benmärgen (och som i allmänhet fordrar narkos hos barn för att komma åt), för att fastställa till exempel behandlingseffekt, eller för fortsatta experiment för att lära mer om de cellulära mekanismerna vid sjukdomen.

Kvarstår gör frågan om ALL-stamcellens identitet och om den går att identifiera skild från mer mogna leukemiska celler. Vi har preliminära data som indikerar att det inte rör sig om *en* typ av leukemiska stamceller utan *flera*, i en och samma patient. Det är till och med möjligt att vid just ALL de flesta eller rentav alla leukemiska celler kan agera stamceller och vidmakthålla sjukdomen, till skillnad från vad som verkar vara fallet vid flera andra tumörformer. Frågan om detta i så fall är goda eller dåliga nyheter för patienter med ALL är i princip redan besvarad – drygt 80% av barnen med sjukdomen botas idag, vilket är överlevnadssiffror de hittils identifierade stamcellsdrivna tumörformerna inte ens kommer i närheten av. Dock återstår de 20% som inte går att rädda, och de utgör ett skäl så gott som något för att fortsätta sträva mot ökad kunskap kring denna sjukdom.

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