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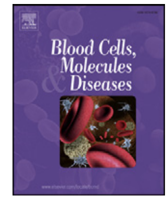

Stem cell clonality — Theoretical concepts, experimental techniques, and clinical challenges

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ABSTRACT

Here we report highlights of discussions and results presented at an *International Workshop on Concepts and Models of Stem Cell Organization* held on July 16th and 17th, 2012 in Dresden, Germany. The goal of the workshop was to undertake a systematic survey of state-of-the-art methods and results of clonality studies of tissue regeneration and maintenance with a particular emphasis on the hematopoietic system. The meeting was the 6th in a series of similar conceptual workshops, termed *StemCellMathLab*,[2] all of which have had the general objective of using an interdisciplinary approach to discuss specific aspects of stem cell biology. The *StemCellMathLab* 2012, which was jointly organized by the Institute for Medical Informatics and Biometry, Medical Faculty Carl Gustav Carus, Dresden University of Technology and the Institute for Medical Informatics, Statistics and Epidemiology, Medical Faculty, University of Leipzig, brought together 32 scientists from 8 countries, with scientific backgrounds in medicine, cell biology, virology, physics, computer sciences, bioinformatics and mathematics. The workshop focused on the following questions: (1) How heterogeneous are stem cells and their progeny? and (2) What are the characteristic differences in the clonal dynamics between physiological and pathophysiological situations? In discussing these questions, particular emphasis was placed on (a) the methods for quantifying clones and their dynamics in experimental and clinical settings and (b) general concepts and models for their description.

In this workshop summary we start with an introduction to the current state of clonality research and a proposal for clearly defined terminology. Major topics of discussion include clonal heterogeneity in unperturbed tissues, clonal dynamics due to physiological and pathophysiological pressures and conceptual and technical issues of clone quantification. We conclude that an interactive cross-disciplinary approach to research in this field will continue to promote a conceptual understanding of tissue organization.

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Introduction

Due to the limited lifetime of most differentiated cells, many tissues, require a constant supply of unique functional cell types. For blood, skin and the intestinal epithelium, where cell turnover is very high, the origin and regulation of mature cell production have been a major topic of investigation for decades. In all of these tissues, the mature cells ultimately derive from a pool of tissue-specific, but undifferentiated, self-sustaining somatic stem cells. The dual ability of these cells to maintain their undifferentiated status (reflecting the operation of a molecular mechanism for self-renewal) and initiate changes that lead to the production of large numbers of differentiated

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[2] Previous *StemCellMathLabs* were held in 2001, 2005, 2007 (Leipzig, Germany), 2008 [1] (London, UK), and 2010 [2] (Dresden, Germany).

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cells (reflecting the activation of a multi-step lineage restriction and eventual differentiation process) constitute their defining biological properties. Somatic stem cells, therefore, are able to guarantee both homeostasis of the tissue and (at least to some extent) its regeneration in response to injury [3,4]. It is this regenerative potential of tissue stem cells that has engendered great interest in the possibility that stem cells will be useful in many future clinical applications. However, although considerable progress has been made in identifying some of the molecular regulators of stem cell self-renewal and differentiation, understanding how these effectuate biological "decisions" at the cell and population level remains poor and conceptual models and theoretical considerations are likewise underdeveloped, particularly at the level of individual cells.

The hematopoietic system is a well-studied example, hence our decision to focus on it as a lead paradigm. Hematopoietic stem cells (HSCs) have been the subject of intense investigation for more than 50 years, and yet, many conceptual issues are not resolved, in particular with respect to the degree of biologic intrinsic heterogeneity that may exist within this compartment. For example, it is still not clear whether the hematopoietic system is fed by a small number of HSCs, which are sequentially activated from a pool of proliferatively inactive (quiescent) "reserve" cells and exhaust over time (i.e., clonal succession theory), or whether there is a constant low cell cycle activity of many (or even *all*) HSCs [5–8]. Another intermediate possibility is that their cycling behavior and self-renewal potential can fluctuate reversibly between these two extreme scenarios depending on the overall systemic need for cell production or other physiologic pressures [9]. Evidence for the latter dynamic adaptation scenario comes from the observation that HSCs can exist in two functional states, which are characterized by a very low and a relatively high cell turnover, denoted as *quiescent* and *active* states, respectively [10,11]. With these ideas in mind it is inviting to assume that in the homeostatic situation, a substantial proportion of HSCs remains in the quiescent ("reserve") state. However, when the system is perturbed, such as following in vivo exposure to agents that kill rapidly dividing cells (e.g., 5-Fluoruracil, 5-FU), almost all of the HSCs can be activated into cell cycle and return to a quiescent "reserve" state over time. Most notably, it has been demonstrated that a transition between the two proliferative states is reversible among the progeny of activated HSCs [10,12].

These results argue for a flexible, adaptive ability of HSCs to change some of their functional properties. However, there is also evidence that other functional properties may be transmitted with fidelity through multiple HSC self-renewal divisions and remain preserved within their progeny. In a simple approximation, the descendants of every HSC constitute a clone, which can include both an expansion of the HSC pool and a contribution of differentiated progeny to the peripheral blood. Cellular properties, inherited by the clonal progeny of individual HSCs, are called *clonal properties*. It is increasingly recognized that there is a considerable clonal heterogeneity among HSCs, e.g. with respect to their engraftment and repopulation potential [13–15] as well as their lineage contribution [16,17]. It has also been reported that the clonal composition of the HSC pool changes in an age-dependent manner both with respect to repopulation potential [18,19] and with respect to lineage contribution [20–22]. However, it is still unclear if and how the heterogeneous properties of individually analyzed HSCs are functionally relevant to the in situ development and aging of the compartment and the types of cells they produce.

In this conceptual framework, it would be expected that malignantly transformed cells, which themselves arise through a process of clonal evolution, might continue to reflect some of the differences in properties of the normal cells from which they arose. In some leukemias, this type of evolutionary process leads to the generation of recognizable subclones that display features different from the original clone, e.g. different degrees of treatment resistance [23,24]. Similar to the homeostatic situation, it is still unresolved, how the clonal composition of evolving malignant populations is affected by the properties of the individual cells/clones (i.e. clonal competition) or by extrinsic parameters.

Many experimentally and clinically observed phenomena can be interpreted and sometimes explained in the context of describing hematopoiesis as a superposition of *clonal processes*. This implies that individual HSCs and all of their progeny define the tissue phenotype over time. Because this concept suggests the coexistence of multiple, non-identical HSC-derived clones, it naturally imposes competition effects to be considered when interpreting system dynamics. The assumption of clonal competition for common resources has proven to be instrumental in modeling a number of phenomena (not just hematopoiesis), including chimeric reconstitution of mouse hematopoiesis [25,26], growth of leukemic populations in vivo [27], and clonal conversion in the intestine [28,29]. Many of these phenomena have been described qualitatively, but a strict quantitative analysis of clonal contributions has been considerably hampered by the limitations in the experimental techniques used that allowed only approximate estimates of clones to be made over time. Marking of individual HSCs prior to transplantation for tracking their clonal progeny produced in vivo has been used for almost 30 years (e.g., using the integration of retroviral constructs in mouse models [6,7,30] or later in gene therapy trials where the viral integration site also served as a marker of clonal identity [31]). However, the results of these studies have been partly contradictory and not adequately quantitative. The use of genetically distinct viruses has also been established for many years [32], but the "re-discovery" of virus-enabled "barcoding" of HSCs in combination with next generation sequencing (NGS) has launched a new era of more efficient and accurate determination of clonal contributions in a time-dependent manner [33–35]. And, although the issue of insertional mutagenesis remains with currently used vectors, the use of barcoding is now facilitating the investigation of hematopoiesis at a clonal level with unprecedented precision and resolution. At the same time, it is important to note that the significant progress in these experimental and measurement techniques for detecting and following clones has introduced new challenges in data analysis and in integrating the results into a conceptually meaningful framework. The *StemCellMathLab 2012* addressed this topic specifically as summarized below.

Definitions, terminology and general concepts

As in many scientific areas, terminology in the field of clonal analysis, is also quite heterogeneous. To allow for constructive cross-disciplinary discussions and to minimize misunderstanding, it is advantageous to define terms as clearly and objectively as possible. To that aim, Ingo Roeder addressed a number of potential pitfalls that could induce a "*Confusio Linguarum*", i.e. a confusion of tongues. In particular, he pointed out that the term "clone" in cell biology is sometimes used to infer that a population of cells sharing a common ancestry will have common properties. However, a more rigorous definition simply requires that all cells of a particular clone derive from the same founder cell. This in turn requires the unambiguous marking of the clonal offspring by the presence or introduction of *unique, heritable clonal markers* that can be detected experimentally (Fig. 1A). Moreover, this definition also accommodates the possibility that the same cell could belong to two (or more) clones, if different founder cells (for example characterized by subsequent clonal marking events) are considered as the clone-defining event. As a consequence, mono-, oligo-, or poly-*clonalities* all have to be regarded as relative properties (i.e., relative with respect to particular marking events) (Fig. 1B). Thus, the clonal progeny of a clone-defining cell can show different functional behaviors due to physiological (e.g. differentiation, maturation) and pathological changes (e.g. subsequent mutations) (Fig. 1C).

For cases where second (and later) clone-defining events are frequent (such as mutations), the term *subclone* is applicable. Subclones are of interest when they display a competitive growth advantage relative to closely related cells of common, but earlier clonal origin. The relative over-representation of a particular clone among others or of a derivative subclone is often referred to as *clonal dominance*.

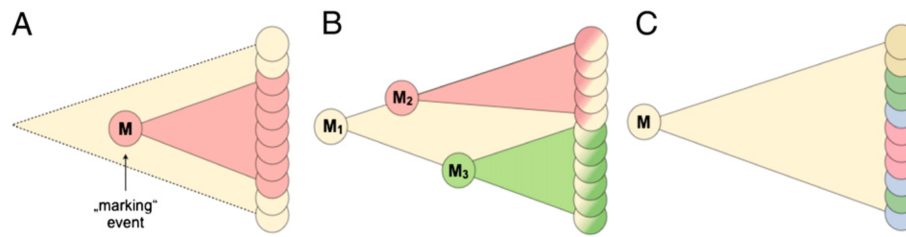

Fig. 1. Schematic representation of clonality patterns. A) Definition of a particular clone (red shade) by a unique, inheritable marking event "M". B) The illustrated cell population can be described as mono-clonal with respect to marking event "M_1", but oligo-clonal with respect to "M_2" and "M_3". This refers also to the fact that cells can simultaneously be members of different clones, due to the definition of a clone relative to a particular marking event. C) A particular clone, here defined relative to the marking event "M", can contain cells with different properties (illustrated by different colors).

Conceptually speaking, clonal dominance quantifies the relative contribution of more abundant clones. The most extreme case of clonal dominance would be apparent mono-clonality, i.e. the outcompetiton of all other cells by a single clone. However, clonal dominance is also encountered in oligo- or polyclonal tissues. It is also generally not possible to conclude from the observation of dominant clones that smaller clones are completely absent, due to the limits of detecting minor clones and sampling constraints.

The clonal pattern, i.e., the clonal composition of a system, can be highly dynamic as its particular behavior strongly depends on the relative "fitness" of each clone. As the relative contributions of particular clones and subclones are dynamic parameters, it is ultimately necessary to monitor clonal contributions over time to characterize the clonality of a system. Such measurements are the prerequisite to perceive trends such as a conversion to oligo- or mono-clonality, which might (though not necessarily) be indicative of their malignant transformation. However, as pointed out by a number of workshop participants (e.g., Hartmut Geiger, Ingmar Glauche, Joerg Galle), similar behaviors are likely to occur naturally in aged organisms. It is therefore critical to establish technological and conceptual tools to distinguish potentially pathological developments from naturally occurring changes.

Leonid Bystrykh pointed out that clonal populations of cells can be identified by a number of different, independent approaches, including analyses of karyotype, gene rearrangements, deletions, insertions or point mutations, X-linked polymorphisms, and integration of viruses into the host cell genomes (see below). However, these methods have different degrees of resolution, with an accompanying degree of ambiguity in the definition of the resulting clones. For example, the use of karyotypic markers allows multiple clonal populations to be analyzed as a group in a heterogeneous environment, whereas viral integration sites allow individual clones of cells carrying unambiguous markers to be discriminated.

Markus Loeffler emphasized the point that the members of a clone need not be biologically similar. In fact, there can be increasing variation in phenotype and genotype as the cells may evolve during their development (cf. Fig. 1C). Using the small intestinal crypt as an important example system to study clonal ancestry in a tissue with high cellular turnover, he illustrated how the size, rate of expansion and conversion of clones may fluctuate over time. In particular, he showed that the application of sophisticated mechanical and molecular mathematical models allow a diverse range of properties of intestinal epithelial cells, such as spatial structure of the crypts, biophysical properties of the cells, migration and molecular signaling, to be integrated into an agent-based framework [28]. Such models have been extremely successful to describe, and thus conceptually understand dynamics, time scales and clonal aspects of crypt homeostasis. Although the models successfully predict that crypts become monoclonal over time, Markus Loeffler pointed out that this only implies a common ancestral relation, but not that there is only one stem cell left. In fact, it indicates that all stem cells of a particular crypt descend from the same initial cell, but they are also still in a state of clonal competition, as repeated marking of the stem cells would again lead to clonal conversion.

Ehud Shapiro broadened this perspective by describing the results of his approach to reconstruct lineage trees of tissues or even complete organisms from an analysis of accumulated mutations in individual cells. These show that upon division, cells acquire a small number of random mutations in their microsatellite repeat loci, making their genomic signature effectively unique. Therefore, the genomic signatures of all cells of an organism implicitly encode a complete cell lineage tree that is defined by a sequence of sequential mutation events that demarcate the origin of new subclones at each cell division. Although this technique requires the sequencing of extensive genomic regions of individual cells, it provides a methodology to completely disentangle the ancestral relation of each single cell in an organism and thus goes far beyond classical clonal analyses. In principle, every cell of higher organisms should be amenable to this type of analysis, and thus allow the complete antecedent divisional history to be derived. However, technical limitations currently preclude such analyses and generally have constrained the use of this approach to tissues in mice with highly mutable microsatellites (short repeats). Nevertheless, several novel aspects of cellular development have been successfully addressed using this approach, e.g. leukemia relapse [36] and crypt dynamics [37].

Clonal heterogeneity

Differences in the behavior of individual stem cells have been well recognized since the first systems became available for analyzing clonal progeny. However, the simplest explanation for the different clonal outputs observed has been that these simply represent different responses of cells with the same intrinsically determined self-renewal probabilities and lineage potentialities but that are activated differently according to the external cues they (and their progeny) receive or by some limiting internal signal that causes a "stochastic" choice behavior. The relevance of such alternative mechanisms to stem cell fate decisions was first considered as potential explanations for the diversity seen in the cellular content of individual spleen colonies derived from multipotent hematopoietic cells and the corresponding acronyms: HIM (hematopoietic inductive microenvironment) and HER (hematopoiesis engendered at random), respectively, were coined to describe these two mechanisms. However, subsequent studies are now revealing that individual tissue-specified stem cells may not exist in a common "ground state", but may display self-perpetuating differences in both their self-renewal and differentiation propertied. Connie Eaves reviewed experimental evidence with single HSC transplants indicating the markedly different lineage outputs that individually tracked HSC-derived clones produce and pass on to successive generations of their clonal HSC progeny. According to their relative contributions to different classes of mature blood cells, different HSCs were classified as α-type (deficient contribution to lymphoid

cells), β-type (balanced contributions to myeloid and lymphoid lineages) and γ- and δ-type (deficient contribution to myeloid lineages) [17]. Although contributions of α and β HSCs into different blood lineages remain stable over long time scales and both types show durable self-renewal activity in secondary transplants; these cells also retain the potential for some interchangeability in their differentiation programs [22]. She suggested, therefore, that this behavior most likely reflects different, epigenomically determined HSC states with only the α and β states being coupled with durable self-renewal potential and external factors exerting their effects on HSC output diversity primarily at the latter stages of hematopoietic cell differentiation. Differential behavior of HSCs was also documented in terms of the *probability* of self-renewal as seen by a comparison of the rate at which HSCs are regenerated in vivo from HSCs of fetal vs adult origin. Although evidence of several intrinsic and extrinsic regulators has been identified, it remains unclear how they work as an integrated mechanism. As one approach to address this complexity, David Knapp illustrated the utility of multiparamater phospho-flow analysis by FACS or CyTOF to reveal heterogeneous patterns of signaling responses within the most primitive, functionally characterized phenotypes of human cord blood cells exposed to different external stimuli suggesting such parameters may also reflect different stem cell features and the extent to which they are activated.

Several workshop contributions focused on the effect of aging on the dynamics of a clonal system. Although changes in clonality/clonal composition are part of multiple theories of stem cell aging, more effort is needed to develop appropriate experimental models in which such changes can be described quantitatively and manipulated. Gerald de Haan provided a summary of earlier developments in this field and presented recent data illustrating that the abundance of functional HSCs in B6 mice increases with age, but in a fashion that is highly variable between different mice [19]. The overall increase in HSC numbers is, however, accompanied by a shift towards HSCs with features of α-HSCs (deficient lymphoid outputs). Clonal analysis of serially transplanted "young" HSCs in which β-HSCs are more predominant leads eventually to a shift in the production of α-HSCs. This shift argues in favor of a hypothesis suggesting that repeated stimulation to divide may underlie some of the effects attributed to "aging" leading to an increase in the overall size of the HSC pool but also a favoring of α-HSC self-propagation or transitioning from β-HSCs [20,21,38,39]. On the other hand Hartmut Geiger pointed to evidence indicating that an aged microenvironment can contribute to the acquired deficiency in lymphopoiesis characteristic of aged HSCs. He also emphasized the potential importance of this effect of the microenvironment in promoting the development of myeloid leukemia, the incidence of which is well known to be age-associated.

Ingmar Glauche took up the idea of an intrinsic heterogeneity in the stem cell compartment and presented a study on the influence of this effect on the speed of clonal conversion. Using earlier estimates on the HSC heterogeneity in individual repopulating cells [15] and within aging cell populations [40], he demonstrated that rate of conversion to mono-clonality increases with the degree of heterogeneity in the population. Although this clonality conversion might be a very slow process, the model-based estimates suggest that a trend to mono-clonality (i.e., decreasing number of contributing clones) could be visible within the lifetime of an animal. However, the sustained function and the observed variability of aged hematopoiesis together suggest that additional processes operate to continue to generate heterogeneity de novo. Thus the extent of heterogeneity within an HSC compartment is not likely to be a consequence of random changes alone, but caused by both stem cell-intrinsic factors and environmental factors.

In his presentation, Joerg Galle addressed the effect of epigenetics in the determination of clonal heterogeneity during aging. Specifically, he presented a theoretical study investigating the hypothesis that clonal heterogeneity in proliferating cell populations can originate from the limited capability of cells to inherit epigenetic information [41]. In this study an artificial genome-based model of transcriptional regulation was combined with a compartment model of stem cell populations. In each of the cells, regulatory machinery is active accounting for transcriptional regulation by cis-regulatory networks, histone modification and DNA-methylation. Thereby, histone modification affects both the promoter activity of genes associated with the modified chromatin regions and the activity of DNA methylases. Both, transcriptional activity and DNA-methylation status are assumed to feed back on the histone modification activity. In simulation experiments with this model, the impact of histone (de-) modification dynamics was investigated, in particular effects of histone (de-) methylation. The presented simulation results suggest that spontaneous loss of histone methylation due to fluctuations on short time scales would give rise to long-term gene silencing by DNA methylation. This silencing is a stochastic process introducing an evolving heterogeneity into the system. The resulting drifts in transcriptional activity would then potentially impair stem cell function and thereby contribute to features associated with aging.

Changing clonal composition due to clonal conversion was not only suggested for the hematopoietic system. It has also been a well-accepted behavior of cells in the crypts of the small intestine. There, the process takes up to about 100 days and can be imaged by the use of multicolor Cre-reporter R26R-Confetti-based clonal marking [42]. Peter Buske presented a simulation study that mimics the mouse intestinal crypt in silico, applying a 3D individual cell based-model. Using this model he showed that the capability of the clonal progeny of any stem cell acquiring crypt dominance strongly depends on its position along the crypt villus axis. Thus the progeny of a cell in the upper part of the crypt would be unlikely to become dominant. According to this model, competitive clones cannot overtake neighboring crypts; they can spread across the tissue by crypt fission only.

These contributions where complemented by presentations describing various specific perturbations that may influence the marrow microenvironment. Katherine Athayde Teixeira de Carvalho showed by flow cytometric analysis that the content of endothelial cells in the marrow increases significantly in female Wistar rats with hypothyroidism. Another group showed that a lack of ovarian hormones (caused by oophorectomy), causes an increase in B precursors/pro-B cells and a significant decrease in mesenchymal stem cells. These provide examples of how modulation of environment with hormones might influence clone formation and/or affect changes associated with aging.

Physiological and pathophysiological clonal dynamics

Basic research on the clonal organization of tissues and related clinical applications such as HSC transplants and cell-based gene therapy protocols are mutually stimulating each other. Basic research provides insights into organizational principles and allows for the testing of novel approaches, which are critical to anticipate safety and efficacy issues important to these clinical procedures. Conversely, clinical studies involving gene-marked cells offer unique opportunities for obtaining clonality information in human recipients of autologous cells.

In his introductory talk Christopher Baum argued that conventional clonal marking using efficient but untargeted integrating vectors generates additional clonal diversity by disruption, silencing or activation of genes near the integration site, a process termed *insertional mutagenesis*. Although this bias might be interesting in clonality studies, it is typically an unwanted effect in clinical trials. Untargeted integrating gene transfer induces a genetically mosaic population, leaving the growth dynamics of the affected cell either unchanged (neutral insertions) or disturbed [43]. While a retrovirally-induced growth disadvantage is usually not a problem and leads to an extinction of the affected clone, the acquisition of a growth advantage can lead to varying degrees of clonal dominance, and in some cases, the outgrowth of a malignant clone [44]. The risk of such malignant transformations, however, can be largely reduced by the choice of optimal viral vectors, which are less likely to integrate in critical genomic regions. Christopher

Baum proposed a candidate for a relatively neutral genetic marking study: an alpha-retroviral vector whose enhancer–promoter sequences are deleted and which uses an internal promoter that lacks strong enhancer activity to express a given marker gene. He points out that a major task in understanding the biology of gene-modified hematopoiesis lies in discriminating between spontaneous (i.e. intrinsic or aging-induced) clonal conversion and the induced clonality resulting from insertional mutagenesis. This requires the development of both sensitive and quantitative methods for the detection of particular clones.

Philippe Leboulch shared an extensive analysis on the sustained lymphoid deficient hematopoiesis obtained in a human gene therapy of β-thalassemia patient. Integration site analysis was performed by LM-PCR and DNA pyrosequencing extracted from both whole nucleated blood cells and purified sub-populations. In this patient, a clone with integration in the HMGA2 locus appeared partially dominant but did not overtake the whole hematopoietic compartment. Interestingly, this dominant clone showed a pronounced myeloid restriction, similar to the α-type HSCs described in mouse experiments. However, it is currently not known how this clonal dominance is maintained. Furthermore, it remains to be verified whether this clone occurred purely at random or not. Since the patient did not show any signs of malignancy, such clonal outgrowth seems to be functionally neutral. It would be important to investigate whether there are cell autonomous mechanisms that protect homeostasis within population of cells against a clonal advantage and compensate for the clonal outgrowth. There is a general agreement that computational models provide valid tools to conceptually address these questions.

Adrian Schwarzer noted that insertional mutagenesis could be the cause of early clonal imbalance leading to the expansion of a premalignant clone in a murine model of T-cell leukemia. Subsequent acquisition of mutations in oncogenes such as Notch1 can rapidly lead to an oligoclonal disease in which a variety of clones induce uncontrolled cell proliferation, followed by clonal progression related to cooperative events through insertional mutagenesis and additional spontaneous mutations. Monoclonality is eventually achieved by the selection of clones that have a high activation of mTORC1 and mTORC2, which both facilitate survival and expansion even in suboptimal environments.

In a related context, Dorothee von Laer and Sebastian Newrzela investigated the molecular mechanisms that control outgrowth of malignant T-cell clones. Interestingly, they observed that the development of leukemia could only be induced in T-cell populations that were monoclonal and not when they were polyclonal, which raise questions about the underlying mechanism(s). Specifically, they want to know what type of characteristics of a mature T-cell must be altered for a T cell clone to overcome clonal control and whether corresponding models of T-cell homeostasis would predict the difference of mono/oligo-clonal and poly-clonal settings in susceptibility to transformation. At present it is not yet clear whether T-cell lymphomas are rare because T-cells require more mutations than other cells to overcome mechanisms of clonal homeostasis that may be particularly strong, or whether the loss of clonal homeostasis is a prerequisite for a transformed T-cell clone to expand, acquire additional mutations and overgrow the other T-cells.

Ute Modlich provided an illustrative summary on clonal aspects of liver regeneration obtained with transplants of vector-labeled hepatocytes. Importantly, integration site analysis to quantify clonal contributions showed a convincing correlation between the number of insertion sites obtained by high throughput sequencing methods and qPCR and retroviral marking did not decrease overall survival nor induce tumor formation. Although the regenerated cells were mostly polyclonal, a few clones were found to have expanded more than others. The provided data nicely illustrates the applicability of integration site analysis to quantify clonal contribution in liver regeneration with an adequate correlation between sequence counts of insertion sites by high throughput methods and quantifications of individual insertion sites by qPCR. Although liver regeneration was not based in stem cell function, clonal succession was similar in the liver compared to the hematopoietic system.

Quantification of clones in experimental and clinical settings

A major and reoccurring theme within the workshop centered on the methodology for the quantification of clone numbers and sizes. As Leonid Bystrykh summarized, the applicability of different methods strongly depends on the particular scientific question being addressed. Retrospectively, all techniques ever reported to be used in clonal studies can be classified on the basis of their clonal resolution. The lowest resolution is achieved using heterozygous allelic markers (binary resolution) followed by color lineage tracing (dozens of distinguishable clones), then barcodes (hundreds to thousands of distinguishable clones) and finally retroviral integration site analysis (potentially unlimited numbers of different clones). Methodologies for undertaking a clonal analysis of malignant and non-malignant tissues or regenerated population need also to be considered differently. Naturally arising mutations offer unique markers to study the evolution of leukemic clones, but are less likely to be applicable to "normal" HSCs where retro- or lenti-viral labeling approaches are the method of choice to follow their dynamics. For genome/transcriptome-based methods, the success of detection and discrimination of a mutant clone is very much dependent on the particular disease. Generally, the analysis and interpretation of data identifying and quantifying clones and subclones present in leukemia populations are complicated procedures, due to the genomic instability of the cells. In this respect, a unique, functional definition of a leukemic clone is difficult to achieve.

In general, there are currently two commonly used methods for high resolution identification and quantification of clones, namely an approach based on the identification of an inheritable viral *integration site*, and an alternative approach in which random, albeit unique sequences (*cellular barcodes*) are contained within the viral vectors. Whereas clonal analysis using integration sites allows for a theoretically endless number of differentially detectable clones, it is sometimes difficult to identify and map all occurring integrations. Furthermore, the necessity to use PCR methods with a variety of restriction enzymes leads to potential biases in their detection and quantitation. In contrast, the cellular barcoding method is generally more consistent in measuring clone sizes over time than integration site analysis (although published data are very limited at this time). Both approaches, however, are similarly prone to sequencing errors, especially when high throughput techniques are applied. In addition, cellular barcoding technique has a limitation in the number of barcodes that may be present in a given library. Therefore, the success in unique labeling of HSCs by this method depends heavily on an in depth examination of the vector library and a reasonably accurate estimate of the number of HSCs to be traced in a given experiment.

Manfred Schmidt pointed out that clinical gene therapy is a rapidly developing field in which new and improved methods for genomic integration (e.g. SIN vectors, transposons, Zinc-finger nucleases, etc.) are applied. Clonal quantification in the context of clinical trials uses primarily next generation sequencing techniques and exploits growing knowledge on the structure of the human genome and the organizational principles of stem cell populations. However, some principal issues and questions remain. The foremost problem concerns the nonspecific integration of the viral vectors still in use which all have the potential for causing insertional mutagenesis and resulting problems. The tight monitoring of such unwanted effects requires continuous, robust and reproducible quantification of the contribution of individual clones. Manfred Schmidt noted that the use of various restriction enzymes and repetitive LAM analysis, a (semi)quantitative analysis for individual clonal contributions can be readily achieved [45]. When applied to serially obtained samples, the same methodology can

provide information about the dynamics of individual clones over time and can guide more detailed quantitative PCR analyses of individual clones. Because integration site analysis includes a determination of the precise location of the vector in the host genome, this can provide very important biosafety and clinical prognostic information.

Current limitations of integration site-based approaches were also addressed by Martijn Brugman. He emphasized the great care that needs to be taken when using deep sequencing to define the number and size of clones identified by genetic alterations in either experimental or clinical material. Noteworthy are biases inherent in LM-PCR or LAM-PCR data, that probably originate from the unequal amplification of DNA sequences of different lengths, which are specific for a particular integration site and result from the usage of restriction enzymes. He showed that this effect can lead to different quantities for specific insertion marks using alternative quantification methods, which is problematic for the robust tracking of fluctuating clonal populations. Although newly developed techniques such as the phage μ-based system [46] or nrLAM [45] might solve restriction issues, their quantitative performance needs careful monitoring [47]. However, this may potentially be achieved by a combination of viral barcoding with classical insertion site analysis.

Kerstin Cornils then provided a specific example illustrating the potentials and limitations of insertion site-based methodologies. She used γ-retroviral and lentiviral vectors in an HSC transplantation setting to assess the clonal composition of peripheral blood at different time points. Next, DNA was extracted and analyzed using LAM-PCR to determine integration sites and to quantify their relative abundance by next generation sequencing. The results showed that this model can recover the kinetics of the marked clones produced, but also demonstrated the limitations of the approach [48]. The PCR-based approach to identify viral integration sites proved to be limited by the access of the restriction enzyme used to the flanking genomic region, and that the length of the PCR fragments generated also severely influences the subsequent amplification and hence quantification of the fragments obtained.

Within the StemCellMathLab, the potentials and especially the technical details of various barcoding approaches were discussed vividly. In brief, these methods rely on the integration of a random "barcode" sequence of fixed length within the original viral constructs to establish viral libraries containing identical virus constructs that only differ in the sequence of their barcode. Upon transduction and transplantation of recipient cells, the resulting population and their offspring can be tracked by quantifying the abundance of specific barcodes. The fixed length and structural similarities largely reduce biased amplification and quantification.

Evgenia Verovskaya reported on the successful implementation of a medium-sized barcode library to study the clonal dynamics of HSCs in mice. Using a sophisticated method for the correction of amplification-induced errors in the barcode sequences, she demonstrated that the method of cellular barcoding could be calibrated in model conditions (e.g., using a series of clonal dilutions) to determine the accuracy and error of the measurements. This validation confirmed the ability of the method to accurately quantify clones representing as little as 0.5% of the total population. She also demonstrated that clones of different sizes could be robustly detected and quantified in experiments in which blood samples were repeatedly obtained over time. This enabled her to then accurately track and compare the clonal variations in HSC contributions to the different mature blood cell types in mice transplanted with young and old bone marrow cells.

Rong Lu also presented results from her recent work on in vivo clonal tracking of barcoded HSCs. She discussed the technical details of her methodology that combines viral barcode labeling and high throughput sequencing [34]. In particular, she emphasized the importance of using a diverse viral library to ensure clonal representation of barcodes since libraries of low diversity may label multiple cell clones with the same genetic barcode. She showed that the diversity is not only related to the number of barcodes in the library, but also to the relative abundance of each barcode. She then presented her recent studies illustrating HSC clonal development through multiple stages of lineage commitment and showed that some HSC clones expand gradually at each differentiation step.

Jeanne Grosselin described her studies of HSC dynamics in transplanted mice using an arrayed barcoded lentiviral strategy. This involved transducing different populations of HSCs with specific, known barcodes, thus allowing for an a priori association between a given barcode and cell population marked by it. She documented a strong maintenance of barcode representativeness over the course of several months after transplantation and successfully recovered the same differentiation patterns as previously described by the Eaves lab (i.e. α, β, δ/γ cells with a robust and sustained deficiency of lymphoid, neither or myeloid cells, respectively) [17].

As a continuation of these findings, Boris Fehse suggested that for certain applications promoter-deficient α-retroviral vectors equipped with individual barcode sequences would represent an ideal clone-specific marker. Although this concept (still) has certain limitations, including the necessity to destroy cell integrity for detecting the barcode and the problem of sufficiently high barcode complexity, it is highly suited to follow clonal contributions in a time-dependent manner while minimizing the problem of insertional mutagenesis.

These technical contributions were complemented by a presentation from Sebastian Gerdes. He used a simple mathematical model of clonal competition to study various challenges in the quantification of relative contributions of individual clones over time. He illustrated how the transplanted cell dose, the true number of HSCs, their cycling behavior and the physiological heterogeneity of these cells influence the observed patterns of clonal compositions (Fig. 2). He also showed a number of available measures to quantify clonal heterogeneity, at the same time demonstrating their inability to robustly distinguish physiological and pathological hematopoieses. However, these approaches point towards a direct impact of mathematical models to investigate and develop tools that are better suited to disentangle these questions.

The discussion during the StemCellMathLab made clear that a consequent description of protocols and technical details on barcode design, library complexity, sample preparation, transduction, transplantation, barcode amplification, sequencing, and error correction are ultimately necessary to truly evaluate and interpret the resulting data. Unfortunately, there are as yet no tools or protocols to standardize the use of this exciting and powerful methodology.

A specific point of the workshop discussion was the potential use of barcoded viral constructs in clinical research and gene therapy. Although it is clear that the use of truly "random" sequences may be limited to model systems, it was interesting to consider the amount of information that might be obtained using a few, clinically approved distinct barcodes. These short sequences could be monitored with much higher accuracy as compared to the viral integration sites. Further studies are required to validate whether such limited-library approach is suited to provide a reliable picture of the overall clonal dynamics and to accurately predict clinically relevant deviations. These questions might well be addressed using computational frameworks.

Summary/conclusions

As described above, the workshop discussions identified several interesting and unresolved questions. *Clonal heterogeneity* is clearly an important topic: Clones of different compositions and behaviors can be observed in even the same situations, but the degree and the reasons causing this variability remain unresolved. The need to quantify the variability of clonal contributions objectively – *measures of clonal heterogeneity* – will be prerequisite not only to understanding normal stem cell populations and their control, but also to distinguish pathologic deviations (e.g., malignant dynamics).

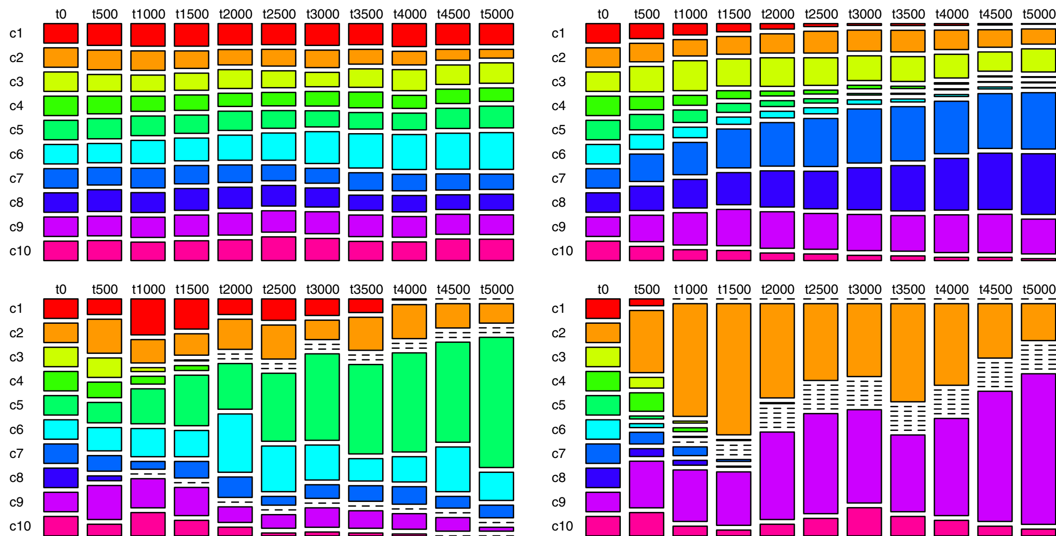

Fig. 2. Dependence of abundance dynamics of 10 stem cell clones on the size of the stem cell compartment and interclonal heterogeneity in a simple model of HSC proliferation. At each time point, a cell is randomly chosen to self-renew, and another to differentiate. Four different scenarios were simulated (in each case 5000 time steps for 10 initially equally abundant clones, and measurements every 500 time steps). In the upper panels, a large stem cell population (n = 1000) has been assumed, and in the lower panels a small stem cell population (n = 100). In the left panels, all cells have the same probability to be selected for self-renewal divisions. In the right panels, this probability depends on clonal membership, inducing heterogeneity between the different clones in the system. These simulations illustrate that the clonal abundance patterns depend crucially on the size of the stem cell compartment, and the degree of heterogeneity between the different clones. In particular, clonal conversion is accelerated for smaller stem cell pools with larger heterogeneity.

Processes that anticipated to increase clonal heterogeneity include mutations and viral integration. However, even without such events, the composition of the clonal repertoire can be expected to change. As agreed by all workshop participants, one general process that affects clonal heterogeneity is *clonal conversion*, which leads to a loss of contributing clones in a system over time due to stochastic fluctuations. As addressed by a number of presentations, this process will depend on many variables, such as the size of the system, local cues (e.g., as provided by stem cell "niches") and the proliferative activity of the cells (which may change in accordance with changing needs of the system). Also, the process of clonal conversion might well be counterbalanced by de novo generation of clonal heterogeneity, e.g. due to genetic mutations or functional adaptations.

If one accepts clonal contribution as a dynamic process and, therefore, the existence of a changing degree of clonal heterogeneity in disturbed but also in normal, healthy systems, it is obvious that the quantitative characterization of clonal contributions poses a major challenge. Along these lines, one has to be aware that deviations from a constant (poly) clonal contribution are not necessarily signs of abnormal or even malignant behavior. This, however, is an essential task also from a clinical perspective, as emphasized e.g. by Christopher Baum. He pointed to the importance of being able to distinguish spontaneous or age-related changes in the clonal repertoire from effects induced by insertional mutagenesis, e.g. in gene therapeutic settings. To objectively identify abnormal clonal patterns, "normal" or "neutral" clonality has to be defined beforehand. Accordingly, an important task will be a thorough characterization of these.

Here, mathematical modeling approaches may be of considerable help, as they are able to systematically assess the effect of different parameters (such as proliferation, available resources, clone size, etc.) on clonal patterns in a quantitative manner, rapidly and *in silico*. In this respect, Ehud Shapiro pointed to an important aspect: The formal (mathematical) analysis of clonality patterns needs suitable methods for data presentation. Because the individual relationship of the cellular progeny is the essential information, clonality structures are best represented by so called *lineage trees* (also referred to as *cellular genealogies*, if the tree structure is complemented with functional annotations). Although genealogical structures can be described by ordered tree graphs in a straight forward way, their analysis poses similar challenges as the advent of string type data (e.g. genome sequences) did 30 years ago. An established and efficient tool box for their analysis ("*bioinformatics of trees*"), however, is currently missing.

Besides the conceptual assessment of clonal patterns, there is also the need for sensitive and quantitative detection techniques. Here the workshop brought together internationally leading experts for a number of these technologies, including L(A)M-PCR (represented by Christopher Baum, Manfred Schmidt) and cellular barcoding (represented by Leonid Bystrykh, Rong Lu, and Boris Fehse). It became very clear that different methods have specific advantages, but also limitations and there was consensus among the workshop participants that a detailed and comprehensive description of the methods applied in any particular experimental or clinical setting will be essential to assess any results obtained.

The benefit of theoretical models for achieving new biological insights was another major topic of the workshop discussions. As brought up by Arne Traulsen, it should be appreciated that (by definition) a *model* is always a simplification of reality and – in this sense – "wrong". However, even though – or sometimes *because* – models are simplifications, they can help considerably to understand complex processes [49]. The British statistician G.E.P. Box formulated this in his famous saying: "Essentially, all models are wrong, but some are useful". Moreover, it should be clear that different problems and different questions call for different answers; thus the models to answer these purposes differ, too. If we want to conceptually highlight different aspects of consequences from stem cell organization and tissue organization, some models may be helpful although they give only crude answers. If instead, we are interested in detailed questions of clonal development, we may require much more detailed analysis, e.g. with information from (complete) genealogical trees. However, if we could have a complete quantitative picture of the clonal contribution during the development of an individual, could we really handle and synthesize the data associated with it? With the advent of methods for accruing increasingly larger data sets, it is becoming obvious that much high-throughput data remains descriptive at best and unclear at worst without proper "mathematical" processing, analysis and interpretation.

As pointed out by Connie Eaves, many experimentalists would like modelers to come up with predictions that can be challenged experimentally. Whereas theoretical predictions should certainly be a driving force of the scientific process, it is, nevertheless, important that

theoretical investigations in biology and medicine are motivated by a specific biological or medical question. The discussion of this point made it clear that there is still the potential of misunderstanding, due to the different perspectives of theoreticians and experimentalists/clinicians. However, there was consensus about Ingo Roeder's statement that we need to *jointly* identify those questions, which are biologically relevant *and* theoretically addressable. Having identified suitable targets for a modeling analysis, one has to ensure that model predictions are formulated in a way that they are experimentally testable – at least in principle. On the other hand, this does not deny the justification of either "purely" experimental or "purely" theoretical studies. Both can lead to new (unexpected) hypotheses, which can again be addressed more specifically. Ideally, our efforts should combine plausible assumptions for making theories, and theoretically acceptable interpretations of experimental data. In any case, only those statements are worthwhile making, which can be experimentally validated and be proven right or wrong.

In summary, the workshop was a successful example of a cross-disciplinary attempt to better understand stem cell organization from a clonality data perspective. However, it also became clear that it is a crucial point to define what we talk about and what we want to learn from our experiments and models, particularly within an interdisciplinary community. Otherwise, we will never escape from the *Tower of Babel*.

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