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Reflections on the European Union Eurythron Network Meeting “Molecular Control of Erythropoiesis,” September 22–23, 2005, Istituto Superiore Di Sanità, Rome, Italy

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ABSTRACT

Red blood cells (RBCs) mediate oxygen transport throughout the body, a function that is essential for life. RBCs are continuously produced via a process called erythropoiesis. Anemias (insufficient numbers of functional RBCs), caused by failure of erythropoiesis, are a major cause of disease worldwide. Hereditary anemias constitute the most common human genetic disorders; they have no effective cure yet. The European research training network Eurythron follows a multidisciplinary approach to clarify the important molecular mechanisms in normal and pathological erythropoiesis, with a view to develop

novel therapies to cure the anemias. The aim is to generate a comprehensive molecular description of mechanisms governing specification of hematopoietic stem cells in embryogenesis, lineage commitment, differentiation, and postmitotic maturation of RBCs. We report on the Eurythron meeting in Rome, in which novel approaches in stem cell and erythroid cell biology, including in vitro expansion of primary cells, biochemistry of receptor/signal transduction complexes and transcription factors, and (epi)genetics, were discussed. *STEM CELLS* 2006;24:2478–2482

Background

The process of hematopoiesis continues to play a central role in establishing the general principles underlying how adult stem cells arise during embryogenesis and the decisions they make in forming fully differentiated mature cells. Historically, the hematopoietic model has prevailed because progenitors are accessible and can be analyzed at different stages of differentiation using well-established high-speed cell sorting and imaging methods. Purified cells can then be characterized using a variety of techniques to determine their transcriptional profiles, epigenetic programs, and responses to alterations in cell signaling. Importantly, many of these processes are well conserved throughout evolution, for instance, in zebrafish, *Xenopus*, mouse, and human, allowing one to extend and extrapolate results from one species to another. Eva Krpelanova and Sjaak Philipsen (Erasmus University Medical Center, Rotterdam, The Netherlands) presented the use of the roundworm *Caenorhabditis elegans* as a tool for functional studies of transcription factor families. Although *C. elegans* does not have a system equivalent to the hematopoietic system of vertebrates, it does contain homologues representing the many and often large vertebrate transcription factor families with hematopoietic func-

tions. *C. elegans* is an attractive model system for the analysis of basic biological problems, including stem cell biology. Unique to this organism is the fact that the origin and developmental fate of every cell are known. This fact, in combination with the complete genome sequence, comprehensive and accessible collections of mutants, and a full arsenal of molecular and genetic tools, makes it feasible to unravel the role of entire gene families in ontogeny and lineage determination of every cell of the organism. Although more time consuming, the use of homologous recombination, transgenic approaches, viral transduction systems, microinjection, single chain antibodies, RNA interference, and morpholinos has enabled comprehensive experimental approaches to address the key issues in vertebrate organisms. The rich and still growing resource of natural and experimentally induced mutations has directed relevant scientific questions and also provided a clinical perspective.

Hematopoietic Stem Cells

How are hematopoietic stem cells generated during development and how are they maintained in adult life? How do they proliferate and control stem cell numbers? How do they choose among self-renewal, lineage commitment, and differentiation?

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First, it has been important to establish the precise cellular origins of hematopoietic stem cells (HSCs) from mesoderm. Mesoderm induction is the first step leading to the specification of embryonic and adult hematopoietic cells. Lineage labeling in *Xenopus* has shown that these two blood populations arise from different parts of the embryo giving rise to transient embryonic (primitive) hematopoiesis and permanent (definitive) hematopoiesis [1] that will generate the adult hematopoietic system. An important aim is to understand the transcriptional circuitry underlying hematopoiesis from stem cell formation to terminal differentiation of the mature cell lineages. Roger Patient (The Weatherall Institute of Molecular Medicine, Oxford, U.K.) reported on the application of new bioinformatics tools to integrate the data from diverse biological and experimental systems into information presented as genetic regulatory networks (GRNs) [2]. The GRNs created and analyzed by the NetBuilder program [3] should help to deal with the ever-increasing data generation. This and similar approaches will be essential to advance the knowledge of fundamental biological processes such as hematopoiesis, because the sheer diversity and amount of data produced daily is beyond comprehension if not put in an accessible framework such as GRNs. A novel approach that combines classic genetics with contemporary genomics, termed "genetical genomics," was presented by Gerald de Haan (University of Groningen, Groningen, The Netherlands). The C57BL/6 and DBA/2 mouse strains display large variation in longevity of hematopoietic stem cells. Such a quantitative trait can be mapped genetically in so-called recombinant inbred lines. Such lines are obtained by crossing DBA/2 with C57BL/6 mice, followed by many generations of brother/sister matings. Eventually, this results in a collection of inbred lines each containing different parts of the DBA/2 and C57BL/6 genomes (BXD lines) [4, 5]. In addition to genetic mapping of the quantitative traits, the mRNA expression levels in purified HSCs isolated these BXD lines were determined using microarray analysis. These data were then combined to identify genes and loci that control HSC function [6]. Overexpression of Enhancer of Zeste homolog 2 (*Ezh2*), a Polycomb group protein involved in histone methylation and deacetylation, prevents hematopoietic stem cell exhaustion. The "genetical genomics" screen was used to identify several novel putative *Ezh2* target or partner stem cell genes. This suggests that a network involved in stabilization of chromatin structure plays an important role in the preservation of HSC potential [7]. Elaine Dzierzak (Erasmus MC) presented the recent identification of the midgestation mouse placenta as a rich source of adult hematopoietic stem cells [8–10]. This raises the exciting possibility that the human placenta is also an abundant source of HSCs, which could be used for therapeutic applications. It is not trivial to extend these observations from the mouse to the human, since there are major differences in the tissue architecture of the human and mouse placenta, and it is unknown at which developmental stage HSCs might be present in the human placenta. It also remains to be determined whether placental HSCs can be expanded in vitro and whether HSCs can be generated in situ in the placenta.

Lineage Determination

Many approaches exist to unravel the process by which a HSC becomes committed to the erythroid lineage. Since the phenotype of a given cell ultimately reflects the genes it currently

expresses or has expressed in the past, research has focused on (a) analyzing global gene expression profiles, both at the transcriptional and the translational levels; (b) the expression of individual genes that are already known to play an important role in hematopoiesis (e.g., the transcription factors *Gata1*, *Gata2*, *Runx1*, and *Tal1*); and (c) emerging signaling pathways (e.g., those activated by integrins; *Wnts*/ β -catenin, Notch, and receptor tyrosine kinases), which may crucially regulate the activity of transcriptional/epigenetic regulators, and thus influence decisions between renewal and commitment. Tariq Enver (The Weatherall Institute of Molecular Medicine) discussed the development of mathematical models to describe how cells make transitions between stable states. It is known that the transcription factors *Gata1* and *Pu.1* have a cross-antagonistic relationship: they are involved in a positive autoregulatory feedback loop but repress each other's activity [11]. Myeloid cells express *Pu.1*, whereas *Gata1* is expressed in erythroid/megakaryocytic cells. Nevertheless, bipotential precursor cells that can give rise to the myeloid- and erythroid/megakaryocytic lineages express both proteins. Intuitively, cells progressing from the bipotential- to the unilineage stage would downregulate one of the factors and simultaneously upregulate the other. The mathematical model suggests another possibility, namely that both proteins are first downregulated before one of the two is upregulated. It will be interesting to determine whether this is a general mechanism in cell fate decisions, and whether it holds bearing on lineage infidelity as has been observed for *Pax5 null* B cells [12].

Erythroid-Restricted Progenitors

Following specification of the bipotent megakaryocyte-erythrocyte progenitor from the multipotent common myeloid progenitor, the earliest recognizable cell committed to the erythroid lineage is the erythroid burst-forming unit (BFU-E). This cell is defined by its ability to form large erythroid colonies in vitro. Under the influence of a variety of cytokines and growth factors, the corresponding progenitors in vivo become further committed to the erythroid lineage, proliferate, and differentiate to mature red cells. There are a number of protocols that enable both human and mouse erythroid precursors to be purified and subdivided into different stages of maturation, and unlike earlier precursors, such cells can be obtained in larger numbers. In addition, protocols have recently been developed for long-term expansion of mouse and human BFU-E-like progenitors [13]. Representing Hartmut Beug (Institute of Molecular Pathology, Vienna, Austria), Marieke von Lindern (Erasmus University Medical Center) presented a novel method of growing large numbers of erythroid progenitors from mouse ESCs [14]. Furthermore, forced expression of the *HoxB4* transcription factor in ESCs can be used to generate mass cultures of primitive hematopoietic progenitors [15]. Clinically, this is an exciting development because ESCs can be used for gene repair by targeting, followed by expansion of genetically defined clones. The use of such clones would avert current safety issues of virally delivered somatic gene therapy [16]. Scientifically, these cell culture systems enable the use of the vast resource of mutant ESC lines (<http://www.genetrap.org/>) for the study of hematopoietic development without the need to generate mice first. These culture models will also be an excellent tool for establishing the transcriptional and translational profiles of the early multipotent

progenitors and erythroid cells at well-defined stages of commitment and differentiation. In addition to knowing the pattern of gene expression at any particular stage of hematopoiesis, it will be important to understand the mechanisms by which cellular memory is established and maintained. These mechanisms include a range of epigenetic modifications, such as nuclear position, the timing of replication in the cell cycle, changes in chromatin structure, multiple histone modifications, and DNA methylation. Claire Francastel (Institut Cochin, Paris) described recent progress in the analysis of nuclear architecture and control of gene expression during erythroid differentiation. It is becoming increasingly clear that nuclear compartmentalization of gene loci and transcription factors change dynamically during cellular differentiation. The β -globin locus relocates to the interior of the nucleus when it becomes actively transcribed during erythroid differentiation, suggesting that nuclear gene position determines the expression status of a gene [17]. The observation that the activity of the transcriptional activator NF-E2 is regulated by sequestration of its subunits into distinct nuclear compartments provides a clue to the mechanism of nuclear localization of active genes [18]. The combined use of molecular biology and advanced confocal microscopy will enable an *in vivo* analysis of the protein domains involved in the dynamics of nuclear localization.

Transcription Factor Complexes

Some key transcription factors involved in erythropoiesis are already known (e.g., Gata1, Gata2, EKLF, NF-E2, Nrf1–3, and Tal1). Newly developed procedures for *in vivo* tagging of these proteins enables the isolation of their interacting partners. John Strouboulis (Erasmus MC) discussed the application of *in vivo* biotinylation tagging of transcription factors, using a small peptide tag recognized by the *Escherichia coli* biotin ligase BirA. Biotinylated factors can be isolated with high efficiency and specificity in a single-step purification procedure, followed by mass spectrometry analysis to identify potential interaction partners [19, 20]. Paul-Henri Romeo (Institut Cochin) showed successful use of biotinylation tagging to identify members of the Eto family of transcriptional repressors, in particular Eto2, as novel interaction partners of the Tal1 transcription factor. ETO is involved in the t(8;21) translocation that is associated with 12%–15% of human acute myelogenous leukemias of the M2 subtype. Binding of Eto2 modulates the activity of the Tal1 complex and prevents differentiation. Two independent studies have confirmed these observations [21] (N. Meier, S. Krpic, P. Rodriguez, J. Strouboulis, M. Monti, J. Krijgsveld, M. Gering, R. Patient, F. Grosveld, A. Hostert, manuscript submitted for publication). Morpholino-mediated knockdown of *eto2* in zebrafish indicates that it is important for the development of the definitive hematopoietic system. Eto2 may respond to extracellular signals by translocating from the cytoplasm into the nucleus, whereas it is downregulated in terminally differentiating cells. This suggests that Eto2 plays a key role in the birth of the definitive HSCs and is required for the subsequent phases of expansion of the different lineages (N. Meier et al., manuscript submitted for publication).

Cell Signaling

An important aspect of understanding normal erythropoiesis is to establish the role of external signals, such as erythropoietin (Epo) and stem cell factor, in controlling proliferation and

differentiation through their receptors, EpoR and cKit. EpoR is known to signal via multiple redundant pathways, which prevent premature apoptosis of the precursors and stimulate their proliferation. cKit plays a major role in controlling proliferation by increasing the Epo responsiveness of the precursors. The characterization of the scaffolding proteins of the EpoR and cKit helps to understand their responsiveness (EpoR) and specificity (cKit) in erythropoiesis. EpoR signaling is very frequently affected in the group of acquired disorders referred to as the myelodysplasias (MDSs) that represent a common, heterogeneous group of blood disorders in the elderly. The incidence of MDSs is estimated to be ~ 14 per 10^5 people per year in the 65–85-year-old age group. A large proportion of these patients develop acute myeloid leukemia with a poor prognosis. Patrick Mayeux (Institut Cochin) presented a proteomics analysis of the EpoR signaling. His data indicate that, after ubiquitination, activated EpoR is quickly degraded by two proteolytic systems. The proteasomes remove part of the intracellular domain while the EpoR is still at the cell surface. Next, the lysosomes degrade the remaining part of the receptor-hormone complex. The efficiency of these processes offers an explanation for the short duration of intracellular signaling activated by Epo. These data have implications not only for the pathophysiological explanation of some types of MDS but also for Epo hyperresponsiveness occurring in polycythemia vera, a condition characterized by overproduction of erythrocytes. The link between cell signaling and translational control of mRNAs was discussed by the laboratory of Marieke von Lindern (Erasmus MC). Previous gene expression profiling analyses of polysome-bound mRNA had shown that translational regulation is common during erythroid differentiation [22]; further work indicated that control of mRNA translation is a major pathway downstream of phosphoinositol-3 kinase in the regulation of progenitor expansion. Furthermore, translational control may be involved in enigmatic diseases such as Diamond Blackfan anemia [23]. It is of interest to note that the 5'-untranslated regions of mRNAs that are subject to translational control are notably absent from the expressed sequence tag databases, likely because of their propensity to form tertiary structures that are not easily copied into cDNA. This has probably contributed to the relative underexposure of the importance of translational control in hematopoiesis.

Terminal Differentiation of Erythroid Cells

During the terminal stages of erythroid differentiation (proerythroblast to reticulocyte) nearly all protein synthesis is directed to the production of hemoglobin. The fully mature, enucleated red cell is a highly concentrated solution of hemoglobin surrounded by a cytoplasmic membrane. In humans, the synthesis of hemoglobin is regulated by the α -globin (Tel- ζ - α -cen) and β -globin (Cen- ϵ - γ - γ - δ - β -tel) loci. Natural mutants of the globin genes underlie the most common inherited diseases throughout the world, the α - and β -thalassemias and sickle cell disease. The two clusters are therefore among the most intensively studied of all mammalian gene loci, but many important questions at the forefront of our attempts to understand gene regulation remain unanswered. One of these questions is the quality control of RNA transcripts, which is surveyed in the nucleus to discriminate normal from aberrant mRNAs. Splicing-defective mutants of globins cause thalassemia, even though the protein-encoding

sequences are completely normal. These defective RNAs accumulate in the nucleus at the site of transcription [24]. Wild-type transcripts recruit the so-called exon junction complex [25], but splicing-defective mutants fail to do so [26]. The laboratory of Maria Carmo-Fonseca (Instituto de Medicina Molecular, Lisbon, Portugal) uses molecular cell biology and three-dimensional microscopic localization to unravel the role of the proteins implicated in the quality control of globin RNA transcripts at the site of transcription. This may aid the development of successful therapeutic approaches entailing rescue of defective splicing, such as oligonucleotide-mediated splice site selection [27, 28]. This work also impinges on the future goal of determining all of the *cis*-acting elements that contribute to fully regulated globin expression from the natural chromosomal environments. Despite extensive work in the past, the proteins that bind these *cis*-elements *in vivo* are still not fully characterized, and approaches to identify the multiprotein complexes that associate with known DNA-binding proteins, such as Gata1, Tal1, Klf1, and Atrx, were presented by Douglas Higgs (The Weatherall Institute of Molecular Medicine). This presentation highlighted the need to develop new web-based graphical interfaces to access the enormous amount and diversity of data that is generated by the integrated use of genomics and proteomics in modern biology. This web tool also needs to take species differences into account. For instance, activation of the human and mouse α -globin gene clusters appears to be subject to quite different regulatory modes, despite the phylogenetic conservation of these loci [29, 30]. Mutation of the ATRX protein affects α -globin expression in humans [31], yet conditional deletion of Atrx in mice has no appreciable effect on α -globin levels. Mice carrying the human α -globin locus in lieu of their endogenous locus may finally provide an appropriate model for the study of human α -globin gene expression and the pathological consequences of defective transcriptional regulation caused by impaired transcription factor function.

Complex Pathology of Diseases Triggered by Defective Hematopoietic Transcription Factors

Molecular defects, either inherited or acquired, in genes encoding lineage-specific transcription factors may trigger a cascade of molecular defects only partially identifiable by gene expres-

sion profiling analysis. An example is Gata1, a transcription factor essential for proper formation of erythroid, megakaryocytic, eosinophilic, and mastocytic cells [32]. Structural alterations of Gata1 lead to X-linked hereditary anemias and/or thrombocytopenias, whereas hypomorphic Gata1 mutations are associated with myeloproliferative disorders and leukemias [33]. Anna Rita Migliaccio et al. (Istituto Superiore di Sanità, Rome) showed that hypomorphic Gata1 mutations in mice induce a syndrome very similar to human idiopathic myelofibrosis that includes development with age of anemia, appearance of teardrop poikilocytes and hematopoietic progenitors in the blood, marrow fibrosis, neoangiogenesis, and extramedullary hematopoiesis [34]. The development of fibrosis is caused by alterations in the cellular interactions established by the megakaryocytes. Hypomorphic Gata1 megakaryocytes express normal levels of P-selectin but reduced levels of Von Willebrand factor, a protein required for proper P-selectin assembly to the α -granules. As a result, P-selectin remains localized on the demarcation membrane system, triggering a pathological neutrophil emperipolesis that results in release of growth factors such as transforming growth factor β (TGF- β) from the megakaryocytes [35]. These high levels of TGF- β induce fibroblasts to produce collagen, thus causing marrow fibrosis. Megakaryocytes are engaged in cell-cell interactions with numerous cell types involved in the regulation of stem cell biology. It is therefore possible that pathological megakaryocyte interactions with osteoblasts, endothelial cells, or other cell types induce the abnormal stem cell trafficking and neoangiogenesis observed in the hypomorphic Gata1 mice. These data emphasize the importance of an integrated approach to the field of stem cell biology, covering the analysis of stem cell emergence, lineage determination, and terminal differentiation through the application of powerful multidisciplinary experimental techniques and model systems.

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DISCLOSURES

The authors indicate no potential conflicts of interest.

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