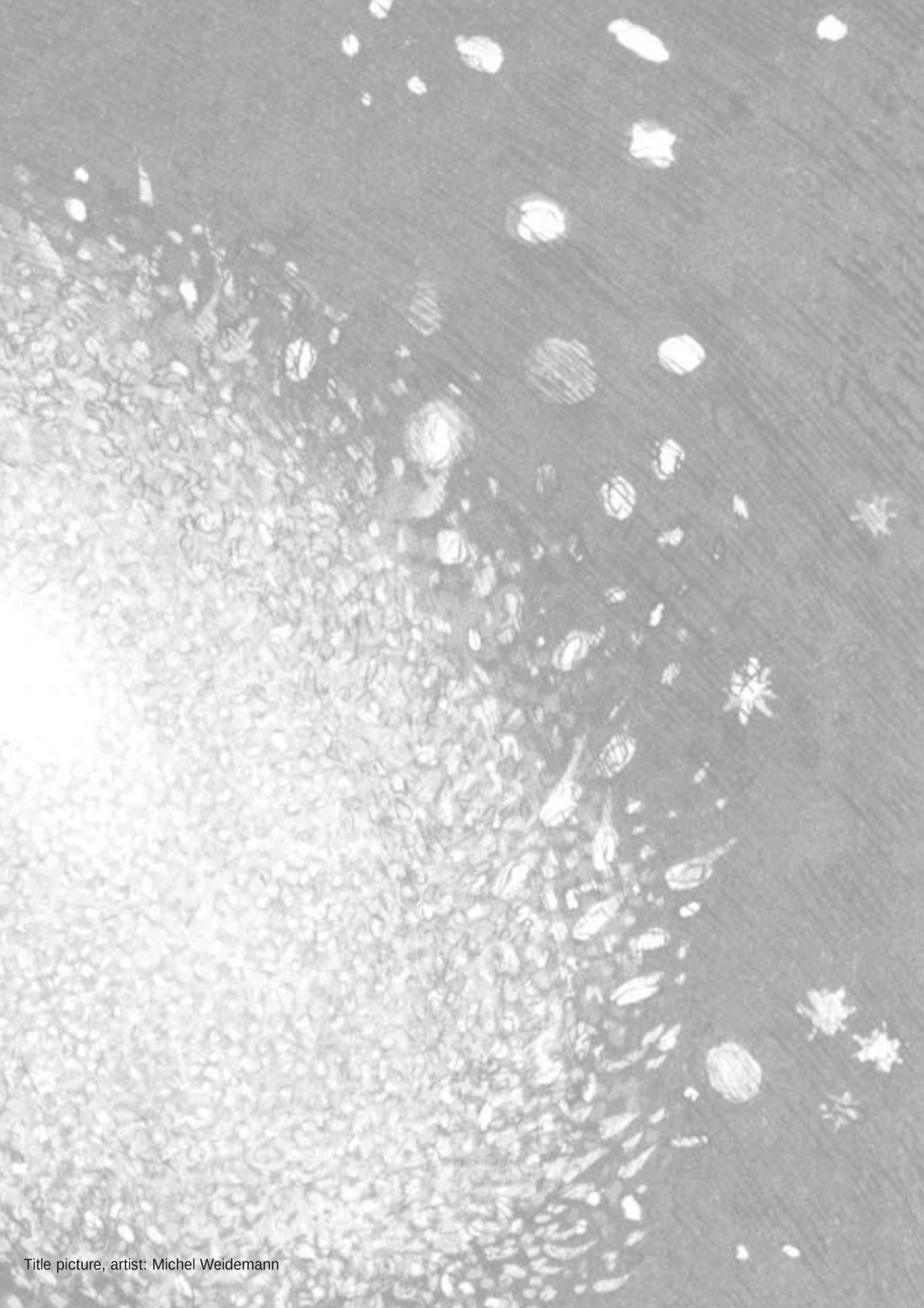
Abstract Book

MODERN TRENDS IN HUMAN LEUKEMIA

XV. Wilsede Meeting / June 14 – 18, 2003



Title picture, artist: Michel Weidemann

Table of Contents

SPEAKER ABSTRACTS

Speaker 1:

Reproductive and Therapeutic Cloning: Promise and Problems

Rudolph Jaenisch

Whitehead Institute for Biomedical Research, and. Department of Biology, MIT, Cambridge, MA 02124, USA

The full term development of sheep, cows, goats, pigs, cats, rabbits and mice has been achieved through the transfer of somatic cell nuclei into enucleated oocytes. Despite these successes, mammalian cloning remains an inefficient process, with a preponderance of reconstructed embryos failing at early to midgestation stages of development. The small percentage of conceptuses that survive to term are characterized by a high mortality rate and frequently display grossly increased placental and birth weights. I will argue that nuclear cloning results in faulty genomic reprogramming and causes gene expression abnormalities and aberrant phenotypes even in normal appearing adult cloned animals.

Nuclear cloning has been proposed as a method to help childless couples to have genetically related offspring (reproductive cloning) as well as an approach to provide an unlimited source of cells that could be used for the treatment of diseases such as Parkinson or Diabetes (therapeutic cloning). The latter approach involves the generation of “tailored” embryonic stem cells from the somatic cells of a patient by nuclear transfer. I will discuss our current understanding of the limitations and promise of these approaches.

Speaker 2:

Regulation by Colony Stimulating Factor-1

E R Stanley, X-M Dai, X Zong, and S Nandi
Albert Einstein College of Medicine, New York, NY, USA

Colony stimulating factor-1 (CSF-1) regulates the survival, proliferation and differentiation of mononuclear phagocytic cells. It is the primary regulator of mononuclear phagocyte production in vivo, where it regulates both locally and humorally. CSF-1 is expressed as a secreted glycoprotein, a secreted proteoglycan and a membrane-spanning, cell-surface glycoprotein. To examine the biological activity of these CSF-1 isoforms in vivo, we have created transgenic mice in which cDNAs encoding each of the different CSF-1 isoforms, driven by the CSF-1 promoter and first intron are expressed on the CSF-1-deficient *Csf1*^{op}*/Csf1*^{op} background. Transgenic mice exclusively expressing each isoform in a normal tissue-specific and developmental pattern exhibit distinct phenotypic differences, indicating differential effects of each type of CSF-1. Preliminary studies with these mice, which are being used to study the role of CSF-1 in development and in disease (atherosclerosis, the enhancement of tumor metastasis and glomerulonephritis), will be discussed. A targeted inactivation of the CSF-1 receptor (CSF-1R), encoded by the c-fms protooncogene, has revealed that all of the effects of CSF-1 are mediated by the CSF-1R. However, the greater severity of the *Csf1r-/Csf1r-* phenotype, compared with the *Csf1*^{op}*/Csf1*^{op} phenotype suggests that the Csf1op mutation is not a complete CSF-1 null, or that there are CSF-1-independent effects of the CSF-1R. On the FVB background, compared with *Csf1*^{op}*/Csf1*^{op} mice, the majority of which survive, osteoclast-deficient *Csf1r-/Csf1r-* mice die within 4 weeks, have frequent spontaneous bone fractures, impaired cortical bone formation, delayed bone mineralization, delayed cartilage resorption, disorganized bone matrix and disorganized and rounded osteoblasts. This phenotype is cell non-autonomous for the osteoblast, since osteoblasts do not express the CSF-1R, embryonic bone mineralization is normal prior to the development of osteoclasts and femoral anlagen transplantation experiments indicate that *Csf1r-/Csf1r-* osteoblasts form normal bones in the presence of wild type osteoclasts. These studies demonstrate an important role of the osteoclast in the regulation of osteoblast function during the bone development.

Speaker 3:

Transcription factors and erythroid differentiation

Frank Grosveld

Erasmus University, Rotterdam, The Netherlands

The differentiation of erythroid cells involves the expression of a number of erythroid specific transcription factors. Here we will focus on a number of functional aspects of some these factors in particular GATA1, which regulates the balance between cell proliferation and differentiation and EKLF, a factor that activates a number of erythroid specific genes including the globin genes. These factors are part of a number of larger protein complexes with very different functions. The biochemical characterization of these complexes will be presented and their role in chromatin modification, including the effects on the three dimensional structure and long range interactions within the beta globin locus in vivo will be described.

Speaker 4:

Retroviral vectors as therapeutic mutagens

C Baum, Z Li, U Modlich, J Meyer, B Schiedlmeier, H Klump, E Will, G Beutel, C von Kalle, B Fehse, J Kraunus, J Bohne, W Ostertag

Medizinische Hochschule Hannover, Hannover, Germany and Cincinnati Children's Hospital Research Foundation, Molecular and Gene Therapy Program, Cincinnati, OH

Transgenes delivered by retroviral (including lentiviral) vectors are efficiently maintained in cells with high replicative potential. Significant progress achieved in vector design and transduction technologies has resulted in the correction of severe monogenic disorders in a few clinical trials and several animal models. However, recent observations suggest that further progress will heavily rely on a systematic evaluation of side effects.

These could originate either from the ectopic expression of the transgene (phenotoxicity) or from genetic damage induced by the gene transfer procedure (genotoxicity). We observed leukemias in mouse models for retroviral gene marking of hematopoietic cells. In one study, insertional activation of the Evi-1 proto-oncogene by the retroviral vector was identified as a key event. However, this is hardly sufficient to explain malignant progression. Circumstantial evidence suggested signal interference induced by the expression of a truncated cell-surface receptor (dLNGFR) as a collaborating factor. We have set up a series of control experiments to verify or falsify this hypothesis. In a new experiment, we obtained a leukemia even when using a retroviral vector encoding a fluorescent marker protein. As in our first study, this was observed after forced expansion of cells by serial bone marrow transplantation. Therefore, an alternative hypothesis arises according to which activation of a single oncogene in combination with strong replication pressure may be sufficient to generate a leukemia (in mice). It will be crucial to investigate to what extent similar unfortunate cooperations (some of which may be disease-specific) underlie the secondary leukemias observed in the first clinical trial performed to correct X-linked SCID.

A related challenge is to adjust transgene expression levels within defined therapeutic windows. Using a retroviral vector expressing HOXB4 to high levels, human hematopoietic stem cells could be expanded in vivo (NOD/SCID mice). However, lymphoid and myeloid differentiation was disturbed depending on the dosage of transgenic HOXB4. These observations are also relevant for other signal molecules and metabolic markers proposed for stem cell selection and disease correction.

Based on these findings, we may have to reconsider our standards for preclinical evaluation of genetic interventions. An open eye for side effects and the identification of the underlying pathomechanisms will be crucial in the definition of therapeutic windows in gene therapy. These insights have immediate practical consequences for technology optimization and also provide new concepts for crucial aspects of stem cell biology and oncogenesis.

Speaker 5:

Suppression of insulin-like growth factor binding protein 3 by Runx1 in the Aorta-Gonad-Mesonephros-derived cells of mouse embryo

Ken Iwatsuki and Takahiko Hara
The Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan

Runx1 (AML1, CBFα2) is known to play a pivotal role in the initiation of hematopoietic stem cells (HSCs) in developing embryos. Runx1 is expressed both in HSCs and some of endothelial cells in the Aorta-Gonad-Mesonephros (AGM) region of mouse embryo. To understand a downstream regulator of Runx1, we first established an endothelial-like cell line, HBL, from AGM region of Runx1 knockout mouse by infection of the retroviral vector carrying a temperature sensitive form of SV40 T antigen. We then introduced Runx1 cDNA under the doxycycline-inducible promoter into HBL cells. By representational differentiation analysis of HBL mRNAs before and after doxycycline addition, we found that mRNA for insulin-like growth factor binding protein 3 (IGFBP-3) is downregulated by re-expression of Runx1 in HBL cells. Adenovirus-mediated production of Runx1 into HBL cells also suppressed IGFBP-3 mRNA expression in a dose-dependent manner. Among six known family members of IGFBPs, IGFBP-3 and IGFBP-6 were detected in the AGM region of mouse embryo and both mRNAs were downregulated in HBL cells by the Runx1 re-expression. When IGFBP-3 was added in a serum free culture of bone marrow-derived HSCs, a total number of myeloid progenitors was decreased. These results suggest that Runx1 may promote amplification of hematopoietic progenitors by suppressing the IGFBP-3/6 expression from endothelial cells in the hematopoietic microenvironment.

Speaker 6:

SCL is essential for normal hematopoietic stem cell function: substantial defects in repopulation and lineage cell fate result from SCL deletion in adult cells

Mark A. Hall*, David J. Curtis*, Donald Metcalf†, Andrew G. Elefanty†, K. Sourris†, Lorraine Robb†, Joachim R. Göthert§, Stephen M. Jane* and C. Glenn Begley§

*Rotary Bone Marrow Research Laboratory , †The Walter and Eliza Hall Institute for Medical Research, Royal Melbourne Hospital Parkville, Vic. 3050, and §Centre for Child Health Research, The University of Western Australia, West Perth 6872, Australia
Current address Amgen Inc, Thousand Oaks, CA 91320, USA.

Gene targeting studies have shown that SCL is critically important for embryonic hematopoiesis, but the early lethality of SCL null mice has precluded the genetic analysis of its function in the adult. We have generated a conditional knockout of SCL using the Cre/lox technology and an interferon-inducible Cre transgenic mouse. Deletion of SCL in adult mice perturbed megakaryopoiesis and erythropoiesis with the loss of early progenitor cells in both lineages. This led to a blunted response to the hematopoietic stress induced by PI-PC, with a persistently low platelet count and hematocrit compared to controls. In contrast, progenitors of granulocyte and macrophage lineages were not affected, even in the setting of stress. Immature progenitor cells (CFU-S12) with multilineage capacity were still present in the SCL null bone marrow, but these progenitors had lost the capacity to generate erythroid and megakaryocyte cells and colonies were composed of only myeloid cells. These results suggest that SCL is critical for megakaryopoiesis and erythropoiesis in steady-state adult hematopoiesis. We have also examined the function of SCL in adult hematopoietic stem cells (HSCs). Loss of a single SCL allele produced a defect in early repopulation of lethally irradiated recipients, consistent with haplo-insufficiency. This decrease in function was evident using competitive repopulation assays. Loss of both SCL alleles mediated by Cre-recombinase demonstrated that SCL-null bone marrow was inferior to wild type marrow for providing radioprotection. Competitive repopulation assays demonstrated a substantial defect in HSC activity beyond that evident with SCL-heterozygous cells. Two distinct mechanisms for the stem cell defect appear to apply – a defect of short-term HCSs that could be rescued by wild-type microenvironment, and a cell-intrinsic defect that skewed differentiation toward a lymphoid cell fate. Overall, these studies demonstrate a substantial and complex role for SCL in HSCs.

Speaker 7:

Hematopoietic stem cells self-renew in coculture with AGM-derived stromal cells

Mitsuo Nishikawa

Pharmaceutical Research Laboratories, Kirin Brewery Co., Ltd., Tokyo, Japan

Definitive hematopoietic stem cells (HSCs) first emerge in AGM – Aorta Gonad Mesonephros – region during mammalian development, and the microenvironment of AGM is considered to be indispensable for hematopoietic stem cell development. We have established AGM-S3 stromal cell line from 10.5 dpc mouse embryo. In addition to the ability to maintain HSC activity for long term, AGM-S3 could generate definitive HSCs from cells derived from yolk sac or paraaortic splanchnopleures when coculture with AGM-S3 cells. Thus AGM-S3 cells have striking features on HSC maintenance or development that mimic microenvironment of AGM region.

On the other hand, we have already demonstrated that the signal through gp130 receptor with early acting cytokines could expand human SCID-repopulating cells. For mouse HSCs, efficient long-term repopulating (LTR-) HSCs expansion could not be achieved with this cytokine mixture.

We considered that long-term repopulating (LTR) HSC self-renewal might be facilitated by the culture combined AGM-S3 cells and the cytokine mixture. Therefore, we evaluated long-term repopulating capacities of the mouse highly purified CD34- c-Kit+ Sca-1+ Lin- (CD34-KSL) HSCs cocultured with the AGM-S3 stromal cells and the cytokine mixture by a competitive repopulation assay. Significantly high donor chimerisms were sustained during 3 months after transplantation compared with that in the case of untreated HSCs or HSCs cocultured with AGM-S3 alone. Actual expansion of HSC confirmed by limiting dilution analysis. These results demonstrated that HSCs self-renew in vitro in combination with AGM-derived stromal cells and the appropriate cytokine mixture.

Speaker 8:

Tracking the signature of self-renewal in homogeneously pure stem cells and their immediate downstream multipotent progeny

N N Iscove, P Benveniste, M Barbara, D Hyam, R Sharma, M Gu, N Winegarden, D Barber

The Ontario Cancer Institute, Toronto, Canada

The heterogeneity of hematopoietic tissues and the small numbers of cells at particular stages of the precursor hierarchy pose significant technical challenges to expression profiling of hematopoietic precursor cells. We have developed generally applicable strategies that essentially solve these issues. Murine long-term reconstituting cells (LTRC) in unseparated marrow engrafted at nearly absolute efficiency in irradiated host mice, a determination essential for the assessment of homogeneity of purified LTRC. In confirmation, 90 % of cells in the Rho123loLy6A+c-kit+CD3-B220- fraction of adult yielded multilineage grafts in vivo after injection of a single cell per mouse, of which 27 % endured permanently. Downstream progeny of stem cells, retaining multilineage potential but lacking in the capacity for sustained self-renewal, were obtained in pure form by sampling from developmentally synchronous clonal starts in cultures each initiated with a single purified stem cell. To accommodate the small numbers of purified cells obtained by either clonal sampling or purification approaches, a method for exponential, global amplification of cDNA, applicable to single cells, was developed and adapted to micro array analysis. Model experiments demonstrated that the abundance relationships characteristic of starting RNA samples as small as 10 pg – the amount present in a single cell – are faithfully retained during exponential amplification. Micro array experiments designed to compare transcripts in stem cells with their immediate downstream multipotent progeny yield dozens of differentially expressed transcription, signalling and membrane-anchored entities of which about half are novel. This groundwork establishes a platform for high resolution micro array-based identification of transcripts potentially involved directly in the process of sustained self-renewal unique to the stem cell stage.

Speaker 9:

The developmental fate of hematopoietic stem cells: the study of individual hematopoietic clones at the level of CFU-S and antigene-responsive B-lymphocytes

N J Drize, N I Olovnikova, M A Ershler, I N Nifontova, E V Belkina, T N Nikolaeva, Y V Olshanskaya, L P Gerasimova, T E Manakova, N L Samoylina, T V Todria, and J L Chertkov.

Hematological Scientific Centre, Moscow, Russian Federation

We have shown previously that hematopoiesis in mice reconstituted with retrovirally marked hematopoietic stem cells (HSC) and sub lethally irradiated mice is provided by multiple, mainly short-lived clones, as measured by retroviral insertion site and radiation marker analysis of individual spleen colony-forming unit (CFU-S)-derived colonies. Polyclonal hematopoiesis has been maintained by simultaneously functioning of 2–15 usually small (1–3 CFU-S) clones. The 90 % clones were observed for not more than 4 months. About 10 % of the clones essentially survived during the whole life of a mouse (20 months). As a rule the size of such long-living clones was 15–20 fold larger then of the short-living. The data show, that the primitive HSC consists of at least 2 categories of precursors – one of them probably does not proliferate from the moment of determination and completely preserves its development potential, whereas the second category of HSC probably undergoes 1–3 mitosis after determination and comes back in a condition of less deep Go from which they can be easier triggered in a cell cycle, though their potential is reduced and they form only short-living clones. Thus, both categories of primitive HSC survive during the whole life, but only one category is capable to maintain hematopoiesis during the whole life of the animal. However, the CFU-S is the relatively early progenitor and the contribution of each CFU-S in the steady state hematopoiesis is uncertain. Here we have studied the fate of individual mature B-cells, as well as CFU-S, representing the progeny of retrovirally transduced primitive HSC. B-cells-generated hybridomas and CFU-S-derived colonies were used to determine the clonal composition of hemato-lymphopoiesis at the single-cell level. Bone marrow cells and splenocytes (approximately 1/3–1/2 of spleen at a time) from mice reconstituted with retrovirally marked syngeneic bone marrow cells were repeatedly collected at 3, 10, and 16 months post-transplant. The percentage of retrovirally marked CFU-S and B-cell-produced hybridomas was about 50 % at 3 months and decreased to 10–15 % at 10 months after reconstitution in spite of stable degree of chimerism. The clonal origin of bone marrow-derived CFU-S and spleen-derived B-cell hybridomas was detected by Southern blot analysis. Overall, DNA obtained from 159 retrovirally marked spleen colonies, 287 hybridomas and 43 bone marrow samples were studied. Multiple simultaneously functioning clones of primitive HSC-derived B-cells were observed. The same individual clones among hybridomas and CFU-S were identified in 3 out of 11 mice. Thus, hematopoiesis is generated by multiple hematopoietic clones some of which can simultaneously contribute to both mature lymphoid cells and myeloid progenitors. These data establish that the stem cell compartment functions by continuously producing progeny, which fully but transiently repopulate all lineages.

Speaker 10:

Liver development and hematopoiesis

Atsushi Miyajima

IMCB, University of Tokyo, Tokyo, Japan

Fetal liver lacks most of metabolic functions of adult liver as a center for metabolism. Instead fetal liver functions as the major hematopoietic tissue until birth. In order to study fetal liver hematopoiesis as well as liver development, we have developed a culture system of mouse embryonic day 14.5 liver cells. In this system, differentiation of fetal hepatocytes can be induced by Oncostatin M (OSM), as evidenced by morphological changes and expression of various adult liver enzymes as well as functions such as ammonia clearance, glycogen accumulation and lipid synthesis. This culture system also supports proliferation and differentiation of hematopoietic stem cells, recapitulating fetal liver hematopoiesis. Significantly, long term repopulating hematopoietic stem cell activity can be dramatically expanded in this culture. As OSM is expressed in hematopoietic cells in fetal liver and stimulates differentiation of hepatocytes, OSM plays a role for coordinating development of liver and hematopoiesis. By using this system, we have been studying the mechanism of liver development and hematopoiesis in fetal liver and recent progress on these studies will be presented.

Speaker 11:

Hematopoietic activity is induced in neural stem cells by chromatin-modifying agents

Albrecht M. Müller

Institut für Medizinische Strahlenkunde und Zellforschung (MSZ), University of Würzburg, D-97078 Würzburg, Germany

To explore whether epigenetic modifications can release the commitment of neural stem cells (NSCs) to the neural lineage we injected treated murine NSCs into murine blastocysts and into irradiated adult recipient mice. Before injection NSCs were treated with compounds that interfere with DNA methylation and histone acetylation. While the tissue distribution of untreated NSCs in chimeric animals developing after blastocyst injection showed colonization preferences for neural tissues, in contrast, following injection of treated NSCs, widespread tissue chimerism was observed, in particular in the hematopoietic system. To investigate specifically the hematopoietic potential of treated NSCs, they were transplanted into irradiated mice. Untreated NSCs showed no hematopoietic repopulation activity, but recipients receiving treated NSCs carried long-term, high-level and multi-lineage NSC-derived hematopoietic repopulation. Importantly, NSC-derived hematopoietic cells showed a 2n chromosome number, indicating that they are not products of cell fusion events. Thus, our data support the notion that upon changing the epigenotype of NSCs they are able to produce hematopoietic cells *in vivo*.

Speaker 12:

Molecular signatures of self-renewal, differentiation and lineage-choice in the haemopoietic system

Ludovica Bruno, Reinhard Hoffman, Fraser McBlane, John Brown, Rajeev Gupta, Chirag Joshi, Stella Pearson, Thomas Seidl, Clare Heyworth, Tariq Enver

Institute of Cancer Research, London, England

The molecular mechanisms governing self-renewal and differentiation remain unknown. We have determined global gene expression profiles of multipotent haemopoietic progenitor cells both under conditions of self-renewal and dynamically throughout their multilineage differentiation. To achieve a statistical robustness lacking in many early studies, data points were acquired in triplicate using unamplified material and stringently quality controlled. Undifferentiated cells displayed striking complexity including the expression of genes associated with different haemopoietic lineage-programmes, indicative of a highly responsive compartment poised to rapidly execute intrinsically- or extrinsically-initiated cell fate decisions. Analysis of 84 datasets reflecting various stages of erythroid, neutrophil, monocyte and megakaryocyte differentiation identified a total of 3650 differentially expressed genes. Early differentiation was broadly characterized by a resolution of complexity through down regulation of gene expression. As expected, effector genes characteristic of mature cells were upregulated late, coincident with changes in cell morphology. Lineage-specific changes in gene expression were however observed prior to this, potentially identifying genes associated with unilineage-commitment. Of particular interest were 347 genes that displayed differential behavior irrespective of the lineage elaborated; the mode behavior, accounting for 83 genes, was rapid down regulation after exposure to a differentiation cue. Genes within this class are candidates for influencing the probability of self-renewal versus differentiation as opposed to lineage-choice.

Speaker 13:

Extrinsic induction of myeloid differentiation by Notch signalling

Timm Schroeder, Hella Kohlhof, Nikolaus Rieber and Ursula Just

Hematopoietic commitment is initiated by and depends on activation of transcription factors. However, it is unclear if activation of lineage-affiliated transcription factors is extrinsically regulated by thus far unknown agents or is the result of a cell-autonomous programme. Here we show that signalling by the Notch1 transmembrane receptor instructively induces and accelerates myeloid differentiation of multipotent hematopoietic progenitor cells along the granulocyte, macrophage and dendritic cell lineage. We demonstrate that activated Notch directly up regulates expression of the transcription factor PU.1 leading to a high concentration of PU.1 protein which has been shown to direct myeloid differentiation. We present data of a cDNA micro array screen, which yielded several additional hematopoietic genes that are regulated by Notch in a positive or negative manner and are known to influence commitment of hematopoietic progenitor cells. These findings identify the Notch signalling system as an extrinsic regulator of hematopoietic commitment.

Speaker 14:

Stress-induced alterations of renewal in hematopoietic progenitors: a process important in leukemogenesis?

Hartmut Beug, Eva Maria Deiner, Helmut Dolznig, Katharina Stangl, Richard Moriggl, Andrea Kolbus, Sebastian Carotta, Sandra Pilat, Uwe Schmidt, Ernst. W. Müllner

Renewal (sustained progenitor proliferation without differentiation) of primary erythroid progenitors reflects their physiological response to stress erythropoiesis. EpoR, c-Kit and the glucocorticoid receptor (GR) cooperate in renewal, requiring signalling through the Stat5 and PI3K pathways. In erythroleukemia, oncogenic RTKs (v-ErbB, v-Sea) substitute for stress signaling via EpoR/c-Kit, activating both Stat5 and PI3K on their own. In this system, erythroblasts from mutant mice lacking components of signaling complexes driving renewal (GR-/-, Stat5-/-, Raf-/-, Btk/Tec-/-) showed interesting defects in renewal and/or terminal differentiation. In addition, red cell differentiation proceeded as a cell-autonomous default program if apoptosis was prevented by the Epo-target gene Bcl-XL. Finally, the human leukemia oncogene MLL (related to the chromatin modifier *trithorax*) required cooperation with c-Kit to transform lymphohematopoietic cells and cause multilineage leukemia.

Currently, we increasingly focus on multipotent progenitor cell systems, and further analyse interesting erythroid cell defects from respective knock-out mice. Besides multipotent progenitors from fetal liver, we also explore hematopoietic progenitors from murine embryonic stem (ES) cells, allowing to characterize possible hematopoietic defects in early embryonic lethal mouse mutants. Results from these approaches will be discussed.

Speaker 15:

The Stress Associated Readthrough Variant of Acetyl-Cholinesterase Enhances Platelet Producation and Megakaryocytopoiesis

V. Deutsch[1], M. Pick[1,2], D. Grisaru[1], E. Naparstek[1] and H. Soreq [2]

[1]Depts. of Hematology, Tel Aviv Medical Center, Sackler Faculty of Medicine, Tel Aviv University,
[2]Dept. of Biological Chemistry, Hebrew University, Jerusalem, ISRAEL

Stress induces overproduction of WBC and platelets, implying stress responsive factors that modulate early myelopoiesis. Acetylcholinesterase (AChE), which terminates neuro-transmission in the synapses, is also found in hematopoietic cells and has long been postulated to regulate megakaryocytopoiesis. The AChE-gene is expressed as three alternatively spliced mRNA variants, resulting in proteins with distinct C-terminal sequences. Since the stress associated readthrough variant of acetylcholinesterase (AChE-R) and its C terminal peptide (ARP) were shown to modulate CD34+ cell growth (Deutsch et al., Experimental Hematology 30:1153-1161, 2002), we studied its relevance to thrombopoiesis. We investigated the effect of ARP on the expansion of MK progenitors (MK-prog) in vitro and on platelet number and BM (MK-prog) in transgenic mice that overexpress this variant and in the NOD/SCID mice transplantation model. Exposure of CD34+ HPC to nanomolar concentrations of synthetic ARP in culture stimulated CD34+ cell proliferation at 24 hours and induced upregulation of AChE-R transcripts at 24 hours, supporting autoregulation of AChE-R. ARP alone or in combination with early acting cytokines supported the survival and expansion of human CD34+ cells, early MK-prog and CFU-MK in two and four weeks cultures in an antisense suppressible manner. ARP was more effective than cortisol in stimulating the proliferation of MK-prog and ARP replaced SCF without compromising expansion of these cells. Transgenic mice overexpressing AChE-R presented elevations in platelet counts and BM MK-prog. Human CD34+ cells expanded ex-vivo with ARP facilitated MK engraftment and thrombopoiesis in NOD/SCID mice following transplantation. Our findings present the AChE-R derived peptide ARP as a new stem cell and megakaryocyte growth factor which may promote stress thrombopoiesis and maybe utilized to facilitate efficient expansion of MK-prog ex-vivo for transplantation.

Speaker 16:

Epigenetic consequences of AML1-ETO action

George A. Follows, Hiromi Tagoh, Pascal Lefevre, Gareth J. Morgan and Constanze Bonifer

Molecular Haemopoiesis and Epigenetics Group, University of Leeds Medical School, Leeds, England

Although many leukemia-associated nuclear oncogenes are well characterized, we know little about the molecular details of how they alter gene expression. Here we examined transcription factor complexes and chromatin structure of the human c-FMS gene in normal and leukaemic cells. We demonstrate by in vivo foot printing and chromatin immunoprecipitation assays that this gene is bound by the transcription factor AML1. In t(8;21) leukaemic cells expressing the aberrant fusion protein AML1-ETO, we demonstrate that this protein is part of a stable complex binding to extended sequences of the c-FMS intronic regulatory region rather than the promoter. The AML-ETO complex does not disrupt binding of other transcription factors, indicating that c-FMS is not stably epigenetically silenced. However, its binding correlates with changes in the histone modification pattern via recruitment of histone deacetylases. Our experiments provide for the first time a direct insight into the epigenetic alterations caused by the binding of AML1-ETO to one of its target genes.

Speaker 17:

Disruption of the PU.1 and C/EBP alpha myeloid transcription factors in adult mice induces specific blocks in differentiation and syndromes partially mimicking acute myelogenous leukemia

Pu Zhang, Junko Iwasaki-Arai, Estelle A Duprez, Pu Zhang, Claudia S. Huettner, Maris Fenyus, Koichi Akashi, Daniel G. Tenen

Harvard Institutes of Medicine, Boston, MA, USA

Acute Myelogenous Leukemia (AML) is the most common form of acute leukemia in adults, including both primary and secondary leukemias. It is characterized by a block in myeloid differentiation. Myeloid transcription factors, including PU.1 and CCAAT/Enhancer Binding Protein alpha (C/EBP alpha), play critical roles in myeloid lineage differentiation. Disruption of PU.1 function in non-conditional knockouts in mice leads to an early multi-lineage block, and C/EBP alpha knockout mice demonstrate a block at the earliest stage of granulocyte differentiation and have myeloid blasts in the blood. Introduction of PU.1 or C/EBPalpha into multi-potential precursor cells can restore lineage specific differentiation. Consistent with these phenotypes, mutations and other disruptions of C/EBP alpha and PU.1 have been found in human AML. However, the embryonic and/or perinatal mortality of both PU.1 and C/EBP alpha non-conditional knockouts has limited our ability to utilize them to model human AML. In order to further characterize the stage at which myelopoiesis is blocked, and to develop animal models of human AML, we have developed conditional knockouts of PU.1 and C/EBP alpha. Analysis of fetal liver from non-conditional and conditional (adult bone marrow) PU.1 knockouts demonstrate an early block at the transition from the hematopoietic stem cell (HSC) to common myeloid progenitor (CMP) and common lymphoid progenitor (CLP) stages. 3 weeks following induction of recombination in newborn PU.1 mice, the bone marrow of the animals has a large number of blasts, similar to what is observed in AML. At this time, the recombination of the PU.1 gene is nearly 100 %. However, after 2 months the bone marrow looks normal, and no recombination of the PU.1 gene can be observed. The conditional PU.1 mice demonstrate a block in the transition from long-term to short term phenotypic HSC, as well as a block from the HSC compartment to the CMP and CLP. However, the long-term PU.1 / HSC appear to have a qualitative defect in self-renewal, and eventually the non-recombined HSC have a selective advantage over HSC in which PU.1 has been disrupted. In contrast, non-conditional (fetal liver) and conditional (adult bone marrow) C/EBP alpha cells are blocked at a later stage, the CMP to GMP (granulocyte/macrophage progenitor) transition. Almost no granulocytic differentiation is observed in the peripheral blood, spleen, and bone marrow of the conditional C/EBP alpha knockout mice. However, erythroid and megakaryocyte development are normal. While CMP cells from C/EBP alpha / mice can differentiate to all different lineages invitro, cells in GM colonies were mainly immature myeloid cells. Loss of C/EBP alpha function selectively blocks myeloid cell differentiation, but does not inhibit the development of other hematopoietic cell types. In contrast to what was observed for PU.1, the blasts persist in the bone marrow and peripheral blood for months. However, the animals do not develop a malignant leukemia. It is possible that additional abnormalities in addition to loss of C/EBP alpha function, such as FLT3 mutations and/or upregulation of anti-apoptotic signals, may be required to induce leukemia in the mouse, and this can be tested using our conditional knockout lines. We acknowledge the collaborative efforts of Julie Lekstrom-Himes, Chamorro Somoza, and Rich Murray in these studies.

Speaker 18:
We Can Accelerate Evolution

Mitsuru Furusawa
Neo-Morgan Lab. Inc., & Daiichi Pharmaceutical Co. Ltd, Tokyo, Japan

When the following two conditions with respect to the *rate, distribution* and *quality* of mutations are simultaneously satisfied, evolution can be accelerated: i) Excess production of mutations accompanying DNA replication beyond the "error-threshold"; ii) Expression of disparity (or unbalanced) mutagenesis.

We have proposed two models by which these conditions are met. One is the leading/lagging strands differential mutagenesis model, in which the fidelity of the lagging strand, for instance, is exclusively decreased far beyond that of the leading strand.[1, 2] The other is the DNA polymerase mixture model, in which both normal Pol and error-prone Pol coexist in appropriate ratios in a single cell.[3] In these models ("disparity mutators"), we can introduce, theoretically, a nearly unlimited number of mutations in a population while avoiding extinction. Computer simulations show that the model organisms can adapt quickly to a non-linear fitness landscape.[3,4]

In living organisms, "disparity mutators" can be made, for example, by introducing one point-mutation in the functional domain of the editing enzyme of Pol δ or ϵ. In addition, several error-prone Pol enzymes have been discovered in higher organisms. These facts together with the distinct feature of the "disparity mutator" would nicely explain the evidence of the punctuation of evolutionary equilibrium – that is, organisms may increase mutation rates beyond "error-threshold" in order to avoid extinction when drastic environmental changes occur. This may result in an acceleration of evolution.

In this meeting, I would like to show how "disparity mutator organisms" can quickly adapt to drastic changes of the environment, and how they can keep their original phenotype irrespective of extremely high mutation rates when environment does not change.[5, 6]

1) Furusawa, M. & Doi, H.; Promotion of evolution: disparity in the frequency of strand-specific misreading between the leading and lagging stands enhances disproportionate accumulation of mutations. *J. theor. Biol.* (1992) 157, 127-133.
2) Furusawa, M. & Doi, H.; Asymmetrical DNA replication promotes evolution: disparity theory of evolution. *Genetica* (1998) 102/103: 333-348.
3) Aoki, K. & Furusawa, M.; Promotion of evolution by intracellular coexistence of mutator and normal DNA polymerases. *J. theor. Biol.* (2001) 209, 213-222.
4) Wada, K., Doi, H., Tanaka, S., Wada, Y. & Furusawa, M.; A neo-Darwinian algorithm: asymmetrical mutations due to semiconservative DNA-type replication promote evolution. *Proc. Natl. Acad. Sci. USA.* (1993) 90, 11934-11938.
5) Tanabe, K., Kondo, T., Onodera, Y. & Furusawa, M.; A conspicuous adaptability to antibiotics in the *Escherichia coli* mutator strain, *dnaQ49. FEMS Microbiology Letters* (1999) 176, 191-196.
6) Goldsby, R. E., Hays, L. E., Chen, X., Olmsted, E. A., Slayton, W. B., Spangrude, G. J. & Preston, B. D.; High incidence of epitherial cancers in mice deficient for DNA polymerase _ proofreading. *Proc. Natl. Acad. Sci., USA*. (2002) 99, 15560-15565.

Speaker 19:

Temporal control of Hox gene expression

Frank H. Ruddle and Aster H. Juan

Yale University, Department of Molecular, Cellular and Developmental Biology, New Haven, CT, USA

The proper expression of Hox genes is necessary for the accurate patterning of the body plan. The elucidation of the genetic basis of transcriptional regulation of Hox genes by the study of their *cis*-regulatory elements provides critical information regarding the establishment of axial specification. In our previous work, we have identified a phylogenetically conserved *Hoxc8* regulatory sequence, the *Hoxc8* early enhancer, that is required for the transcriptional control of *Hoxc8* transgenes. In the present work, we have deleted this regulatory region *in vivo* using ES cell technology, and we have shown that this enhancer is necessary for the correct transcriptional regulation of *Hoxc8* expression in both activation and maintenance phases, but particularly in the former. Our results are consistent with emerging evidence that the precise temporal expression of Hox genes is critical for establishing regional identities, that the deletion of the *Hoxc8* early enhancer does not completely eliminate *Hoxc8* suggesting the existence of a *Hoxc8* regulatory network to some degree independent of the *Hoxc8* early enhancer, and that the anterior boundary of the *Hoxc8* expression domain is established normally, but the posterior boundary is not maintained at certain developmental periods in the mutant embryos, indicating separate mechanisms regulate anterior and posterior boundary formation. Finally, our data suggest that discrete regulatory pathways are responsible for the expression of *Hoxc8* in different structures such as somite, neural tube, and limb bud.

Speaker 20:

Establishment and maintenance of gene expression in lymphocytes

Veronique Azuara, Karen Brown, Jon Baxter, Matthias Merkenschlager and Amanda Fisher

MRC Clinical Sciences Centre, Hammersmith Hospital, Du Cane Road, London W12 ONN, UK

Our group has a longstanding interest in understanding how patterns of gene expression are established, maintained through cell division, and changed in a progressive and ordered fashion during development. In addition to identifying candidate genes involved in lineage choice, we have performed studies to examine the influence of nuclear location for gene expression. Our experiments combine techniques for fluorescence in situ hybridization (FISH) that preserve nuclear structure with high resolution fluorescence microscopy. These studies have shown that transcriptionally inactive genes are often positioned close to constitutive heterochromatin in the nucleus of dividing lymphocytes[1]. This functional "compartmentalization" is achieved as cells enter the cell cycle and appears to be important in maintaining the heritable repression of a subset of genes[2-4]. We have now applied a PCR-based approach to examine the replication timing of developmentally regulated loci in relation to their expression status and to their position within the nucleus. Contrary to expectations, we regularly observe early replication of genes that are silent and positioned (in trans) close to constitutive heterochromatin. However, late replication is seen for inactive transgenes integrated (in cis) in a heterochromatic environment. Hence, replication timing may indicate whether candidate loci reside in an "open" or "closed" chromatin environment. We are using this approach to compare the chromatin status of key genes that determine lineage fate in cells with different lineage potential; totipotent ES cells, multipotent haemopoietic precursors and unipotent lymphocytes. The data so far are consistent with lineage restriction being underpinned by a progressive ‘shutting down’ of the genome. The implications of this work both for tracing lineage fate-maps and for our understanding of stem cell plasticity are discussed.

1) Brown, K.E. et al. Association of transcriptionally silent genes with Ikaros complexes at centromeric heterochromatin. Cell 91: 845-54 (1997).
2) Brown, K.E. et al. Dynamic repositioning of genes in the nucleus of lymphocytes preparing for cell division. Mol. Cell 2: 207-17 (1999).
3) Brown, K.E., Amoils, S. et al. Expression of a- and b-globin genes occurs within different nuclear domains in haemopoietic cells. Nature Cell Biol. 3: 602-606 (2001).
4) Skok, J.A. et al., Non-equivalent nuclear location of immunoglobulin alleles in B lymphocytes. Nature Immunology 2: 848-854 (2001).

Speaker 21:

Are pre B I cells of PAX 5-deficient mice hematopoietic stem cells?

Fritz Melchers

Biozentrum of the University of Basel, Dept. of Cell Biology, Basel, Switzerland

Pre B I cells from PAX 5- deficient mice ,in contrast to their counterparts in wild type mice, have properties of self renewal "in vitro" and "in vivo", of pluripotency and of long-term reconstitution which are similar but not identical to those expected from long-term reconstituting pluripotent hematopoietic stem cells of fetal liver or bone marrow of normal mice. Possible reasons for these differences will be discussed

Speaker 22:

Sampling and connecting the genes of the B-cell line DT40

Jean-Marie Buerstedde[1], Hiroshi Arakawa[1], Piero Carninci[2] and Yoshihide Hayashizaki[2]

[1]Institute of Molecular Radiobiology, GSF, 85758 Neuherberg, Germany
[2]Laboratory for Genome Exploration Research Project, Genomic Sciences Center and Genome Science Laboratory, RIKEN Tsukuba Institute, 3-1-1 Koyadai, Tsukuba, Ibaraki 305-0074, Japan

Thousands of new genes have been discovered by genome sequencing and their cellular functions need to be clarified. An unusual high homologous recombination activity makes the chicken B cell line DT40 suitable for the study of vertebrate gene function by gene disruption. To facilitate these studies we have build a comprehensive bursal B-cell EST database which currently holds over 24,000 ESTs. Sequences corresponding to interesting candidate genes can be identified by on-line BLAST or keyword searches. One of our bursal cDNA libraries (riken1) contains a high percentage of full length cDNA inserts. Based on the available 5' reads, we have chosen 3000 new full length cDNAs for sequencing by primer walk. It will be interesting to align these full length-coding regions to the chicken genome sequence, which is expected to be release in autumn 2003. The collection of full-length coding sequences will also be a valuable resource for systematic gene expression and protein interaction studies. The biological interest of the laboratory concerns lymphoid recombination and DNA repair. We have recently found that the disruption of the chicken *AID* gene completely blocks immunoglobulin (Ig) gene conversion in DT40. The *AID* master gene therefore tightly controls all B cell specific modifications of vertebrate Ig genes. To clarify the mechanism of AID action and the regulation of Ig gene diversification we will search for additional transacting factor and cis acting regulatory sequences within the Ig light chain locus. Details of the bursal EST sequencing project and other resources can be found via the DT40 web site (http://swallow.gsf.de/dt40.html).

Speaker 23:

RhoGEFs regulate lymphocyte activation and homeostasis

Klaus-Dieter Fischer

Dept. of Physiological Chemistry, Universität Ulm, Germany

Rho GTPases catalyze many cellular processes, but predominantly rearrangements of the cytoskeleton, and are activated by RhoGEFs. RhoGEFs often contain many different signaling domains, which may enable them to activate additional signaling pathways in concert with RhoGTPases. We are analyzing several RhoGEFs that are highly expressed in the immune system, including Lsc, an RGS-domain RhoGEF, and the Vav family of RhoGEFs. Lsc is activated downstream of serum lipids, which activate Galpha12/13-coupled G-protein receptors. Lsc is required for immune responses and marginal zone (MZ) B cell homeostasis. In contrast, Vav family members act downstream of antigen receptors and are required for lymphocyte development and activation. Recent findings on our functional analysis of RhoGEFs in the immune system will be presented.

Speaker 24:

Early lymphocyte development and leukemia

Harald von Boehmer
Harvard Medical School, Boston MA, USA

While it has been convincingly shown that the adult bone marrow contains pluripotent hemopoietic stem cells (HSC) as well as lymphoid-restricted precursors (CLP) there is a ”missing link“ between such cells and the first intrathymic precursors that eventually produce T cells. We have identified the "missing link" with the aid of reporter mice that express a surface marker controlled by the 5' promoter and enhanced region of the pre-T cell receptor alpha locus (pre-TCRalpha). In further studies we have analyzed the role of the pre-T cell receptor as well as its signaling in acute lymphoblastic T cell leukemia (T-ALL). The results show that in some forms of leukemia continued pre-TCR upregulation and cell-autonomous pre-TCR signaling are part of the transformation process. In other types of T-ALL, mechanisms that mimic pre-TCR signaling are responsible for transformation.

Speaker 25:

Susceptibility to infection in children with malignancy due to deficiency of mannose-binding lectin

Olaf Neth, Malcolm W Turner and Nigel J Klein

Immunobiology Unit, Institute of Child Health and Great Ormond Street Hospital NHS Trust, 30 Guilford St, London WC1N 1EH

Infection is a major cause of morbidity and mortality in children with malignancy. This is predominantly due to chemotherapy and disease induced neutropenia. Although all patients have neutropenic episodes, not all suffer equally from infectious complications. The reasons for this are unclear.

In the last few years, it has been shown that mannose-binding lectin (MBL), a secreted pattern-recognition molecule of the innate immune system plays an important role in susceptibility to infections in immunocompromised individuals. MBL binds carbohydrates on the surface of clinically relevant organisms, activates complement and facilitates phagocytosis (1, 2). Polymorphisms in the MBL gene or its promotor give rise to low levels of circulating protein, and are present in up to 40 % of the general population. There are now three studies demonstrating a role for MBL in protecting neutopenic patients from infection. Two studies, one in children (3) and one in adults' (4) show that MBL deficiency predisposes patients to severe or prolonged infections. The third study demonstrated, that in patients receiving allogeneic haemapoietic stem cell transplantation, MBL deficiency in both donor and recipient was independently associated with an increased risk of major infection (5). Interestingly, deficiency of MBL has also been associated with an increased risk of childhood ALL, particularly with early age onset (6).

These studies suggest that determination of the MBL status of all patients receiving chemotherapy / stem cell transplantation (including donor) would be beneficial to identify those at particular risk of infection. MBL replacement could represent a new therapeutic approach in the management of chemotherapy and transplantation induced complications in this population of patients.

References:

1) Neth et al, *Infection and Immunity,* 2000, 68:688-693
2) Neth et al, *Journal of Immunology,* 2002, 169:4430-4436
3) Neth et al, *Lancet,* 2001, 358:614-618
4) Peterslund et al, *Lancet,* 2001, 358:637-638
5) Mullighan et al, *Blood,* 2002, 99:3524-29
6) Schmiegelow et al, *Blood,* 2002, 100:3757-3760

Speaker 26:

Fratricide: A Mechanism for T Memory Cell Homeostasis

R Callard, A Yates and J Stark
Institute of Child Health, UCL, London, UK

Immunological memory is mediated by a self renewing pool of antigen specific T memory (Tm) cells but the homeostatic mechanisms that maintain the size and diversity of the pool are largely unknown. Competition for space or growth factors has been suggested as a mechanism but there is little or no experimental evidence to support this concept. We suggest that fratricide via Fas mediated apoptosis within the small subpopulation of cycling Tm cells results in a density dependent death rate that controls the size of the pool without the need of an external quorum sensing device or competition for space or growth/survival factors. A mathematical model based on this concept predicts the known behaviour of the Tm pool including the observed difference in heterogeneity of the CD4 and CD8 compartments. It also highlights the importance of reduced apoptosis rather than enhanced proliferation for uncontrolled growth of leukaemic T cells. The model may also provide a paradigm for homeostasis of other haematopoietic cell populations.

Speaker 27:

Studies in MDS: DNA Microarrays, DLK, Paricalcitol and Lyp

W-K Hofmann, T Kumagai, W Chien, N Tidow, B Teisner, C Harken Jensen, S Sakajiri, L Jones and H P Koeffler

We examined the gene expression profile of CD34+ hematopoietic stem cells. Seven patients with low-risk myelodysplastic syndrome (MDS) and 4 patients with high-risk MDS were compared with expression data from CD34+ bone marrow cells from 4 healthy control subjects. Class membership prediction analysis selected 11 genes. Using the expression profile of these genes, we were able to discriminate patients with low-risk from patients with high-risk MDS and both patient groups from the control group by hierarchical clustering (Spearman confidence). The power of these 11 genes was verified by applying the algorithm to an unknown test set containing expression data from 8 additional patients with MDS (3 at low risk, 5 at high risk). Patients at low risk could be distinguished from those at high risk by clustering analysis. Using real time RT-PCR, we have identified a number of up- and down-regulated genes. One gene that was overexpressed in the CD34+ cells is DLK1. This is a secreted protein that has epidermal growth factor motifs. We found high DLK1 expression in the CD34+ hematopoietic stem cells from individuals with low grade MDS as well as agnogenic myeloid metaplasia. In addition, we looked at serum levels of DLK in MDS and myeloproliferative disorders (approximately 50 samples) and found elevated levels of DLK in the serum in 40 % of low grade MDS samples compared to levels observed in over 70 normal serum samples. Further studies showed DLK to be expressed in human erythroleukemia and megakaryocyte cell lines. Induction of differentiation of K562 cells either towards erythroid or more mature megakaryocyte precursors was associated with a decrease expression of DLK. Forced overexpression of DLK in K562 cells did not change their phenotype although these cells grew more quickly than control cells in low fetal bovine serum. We are now studying hematopoesis in DLK knock-out mice.

In further studies, we have found that a vitamin D analog (paricalcitol) approved by the FDA for secondary hypercalcemia has marked antiproliferative effects against myeloid leukemia and several solid tumor cell lines. Paricalcitol inhibited the growth of myeloid leukemia cells in vitro and had prominent anti-cancer effects on human cancer xenografts grown in nude mice. We have begun a clinical trial of paricalcitol for treatment of patients with MDS.

Another investigation concerns the BCR-ABL protein tyrosine kinase implicated in the development of chronic myeloid leukemia (CML). The potential role of protein tyrosine phosphatase in the regulation of BCR-ABL signaling was explored. First, expression patterns of tyrosine phosphatases in leukemic cell lines were investigated using degenerate primers for RT-PCR followed by cloning and sequencing of the cDNA. Distinct patterns of distribution of phosphatase were found in erythroid and myeloid leukemic cell lines. Whereas some phosphatases were ubiquitously expressed, others were limited to specific cell types. Surprisingly, a previously cloned "lymphocyte-specific" phosphatase, Lyp, was frequently detected in a number of myeloid cell lines. Lyp was localized to the cytosol and was associated with the proto-oncogene cbl. Overexpression of Lyp caused dephosphorylation of multiple tyrosyl phosphoproteins in KCL22 CML blast cells including CBL, BCR-ABL, ERK1/2 and CRKL. Co-expression of Lyp and BCR-ABL in Cos-7 cells resulted in reduced protein expression of BCR-ABL, GRB2, and MYC. KCL22 overexpressing Lyp had decreased colony growth in soft agar compared to wild-type KCL22 cells. Taken together, the data suggest that Lyp may play an antagonistic role in signaling by the BCR-ABL fusion protein.

Speaker 28:

Pathogenesis of CML at the Stem Cell Level

Connie Eaves, Xiaoyan Jiang, Yves Chalandon, Yun Zhao, Gerry Krystal and Allen Eaves

Terry Fox Laboratory, BC Cancer Agency and University of British Columbia, Vancouver, BC, Canada

Chronic myeloid leukemia (CML) is a multi-lineage human myeloproliferative disorder that arises by clonal expansion of a genetically altered hematopoietic stem cell. An early initiating event is the formation of a BCR-ABL fusion gene and its expression as an oncoprotein with elevated tyrosine kinase activity. In patients with chronic phase disease, the clonally expanded BCR-ABL+ progenitors show altered survival and turnover properties but execute normal differentiation programs. This has enabled the use of assays for normal progenitors to be applied to the investigation of perturbations that accompany the development of CML. Previous studies of chronic phase patient cells analyzed either directly, or after being placed in culture, have suggested that the self-renewal ability of CML stem cells is compromised. Thus expansion of the clone appears largely due to a continuing increased proliferative activity of BCR-ABL+ cells at later stages of differentiation along all lineages, although consequent increases in terminal cell output are restricted primarily to the granulopoietic lineage. The increased turnover and decreased self-renewal ability of CML stem cells leading to an expansion of early progenitors has been attributed to a unique differentiation-associated autocrine IL-3/G-CSF mechanism that is consistently activated in these cells. Notably these changes in the biological properties of primitive CML cells mirror the effects of exposure of primitive normal hematopoietic cells to excess levels of IL-3 and G-CSF in vitro. In recent experiments we have further demonstrated that up-regulation of IL-3 and G-CSF expression is a rapid consequence of forced overexpression of BCR-ABL in primitive murine cells and that this autocrine mechanism contributes to the acquisition of factor-independence in vitro and leukemogenicity in vivo. In addition, we have found that the frequency of transplantable multi-lineage leukemia-initiating cells (L-IC) in a highly stem cell-enriched fraction of mouse bone marrow cells analyzed immediately after transduction with BCR-ABL is 7-fold lower than the expected frequency of normal stem cells. Moreover, characterization of the WBCs regenerated in recipients of <1 L-IC indicated no residual long-term normal stem cell activity, suggesting the expression of BCR-ABL in these cells had abbreviated their self-renewal capacity in vivo. In primitive naive human hematopoietic cells, overexpression of BCR-ABL also rapidly up-regulated IL-3 and G-CSF production and a factor-independent phenotype but also induced erythropoietic differentiation both in vitro and in vivo. However, induction of features of myeloproliferative disease in vivo (in transplanted immunodeficient mice) was rare and required a latent period of >4 months. To examine potential candidate mediators of these effects, we have examined the levels of expression of BCR-ABL and SHIP in primary CML cells at different stages of differentiation. Interestingly, these have shown both to be higher in the more primitive CD34+ CML cells and to decrease several-fold by the time the progeny of these cells have entered the predominant CD34- compartment of terminally differentiating cells. These findings illustrate how a mutation originating in a hematopoietic stem cell can have complex effects on the ultimate generation of a leukemic clone because the differentiation process may alter its expression as well as that of potential downstream targets.

Speaker 29:

Disruption of Myeloid Differentiation in AML

Maike Schwieger, Jürgen Löhler, and Carol Stocking
Molecular Pathology, Heinrich-Pette-Institut, Hamburg, BRD

The hallmark of acute myeloid leukemias (AML) is the clonal, neoplastic proliferation of immature hematopoietic cells. The observed inhibition or complete arrest of differentiation can conceivable be the indirect outcome of mutations that disrupt cell cycle controls and/or be directly due to mutations in proteins that belong to or interfere with the differentiation program. We have recently shown that AML1-ETO, the fusion product of the (8;21) translocation, impairs maturation of myeloid, erythroid, and lymphoid lineages in a retroviral-transduction mouse model. This is accompanied by increased granulopoiesis and reduced erythropoiesis in the bone marrow. The impaired myeloid differentiation resulted in the accumulation of myeloblasts in bone marrow (10 % vs 4 % in controls), and a striking increase in immature myeloid colonies *in vitro* [Schwieger *et al.* (2002)]. AML1-ETO transformation is postulated to be caused by transcriptional repression of genes directly or indirectly regulated by AML1, the DNA-binding subunit of the core-binding factor (CBF) transcription factor complex. Recent studies have shown that several genes that encode cell-cycle regulators and transcription factors are down-regulated by AML1-ETO. Of special interest is the observed down-regulation of the CCAAT/ enhancer binding protein-α (C/EBPα), a major regulator of myeloid differentiation. In support of its importance in blocking differentiation in AML, several independent studies have identified mutations in the gene encoding C/EBPα in approximately 8 % of AML. These mutations are found in AMLs with an immature granulocytic phenotype (FAB M1 and M2) but that do not carry t(8;21). N-terminal mutations of C/EBPα were shown to generate a dominant negative protein [Pabst *et al.* (2001)]. To explore the significance of down-regulation of C/EBPα in the manifestation of AML, a N-terminal mutant was transduced into normal mouse bone marrow cells and subsequently transplanted into irradiated donors. Surprisingly, bone marrow cells expressing the C/EBPα mutant showed significantly increased levels of mature granulocytic forms as compared to FMEV/GFP-infected controls, even under culture conditions that promote proliferation and inhibit differentiation, suggesting that the mutant stimulated rather than inhibited granulopoiesis. In striking contrast, expression of the mutant C/EBPα in human progenitor cells isolated from umbilical cord blood dramatically inhibited differentiation of both myeloid and erythroid lineages. Histochemical and immunohistochemical analysis demonstrated the co-expression of myeloid and erythroid markers in the immature transformed cells. These results underline the intrinsic differences between hematopoietic controls in mouse and human, but add support to the hypothesis that mutations i*n CEBPA a*re critical events in the disruption of myeloid differentiation observed in many AMLs. This work was supported by funds of the José Carreras Leukämie-Stiftung.

Speaker 30:

The study of multiple NUP98-HOX fusion genes reveal common HOX dependent proliferative effects and leukemogenic potential in concert with Meis1

Nicolas Pineault, Ph.D.[1], Hideaki Ohta, MD[1], Carolina Abramovich, Ph.D.[1],Christian Buske, MD[2], Rhonna Gurevich[1], Peter D Aplan, MD[3], Michaela Feuring-Buske, MD[2], Donna E Hogge, MD, Ph.D.[1] and Keith R Humphries, MD, Ph.D.[1].

[1]Terry Fox Laboratory, BC Cancer Agency, Vancouver, BC, Canada, V5Z 1L3;
[2]National Research Center for Environment and Health, Ludwigs-Maximilian University Munich, Munich, Germany, 81377 and
[3]Center for Cancer Research, NCI, Gaithersburg, MD, 20892-4605.

An increasing number of NUP98-HOX fusion genes have been characterized in de novo and/or therapy related cases of AML, MDS and CML. Thus far, all HOX genes found rearranged with NUP98 (11p15) are members of the A, C or D cluster belonging to paralog group 9 to 13 raising the possibility of unique properties of AbdB- like HOX genes as leukemogenic partners of NUP98. To test this, we have created and compared the functional properties of a number of novel fusion genes consisting of the common NUP98 portion and the homeodomain (HD) containing 3' exons of both an AbdB-like HOX, HOXA10 (NUPA10) not previously reported in NUP98 translocations, and of the non-AbdB HOX genes, HOXB3 (NUPB3) and HOXB4 (NUPB4) the latter with or without its PBX interacting motif (NUPB4-PIM). The various fusion genes were expressed in BM cells through retroviral transduction and the empty vector (MSCV-IRES-GFP) was used as negative control and the AML-associated genes NUP98-HOXD13 (NUPD13) as a positive control. The infected BM cells were first tested in methylcellulose assay where larger colonies were constantly observed for all HOX and NUP98-HOX fusion cells compared to GFP control. The growth enhancing effect was also apparent in liquid culture expansion, as the proportion of GFP+ transduced cells increased over time for all fusion genes (eg HOXB4, NUPB4 and NUPB4-PIM cells rose from input levels of 15-29 % to >95 % within 2 weeks (n=2)). Moreover, the growth stimulating effect was also evident on primitive cells, as all fusion genes induced a marked increase in the output of primitives CFU-S cells following 1 week of liquid culture (>1,000-fold over control GFP transduced cells), highly similar to that seen with HOXB4 and HOXA10 (n=2). The impacts of the genes were next studied in vivo by reconstituting irradiated mice. Both NUPA10 and HOXA10 significantly perturbed hematopoietic differentiation, and common to both genes was a marked impairment in B lymphopoiesis. This phenomenon is commonly observed with HOX genes and has also been previously documented with NUPD13. In contrast, no obvious effect on differentiation by NUPB4 and control HOXB4 was observed. However, for all fusions tested, their co-expression with Meis1 was highly oncogenic, causing AML with a disease median latency ranging from as little as 66.5 days for NUPA10 + Meis1 to 170 days for NUPB4 + Meis1. Together, these results reveal a high degree of functional over-lap/redundancy and striking leukemogenic potential of a wide range of HOX genes when expressed as fusion partners of NUP98 and thus argue that HOX may act through a limited number of common, key target pathways in exerting their leukemogenic potential. This is further reinforced by our findings the homeodomain alone is sufficient for the leukemogenic/proliferative acitivity of NUP98-Hox fusions and that homeodomains from several Hox are interchangeable in this regard.

Speaker 31:

Development of SAG (Selective Amplifier Gene) system for hematopoietic stem cell gene therapy of chronic granulomatous disease

Keiya Ozawa, Yutaka Hanazono, Akihiro Kume, Takeyuki Nagashima, Yasuji Ueda, Keiji Terao and Mamoru Hasegawa

Divisions of Genetic Therapeutics and Hematology, Jichi Medical School, Tochigi; DNAVEC Research Inc., Ibaraki; Tsukuba Primate Center, Ibaraki, Japan.

Chronic granulomatous disease (CGD) is a rare inherited disorder of phagocytes. Responsible mutations are found in one of the four genes encoding essential subunitsof the phagocyte NADPH oxidase. Phase I clinical trials of gene therapy have failed due to the low-level gene transfer. To overcome the low efficiency of gene transfer into hematopoietic stem cells, we have developed a novel system for selective expansion of transduced cells. The system utilizes "SAGs (selective amplifier genes)" which encode fusion proteins consisting of a growth-signal generator and its molecular switch. The first generation SAG encodes a fusion protein between the granulocyte colony-stimulating factor receptor and the steroid binding domain, and a drug-induced expansion of hematopoietic stem/progenitor cells was achieved. We have also developed the second-generation SAG, which encodes chimeric receptor between extra cellular and transmembrane portion of erythropoietin receptor (EpoR) and intracellular portion of the thrombopoietin receptor (Mpl). Erythropoietin (Epo) dimerizes the EpoR-Mpl fusion protein to generate a potent growth signal. The efficacy of the SAG system was evaluated in an X-CGD mouse model. We constructed a bicistronic retrovirus MGK/gp91-ires-EpoR-Mpl encoding the human gp91 for therapeutic use and the EpoR-Mpl SAG for cell expansion. Lethally irradiated X-CGD recipients were transplanted with transduced X-CGD marrow. After hematological recovery, superoxide production was monitored by flow-cytometry measuring reduced dihydrorhodamine-123. In the animals treated with Epo, the percentage of the oxidase-positive granulocytes was 27.5 ± 6.8 % (n = 4), which was significantly higher than that in the unstimulated mice (8.4 ± 3.1 %) (n = 3) ($p < 0.03$). The results suggest that the second generation SAG would augment the efficacy of gene therapy for CGD.

Speaker 32:

The leukemia disease gene Cb2 encodes a G-protein coupled receptor which blocks neutrophilic differentiation

Ruud Delwel and Meritxell Alberich-Jorda
Erasmus University, Rotterdam, The Netherlands

Aberrant expression of G protein coupled receptors (GPCRs) has been demonstrated in various forms of cancer. The majority of these transforming GPCRs belong to the class that couples to the Gas-complex, proteins, which have a positive regulatory effect on the cAMP/PKA pathway. By means of retroviral insertional mutagenesis, we identified a novel common virus integration site (Evi11) in murine leukemias, and demonstrated that the Cb2, encoding the peripheral cannbinoid receptor, is the potential proto-oncogene. This GaiPCR, which is nomally expressed on B-lymphocytes, has been shown to be abnormally expressed in certain myeloid leukemias carrying proviral insertions near the gene. To elucidate the role of Cb2 receptor in leukemic transformation we generated Cb2-EGFP cDNA constructs which were introduced into the 32D/G-CSF-R in vitro leukemia model. 32D/G-CSF-R cells require IL-3 to proliferate in vitro. In the presence of G-CSF the cells fully differentiate towards mature neutrophils. In a transwell assay, it was demonstrated that Cb2 expressing cells migrate in response to the endocannabinoid 2-arachidonoylglycerol (2-AG). A Cb2-specific inverse-agonist, SR144528 fully blocked 2-AG induced migration. Interestingly, a full block of neutrophilic differentiation was observed when Cb2/EGFP expressing 32D/G-CSF-R cells were cultured with G-CSF and another Cb2 ligand, CP55,940. 2-AG did not block G-CSF induced differentiation. Moreover, SR144528 completely recovered CP55,940 mediated neutro-philic differentiation of 32D/G-CSF-R/Cb2-EGFP cells. Interestingly, normal bone marrow cells infected with Cb2-EGFP also showed a CP55,940 mediated block of differentiation which could be counteracted by SR144528. We investigated which signal transduction pathway may be involved in the 2-AG stimulated migration and CP55,940 mediated block of neutrophilic differentiation in 32D/G-CSFR/Cb2-EGFP cells. Inhibitors the MEK/ERK pathway (PD98059 or U0126), interfered with 2-AG induced migration and they full recovery G-CSF induced neutrophilic differentiation. Addition of dbcAMP blocked migration of the GaiPCR Cb2, but it did not restore differentiation. Our data demonstrate that aberrant expression of the oncoprotein Cb2 in myeloid progenitor cells has two effects, i.e. stimulation of migration and block of neutrophilic differentiation. Interestingly, these two effects depend on the activation the Cb2 receptor by different cannabinoid ligands. Moreover, Cb2 mediated migration involves the cAMP/PKA and the MEK/ERK pathways. Interference of neutrophilic differentiation involves only the MEK/ERK pathway. Moreover, Cb2 mutants which are incapable of interfering with the cAMP/PKA pathway that we have generated are incapable of stimulating migration, but fully block neutrophilic differentiation. Our data suggest that we identified a novel mechanism of leukemic transformation by the GaiPCR Cb2.

Speaker 33:

Minor Histocompatibility antigens: Key molecules for stem cell based immunotherapy of malignancies

E. Goulmy

University Medical Center, Leiden, The Netherlands

Minor Histocompatibility (H) antigenic disparities between Stem Cell (SC) donor and recipient are capable of inducing allo immune responses that participate in both the development of Graft-versus-Host Disease and in the Graft-versus-Leukemia activity after SC transplantation (SCT). Sofar, minor H antigens show low allelic polymorphisms with in general only one immunogenic T cell epitope. Their immunogenicity is best reflected by the cellular alloimmune responses minor H antigens elicite in vivo after HLA identical SCT; also in vitro minor H antigen cytotoxic T cells (CTLs) can be generated. Minor H antigen exhibit either broad (i.e. on all body cells and tissues) or limited (i.e. solely on cells of the haematopoietic system) expression.

CTLs specific for broadly expressed minor H antigens are capable of massive destruction of skin tissues, in an ex-vivo skin model, resembling GvHD grades III-IV. Thus, broadly expressed minors are the prime targets for GvHD. CTLs specific for the haematopoietic system restricted minors efficiently lyse blood cells and various types of leukemic cells but leave the skin intact. Interestingly, the haematopoietic system specific minor H antigen HA-1 shows aberrant expression on epithial cancer cells, while absent on nonmalignant epithelial cells.

The combined information on the genetics, immunogenicity, tissue and cell specific expression together with the knowledge on their chemical composition, minor H antigens form the basis for adoptive cellular immunotherapy or vaccination strategies for haematological malignancies and solid tumors in combination with SCT. The timing post SCT for either strategy, i.e. donor type hematopoiesis is crucial.

For references the reader is referred to:

E. Goulmy. Human minor Histocompatibility antigens: new concepts for marrow transplantation and adoptive immunotherapy. Imm. Rev. 1997, 157: 125-140.

T. Mutis, E. Goulmy.Hematopoietic system specific antigens as targets for cellular immunotherapy of hematological malignancies. In: Seminars in Hematology, 2002, 39, 23-31.

A.M. Dickinson, X.N. Wang, L. Sviland, F.A. Vyth-Dreese, G.H. Jackson, T.N.M. Schumacher, J.B.A.G. Haanen, T. Mutis, E. Goulmy. In situ dissection of the graft-versus-host activities of cytotoxic T cells specific for minor histocompatibility antigens. Nature Medicine 2002, 8: 410-414.

C. Klein, M. Wilke, J. Pool, C. Vermeulen, E. Blokland, E. Burghart, S. Krostina, N. Wendler, B. Passlick, G. Riethmüller, E. Goulmy. The hematopoietic system specific minor Histocompatibility antigen HA-1 shows aberrant expression in epithelial cancer cells; relevance for immunotherapy of solid tumors. J. Exp. Medicine 2002, 196, 359-368.

Speaker 34:

Tumor stroma: The Achilles heel of the tumor

Thomas Blankenstein

Max-Delbrück-Center for Molecular Medicine, Robert-Rössle-Straße 10, 13092 Berlin, Germany

It is established that most experimental and probably many human tumors express antigens against which an immune response can be induced. Immunity to transplanted tumors usually relies on T cells, often both $CD4^+$ and $CD8^+$. Their activation requires antigen cross-presentation by host antigen presenting cell (APC) in draining lymph nodes. Following activation T cells migrate to the tumor site and, when they arrive in time, can reject the tumor. In some tumor models $CD4^+$, in other $CD8^+$ T cells are the main effectors. Both cell types reject tumors by a similar mechanism. Most importantly, T cells must express IFNγ that acts on non-bone marrow derived tumor stroma cells resulting in angiostasis and inhibition of rapid tumor burden. This may allow residual tumor cell elimination by other mechanisms such as direct killing, yet in several models perforin expression by T cells is not necessary. Together, the primary target during tumor rejection is the stroma that is essential for all solid tumors and appears to be the most vulnerable component. IFNγ producing T cells inhibit the establishment of the stroma, e.g. vascularization, but usually fail against established tumors which could explain why therapeutic vaccination is so notoriously unsuccessful, simply because the T cells come too late.

Speaker 35:

TCR gene therapy of tumors

H. J. Stauss, S. Xue, L. Gao, R. Gillmore , A. Holler, Y. Ghani, A. Downs, G. Bendle, K. Vento, F. Ramirez, E. Morris

Imperial College London, Hammersmith Hospital, Department of Immunology, London W12-0NN, UK

We have previously described the allo-restricted CTL approach to circumvent immunological tolerance to self-proteins expressed at elevated levels in tumor cells. In vitro studies have shown that human and murine allo-restricted CTL can selectively kill tumor cells, whilst expression levels of the target antigen in normal cells are insufficient to trigger CTL killing. A major limitation of immunotherapy in the human setting is that infusion into MHC mismatched patients would stimulate an immune response against alloantigens expressed by the infused CTL. We are using the murine system to explore a strategy that takes advantage of the unique specificity-specificity of allo-restricted CTL, and avoids the complication of recipient immune responses against alloantigens. The T cell receptor (TCR) of murine allo-restricted CTL specific for mdm2, a nuclear protein often overexpressed in tumors, were cloned into retroviral vectors with the aim to transfer the TCR into normal CD8 T cells. We can now use established in vivo models to compare the specificity protective potential of TCR-transduced recipient CTL and allogeneic CTL expressing the same TCR.

Speaker 36:

Surprising findings of clonal contribution to models of gene modified hematopoiesis under selection pressure

Christof v. Kalle

Cincinnati Children's Hospital Research Foundation, Molecular and Gene Therapy Program, Cincinnati, OH

Retrovirus gene transfer allows the addition of stable genetic information to the cellular genome. Interestingly, semi-random vector integration establishes stable genetic markers of clonal derivation are established by, because every transduced stem or progenitor cells will pass on its unique insertion site to all its progeny cells. This approach has been a very interesting tool to decipher hematopoietic stem cell contribution to the peripheral blood. Due to technical limitations, no prior study has directly uncovered the clonal composition of marked peripheral blood beyond small animals transplanted in restricting dilution. Recently, very sensitive detection and sequencing of insertion sites has enabled us to determine the number and contribution of genetically modified stem cells by minimally invasive analysis of the peripheral blood in animal models and clinical gene transfer trials. By displaying the LAM-PCR amplified restriction length polymorphism of retrovirus insertion sites, an analysis of the current clonal contribution to hematopoiesis could be generated for each sampled individual in different scenarios. After transplantation of high numbers of gene modified cells in both the rhesus model (in collaboration with C. Dunbar, NIH, Bethesda) and the baboon model (in collaboration with H. P. Kiem, FHCRC, Seattle), continued polyclonal contribution was observed after peripheral blood and bone marrow stem cell modification, respectively. The stability of oligoclonal stem cell contributions over time could be confirmed in more oligoclonal transplant recipients both of dog and primate models. Sequencing of the insertion sites and semi-quantitative PCR tracking of single insertion sites has allowed us to reconstruct the contribution of particular clones over different time points. These studies have demonstrated that even at the single clone level, long term contribution of pluripotent stem cells can be stable for the entire duration of the observation period, so far for up to 3 years after transplantation. In all long term clones studied systematically in three different primates, the stable contribution to the peripheral blood did not reach detectable levels until six weeks after transplantation. We have now observed stable progenitor cell contribution in a clinical trial for up to 9 years (Schmidt et al. Nat Med 2003). With increasing efficiency of gene transfer, the first ever serious side effects have been observed as clonal cell proliferations induced by replication incompetent retrovirus vectors, first in a mouse model (in collaboration with C. Baum, Hannover, Germany / Cincinnati) and unfortunately, in two patients of the French clinical X-SCID gene therapy trial (in collaboration with A. Fischer, M. Cavazzana-Calvo, Hopital Necker, Paris). We were able to detect that insertional mutagenesis in all three of these cases had most likely initiated the development to leukemia-like disease by insertional activation of a cellular oncogene. Both X-SCID cases demonstrate a strikingly similar molecular origin, pointing to possibilities of circumventing these problems by vector and trial design for ongoing and future gene therapy.

Speaker 37:

Short term incubation with linear polyamine copper chelators preferentially expand cord blood CD34+38- and CD34+ Lin- progenitor cells

Arnon Nagler

BMT and CBT Chaim Sheba Medical Center, Tel Hashomer, Israel

Expansion of self-renewal hematopoietic cells in ex-vivo cultures is limited. Using ex-vivo CD34+ cell enriched cultures we have previously demonstrated that cellular copper is involved in the regulation of hematopoietic progenitor cell proliferation and differentiation and it's cellular concentrations modulates the balance between the two. We demonstrated that adding a polyamine copper chelator to media containing cytokines (Flt3, IL-6, TPO, SCF) extend the long-term expansion of cord blood derived stem/progenitor cell cultures resulting in amplification of total cell proliferation, long term CFU and CD34+ cells. The potentiating effect initiated by the linear polyamines copper chelators required only 3 weeks (3w) of incubation, which is of major clinical importance. After three weeks in culture, there was no visible immediate difference in the total cells, CD34+ cells and CFU numbers. However, following additional 5w in polyamine free media, a dramatic difference was observed. The content of CD34+ cells as well as LTC-CFUc was 10-100 higher in the polyamine pre-treated cultures as compared to the con-tent of the above populations in the control cultures. These results indicated that 3w treatment with a polyamine chelator favoring extensive proliferation of a small subset of cells with higher self-renewal potential, resulted in extension of the long-term duration of cord blood derived CD34+ cell cultures. To understand the short term effect of the chelators, we measured the proportions of early progenitor cells in enriched populations of CD34 positive cells following three weeks of cultures. FACS analysis demonstrates that the copper chelator supplemented cultures contained higher percentages of CD34+CD38- and CD34+lin- cells (21±3.8; 7.0±1.9, n=9), respectively as compared with the content of these subpopulations in the control, only cytokine supplemented cultures (2.6±1; 0.9±0.3, n=9). The short incubation with this differentiation inhibitory agent in combination with the early acting cytokines resulted in 268±52 and 67±21 (n=18) fold expansion of CD34+ CD38- and CD34+ Lin- cell subsets, respectively (Table).

In vivo experiments in SCID mice demonstrated that engraftment of cells obtained following 3w expansion with the polyamine copper chelators was about 10 fold higher as compared to the level of engraftment of the same number of uncultured cells. Since a graft enriched with early progenitor cells may have clinical benefits, we developed a large scale cord blood derived ex vivo expanded progenitor cells transplant product using AC133 separation device, tissue culture bags and GMP ingredients.

Further studies of the molecular events during the 3w short-term exposure will enable better control of self-renewal and proliferation in ex-vivo conditions. The polyamine chelator based method of extension of self-renewal potential of stem/progenitor cells in short term cultures will be utilized for improving and facilitate engraftment in clinical cord blood transplantation.

Table: Three week cultures: CD34+ cells and subsets analysis

TEPA (µM)	34+ (%)	34+/38-(%)	34+/Lin (%)
0*	4.65 ±0.82	2.66±0.97	0.93±0.30
5*	6.3 ±0.90	21.4±3.80	7..84±1.99

Speaker 38:

Longterm Monitoring of Mpl-Based In Vivo Selection Using Chemical Inducers of Dimerization

Robert Richard, Michael Weinreich, Sylvia Chien, Masayoshi Masuko, James Yan, Tobias Neff, Peter Horn, Laura Peterson, Julie Morris, Manfred Schmidt, Hans-Peter Kiem, Christof von Kalle, Tim Clackson, and C. Anthony Blau.

University of Washington and the Fred Hutchinson Cancer Research Center, Seattle, WA, University of Cincinnati, Cincinnati OH, and ARIAD Pharmaceuticals, Cambridge MA.

Artificial ligands, called chemical inducers of dimerization (CIDs), can give transduced cells a reversible growth advantage in vitro or in vivo. CID-responsiveness is conferred by modified receptors that are activated not by natural ligands, but by the CID. We have previously shown that marrow cells transduced using a vector encoding a modified thrombopoietin receptor, F36Vmpl, expand in transplanted mice and dogs following CID administration in vivo. Here we describe the response to repeated courses of CID treatment. Among 38 mice treated with one or more courses of the CID, AP20187, 35 demonstrated rises in transduced cells, with a preferential expansion of cells expressing the highest levels of the F36Vmpl fusion. Red blood cells responded markedly, platelets moderately and granulocytes only slightly. Repeated administrations of AP20187 were required to maintain persistently high levels of transduced red cells. Concomitant administration of granulocyte colony stimulating factor (G-CSF) or c-kit ligand (KL) failed to alter the profile or persistence of AP20187's effect. In some mice, the response to CID waned with repeated treatments, whereas in other mice responsiveness persisted for up to six treatments. Three dogs transplanted with F36Vmpl-transduced marrow cells have been monitored for up to 30 months. Two of the three dogs have been treated with 3 cycles of AP20187 without adverse effects, other than for mild to moderate thrombocytopenia that resolved after discontinuation of the drug. Similar to our results in the mice, AP20187 administration in the dogs produced a significant rise in transduced red cells, an intermediate rise in transduced platelets, and only a slight rise in transduced neutrophils. Unlike mice, mpl signaling in the dogs promoted a dramatic, CID-dependent expansion of transduced B cells. Integration site analysis documents an oligoclonal expansion of transduced cells in response to CID treatment. Longterm monitoring will be required to evaluate potential late toxicities.

Speaker 39:

Rac GTPases regulate the engraftment phenotype and marrow localization of hematopoietic stem cells

Yi Gu, Marie Dominique Filippi, Jose Cancelas, Rethinasamy Prabhakar and David A. Williams

Cincinnati Children's Hospital Research Foundation, Molecular and Gene Therapy Program, Cincinnati, OH

Rho family Guanosine Triphosphatases (GTPases), including Rho, Rac and Cdc42, are critical regulators of multiple cell functions in mammalian cells, although their role in blood cells is still largely unknown. Genetic deletion of the murine alleles of Rac1, Rac2, or both revealed that Rac GTPases regulate the hematopoietic engraftment phenotype, since deficiency of both Rac1 and Rac2 leads to massive mobilization of hematopoietic stem/progenitor cells (HSC/P) into the peripheral circulation and Rac1-/-;Rac2-/- stem cells are devoid of reconstitution capacity in irradiated recipient mice. We studied the specific roles of the highly homologous Rac1 and Rac2 proteins, which share >92 % amino acid identity, in both primitive and differentiated hematopoietic cells. In cytokine-stimulated HSC/P, Rac1 regulates proliferation via Cyclin D1 and p27expression. In contrast, cell cycle progression is normal in Rac2-/- HSC/P, but apoptosis is significantly increased due to reduced Akt activation. Although Rac1 and Rac2 share overlapping roles in cell adhesion and migration in hematopoietic cells, both activating Erk1/2, each GTPase plays a distinct role in the localization of filamentous actin and cell shape. In fully differentiated neutrophils Rac2, but not Rac1, is necessary for N-formyl-methionyl-leucyl-phenylalanine-induced superoxide production, a key functional component of phagocytic cells and for cell migration. Thus in spite of the high degree of sequence homology, Rac1 and Rac2 play both unique and overlapping roles in blood cell development and function via shared and distinct signaling pathways but are critical for localization of HSC to the microenvironment and subsequent reconstitution of the blood system.

Speaker 40:

Endothelial injury mediated by cytotoxic T lymphocytes and microvessel loss in human graft versus host disease

B. C. Biedermann[1], S. Sahner[2], M. Gregor[3], D. A. Tsakiris[3], C. Jeanneret[1], J. S. Pober[4], A. Gratwohl[3]

[1]Department of Medicine, University Hospital, Bruderholz, [2]Cantonal Institute of Pathology, Liestal, and [3]Division of Hematology, University Hospital, Basel, Switzerland. [4]Yale Medical School, New Haven CT.

We tested the hypothesis that chronic graft versus host disease (GVHD) after allogeneic stem cell transplantation (SCT) results from ongoing vascular injury leading to microvessel rarification in the affected tissues. We quantified microvessel density in lesional skin biopsies from 15 patients with acute and chronic GVHD and in 6 normal skin biopsies. As an indirect marker of vascular injury, von Willebrand factor (vWF) was measured in plasma of 18 patients with acute and chronic GVHD, in 9 SCT recipients without GVHD and in 9 healthy controls. We found extensive microvessel rarification in late acute and chronic GVHD ($p<0.001$). Elevated vWF levels were found in patients with GVHD but not in patients without GVHD ($p<0.001$). CD8 T cell infiltrates in skin correlated with vWF plasma levels in patients with GVHD ($r2=0.687$, $p=0.02$), and activated cytotoxic T cells (CTL) and endothelial injury were present in these infiltrates. We conclude that host endothelial cells are a target of CTL-mediated injury following allogeneic SCT. Progressive microvessel loss, in turn, results in tissue fibrosis, the hallmark of chronic GVHD. Disruption of this pathogenetic sequence may prevent disease progression.

Speaker 41:

Imprinting and epigenetic reprogramming in mammalian development

F Santos, K Mitsuya, H Morgan, J Walter, W Dean, W Reik

Babraham Institute, Cambridge UK

Imprinted genes generally possess differential methylation in particular regions (DMRs) between parental chromosomes which is often acquired in the parental germ cells. Imprinted genes tend to occur in clusters in the mammalian genome, and some DMRs in the cluster appear to be imprinting centres (ICs), which regulate imprinted expression and epigenetic modifications of genes in the cluster in cis. We are particularly interested in the distal chromosome 7 imprinting cluster in the mouse, which is crucial for the regulation of fetal growth and placental function. We have found that a number of genes in this cluster are exclusively imprinted in the placenta and do not have differentially methylated regions. We have tested the effect of loss of methylation on imprinting of these genes and found that their imprinting is largely refractory to loss of maintenance methylation, suggesting that epigenetic marks other than DNA methylation may play a major role in imprinting in the extraembryonic tissues.

While epigenetic marks in somatic tissues are generally stable, there are two phases in mammalian development in which major epigenetic reprogramming occurs. This is in primordial germ cells and in early preimplantation embryos. In both, methylation is probably actively erased from the DNA but the mechanisms are not clear. In cloned embryos, high levels of DNA methylation and histone modifications characteristic of somatic cells are maintained, suggesting a general failure to reprogramme the somatic genome to totipotency. However, epigenetic heterogeneity exists in certain donor cells used for cloning and in cloned embryos, and is associated with cloning success.

Speaker 42:

Characterization of the niche for c-Kit-dependent stem cell systems

S. I. Nishikawa and M. Osawa

Stem Cell Research Group, Riken Center for Developmental Biology, Kyoto, Japan

In most adult tissues such as hematopoietic tissues, mature cells are lost continuously or intermittently throughout the life of the organism. To maintain homeostais during adult life this loss is replenished from an established stem cell system. The stem cell system consists of at least three distinct compartments; stem cell compartment, which is slowly cycling, transit amplifying compartment which is rapidly cycling, and differentiated compartment. Previous BrdU or 3H-thymidine labeling studies of cycling tissues such as intestinal epithelial cells and epidermal kerationocytes implied that resting or slowly cycling compartment are present in a particular region of the tissues that is designated as niche, which is responsible particular region called niche. In this talk, we will first show that stem cells of the melanocyte lineage are present in the bulge area of mouse hair follicles throughout the hair cycle. As compared with melanocytes in other area, they are characterized in their resistance to anti-c-Kit mAb and also an ability to reconstitute the new melanocyte stem cell systems.

After defining the localization of each compartment of melanocyte stem cell system in the hair follicle, we have analyzed expression of various molecules to obtain the molecular probes that can be used to specify each compartment by their gene-expression profiling. Among molecules analyzed to date, Sox10 expression was shown to delimit the boundary between resting and activated stem cells. Thus, it is clear that melanocyte stem cells display a distinct molecular expression profile from that of activated melanocytes, indicating discrete genetic programs between two populations.

This profiling was further extended to the production of cDNA library of single cells. We will describe our recent results in this attempts together with a new method that can avoid amplification of genomic DNA. The detected differences between stem cell and activated compartments now allow us to determine the mode of stem cell maintenance. Our results will show that a widely accepted view of stem cells that diverge to self-renew and differentiate is not the mode of melanocyte system. In this system, stem cells, upon activation, enter to the next stage without leaving stem cells, but a portion of activated cells revert to the stem cell stage, thereby replenishing the stem cell pool.

This system also allow us to address the issue of stem cell plasticity. By using a K14-SCF transgenic mouse, we were able to show that some cells in the transit amplifying compartment can be reintroduced to the resting stage in the niche suggesting the plasticity of these two compartments mediated by niche. While it is not clear whether or not the findings accumulated in the melanocyte stem cell system can be extrapolated to the hematopoietic system, we believe both systems share many common features. Hence, the results that will be presented in this symposium may be useful also for investigation of the niche for hematopoietic stem cells.

Speaker 43:

Cell diversification in the mammalian retina

M. Cayouette, A. Whitmore, M. Raff

MRC Laboratory for Molecular Cell Biology, London, England UK

How do individual neuroepithelial cells in the developing CNS choose between alternative fates? We have been addressing this question in the rat retina. We have obtained evidence that asymmetrical segregation of m-Numb and intracellular programmes both contribute to the cell diversification process. Whereas most neuroepithelial cells in the developing rat retina divide horizontally, with their mitotic spindle aligned parallel to the plane of the neuroepithelium, some divide vertically, with their spindle oriented 45°-90° off this plane. As m-Numb is concentrated at the apical side of retinal neuroepithelial cells, it is preferentially inherited by the apical daughter cell in vertical divisions but is inherited by both daughters in horizontal divisions. Long-term time-lapse video studies of GFP-labelled neuroepithelial cells in newborn retinal explants suggest that the two daughter cells of vertical divisions usually have different fates, whereas the two daughters of horizontal divisions usually have similar fates. Moreover, overexpression of m-Numb in the neuroepithelial cells decreases the diversity of cell types that the neuroepithelial cells give rise to. Together, these findings suggest that the asymmetrical segregation of m-Numb influences cell fate choice. Finally, proliferation and diversification of embryonic day 17 retinal neuroepithelial cells in clonal density dissociated cell cultures is very similar to that in explant cultures, suggesting that the neuroepithelial cells are already specified by this age and step through an intrinsic developmental programme that determines how many times they divide and what cell types they become.

Speaker 44:

”Stem Cell Nephroplasty“, potentially novel strategies for the treatment of Acute Renal Failure (ARF)

Christof Westenfelder, Florian Tögel, Zhuma Hu, Robert L. Baranowski
University of Utah, Salt Lake City, Utah USA

Clinical ARF from all causes remains a common complication associated with high, and largely unchanged morbidity and mortality. Effective new therapies for its prevention and treatment are urgently needed. Central to the pathophysiology of ARF is severe vasoconstriction, filtration failure, injury and death of tubular and vascular endothelial cells, and inflammation. Preservation of and recovery of adequate kidney function is directly dependent on cell survival and cellular repair, respectively. Repair of cell injury in ARF is carried out by surviving tubular and vascular cells, and is possibly supported by as yet undiscovered kidney-specific stem cells (SC) and SC delivered via the circulation. It was recently reported [Orlic, PNAS 2001] that mobilization of HSCs with SCF and G-CSF prior to the induction of myocardial infarction in mice resulted in improved function and tissue repair, suggested to be mediated by mobilized HSCs. There is early evidence that both HSCs and MSCs are able to differentiate, to variable degrees, into glomerular mesangial, vascular endothelial and renotubular cells. Based on the apparent kidney-specific plasticity of HSC and MSC, we tested (1) whether HSC mobilization (Cytoxan 200 mg/kg, followed by G-CSF, 250 µg/kg b. wt., given in two daily doses x 4 days) prior to the induction of ARF (60 min bilateral renal pedicle clamp on day 6 after cytoxan or vehicle) in FVB mice would improve outcome; (2) whether administration of vascular endothelial cells (EC) derived by differentiation with VEGF from MSC was as renoprotective as administered aortic EC in rats with ARF; (3) whether MSC, administered immediately or 24 hrs following induction of ARF altered subsequent outcome; (4) whether SDF-1 and its receptor, CXCR4, are involved as potential mediators of intrarenal SC homing, migration and engraftment. We found that (1) the mobilization-induced peak of circulating HSC (CFU-C) and WBC numbers (70,000/µl with granulocyte-predominant pattern) occurred on day 6. Subsequent induction of ARF caused, vs. controls, significantly more severe renal insufficiency and higher mortality. Granulocytopenia resulted in amelioration of ARF compared to the mobilized group; (2) the administration of aortic EC to rats with ARF was significantly renoprotective. MSC were readily differentiated into EC in vitro, and their ability to exert comparable renoprotection is under investigation; (3) administration of MSC or HSC per se to rats with ARF resulted in variable outcomes. In some animals renal function remained unchanged, in others there was improvement or deterioration. Administered cells could be tracked in the injured kidney; (4) SDF-1 expression appears upregulated in injured cells in ARF by in-situ hybridization. In conclusion: (1) HSC mobilization prior to induction of ARF greatly aggravates ARF via mobilization-associated leukocytosis and should thus not be utilized clinically unless strategies that increase the delivery of HSCs without associated leukocytosis are developed and proven to be renoprotective; (2) EC administration appears highly renoprotective but collection of EC from patients is too difficult. However, if EC derived from MSC are found to be equally beneficial, this technique may be a practical new approach to treat ARF; (3) further refinement of ARF treatment protocols with HSC or MSC may yet lead to the development of beneficial strategies; (4) the renal SDF-1 expression pattern in ARF together with CXCR4 expression in MSC, if confirmed, raise the possibility that this chemokine system may participate in intrarenal homing of MSC towards regions of parenchymal injury.

Speaker 45:

Transdifferentiation potential of hematopoietic stem cells

Makio Ogawa and Christopher J. Drake

Experimental Hematology, Medical University of South Caroline, Charleston, SC, USA

It has recently been reported that a variety of stem cells, including those of hematopoietic, liver, muscle and neuronal origins are "plastic" in that they have the potential to "transdifferentiate" into tissues other than those specified by their origin. Since most of the transplantation studies have been conducted using populations of cells, it is possible that the apparent multi-potentiality of the stem cells is from engraftment by multiple separate populations of mono-potential cells. In order to seek definitive information on the tissue/organ engrafting capabilities of mouse hematopoietic stem cells (HSC), we developed an efficient method to create mice with clonal hematopoietic engraftment by combining FACS cell sorting and short-term suspension culture of bone marrow cells. We used transgenic CD45.2 C57BL/6 (B6) mice that ubiquitously express enhanced green fluorescent protein (EGFP) as the source for donor HSC and congenic CD45.1 B6 mice as recipients. The contribution of EGFP-labeled donor HSC to various organs/tissues of the recipient mice was assessed using laser scanning confocal microscopy and conventional epifluorescence microscopy in combination with differential interference contrast (DIC) microscopy. This approach was successful in determining the hematopoietic origin of glomerular mesangial.

We have also been studying human “transdifferentiating” stem cells using a murine/human xeno-transplantation assay we recently developed. The assay involves transplantation of human cord blood cells through the facial vein of newborn NOD/SCID/β2-microglobulin-null (NOD/SCID/BMGnull) mice that had been “conditioned” with 37 mg/kg of 5-fluorouracil and 30 mg/kg of blocking anti-mouse c-kit antibody, Ack-2. This model allows observation of high-level engraftment by human hematopoietic cells for up to 4 to 5 months. Using fluorescence in situ hybridization (FISH) to identify human cells and a variety of techniques including immunohistochemistry, RT-PCR, and in situ hybridization for mRNA to determine their lineages, we found the presence of human hepatocytes in the engrafted mice.

Speaker 46:

Clonal Analysis of Hematopoietic Stem Cell Differentiation

Margaret A. Goodell

Baylor College of Medicine, Houston TX, USA

Recent studies have suggested that adult-derived stem cells may differentiate outside their tissue of origin. Lacking is definitive data that a single adult stem cell can generate more than one tissue type. We will discuss recent data that demonstrate hematopoietic stem cells can regenerate blood as well as functional hepatocytes and skeletal muscle. Cell fusion appears to account for some but not all of the apparent "transdifferentiation" activity. Our results unequivocally identify the hematopoietic stem cell as responsible for the majority of the myogenic and hepatogenic activity in murine bone marrow.

POSTER ABSTRACTS

Oncogenesis

Poster 1:

AML1/ETO prolongs the survival of hematopoietic progenitors in NOD/SCID mice but does not abrogate their lineage specific differentiation

Jörg Bäsecke[1], Maike Schwieger[2], Frank Griesinger[2], Christopher Baum[2], Gerald Wulf[1], Lorenz Trümper[1], Carol Stocking[2]

[1]Dept. of Hematology and Oncology, University of Göttingen, Göttingen, Germany
[2]Heinrich-Pette-Institut, Hamburg, Germany
[3]Dept. of Hematology and Oncology, Medical School Hannover, Germany

We used the SF91-wPRE vector for retroviral transduction of the fusion gene AML1-ETO (CBFA2-MTG8), associated with acute myeloid leukemia FAB M2, in human cord blood derived CD34+ hematopoietic progenitors. Efficiency was 70 % for the green fluorescent protein(GFP)-control vector and 30 % for the AML1/ETO-GFP vector. Following selection by FACS, infected (GFP+)-cells were subjected to in vitro assays. In liquid culture (LC), using serum free medium, supplemented with FLT-3, SCF, IL-6, IL-3 and TPO, AML1/ETO-GFP transduced cells (AE-G cells) were expandable for more than 10 weeks, whereas the control terminally differentiated within 4 weeks. The AE-G cell population in LC retained an cytological and immunophenotypical immature morphology of predominating myeloblasts with high expression of CD34, HLA-DR, CD13 and CD 33. In spite of significantly prolonged survival, AE-G cells gave rise to normal colonies in the CFU-assay, indicating that terminal maturation was not impaired.
The NOD/SCID-mice were kept up to 14 weeks for extended observation. The median chimerism was 17 % in the control and 46 % in the AE-mice. In no animal was acute myelogenous leukemia diagnosed. Immunophenotyping showed that AE transduced human CD34+-cells engraft the myeloid (CD 15, CD33), B (CD19)- and T(CD3)-lymphoid, NK-cell (CD56) and erythroid (GPA) lineage. Though able to engraft the major lineages, mononuclear cells of AE-mice exhibit a higher expression of CD34 and CD33 whereas the expression of CD38 was markedly reduced. A high transcription level of AML1/ETO in the AE-mice was confirmed by real-time PCR.
Our findings indicates that AML1/ETO seems to prolong the survival of hematopoietic progenitors in liquid culture and the xenograft model but does not abrogate their differentiation along the main lineages.

Poster 2:

Dose-dependent effects of Bcr-Abl expression on functional properties of murine cell lines

David J. Barnes, John M. Goldman, Junia V. Melo

Chronic myeloid leukaemia (CML) is a haemopoietic disorder characterized by excessive proliferation of bone marrow stem cells. Unlike most other human neoplasia, the presence of a single oncogene, BCR-ABL, is necessary and sufficient for the transformed phenotype. In some patients with CML, disease progression is accompanied by the appearance of progenitor cells in which the expression of Bcr-Abl, the tyrosine kinase encoded by the oncogene, is elevated. In order to model variable expression of Bcr-Abl, we transfected the murine myeloid cell line, 32D, with a bi-cistronic vector containing both BCR-ABL and the gene for the enhanced Green Fluorescent Protein (eGFP). Cell sorting by flow cytometry was employed to select six clones that expressed different levels of Bcr-Abl. In contrast to previously established 32D/BCR-ABL cell lines, our lines were always maintained in the presence of IL-3, avoiding in this way an enforced selection for artificially high Bcr-Abl overexpression. In two different assays the specific properties of these clones was related to the level of Bcr-Abl expression. First, cellular motility, assessed by migration through the pores of a Transwell, correlated with expression of Bcr-Abl in the clones. Whereas 50 % of cells of the clone expressing the highest level of Bcr-Abl migrated through the Transwell, only 7.8 % of cells of the clone expressing the lowest level of Bcr-Abl underwent migration. Second, elevated expression of Bcr-Abl also enhanced the adhesion of clones to the extracellular matrix protein, fibronectin. In assays in which cells were allowed to attach to the fibronectin-coated wells of a microtitre plate, almost all of the cells of the highest expressing clone remained adherent following washing to remove unattached cells. In contrast, two-thirds of the cells of the clone expressing the lowest level of Bcr-Abl became detached upon washing. Significantly, we were able to demonstrate that clones expressing low levels of Bcr-Abl exhibit deficient adhesion to fibronectin, compared to parental 32D cells, when assayed in the presence of IL-3. Although previous studies have indicated that transformation with BCR-ABL tends to enhance the adhesiveness of cell lines, it is well established that primary CML progenitors exhibit reduced adhesive properties, relative to normal progenitor cells, and this has been implicated as one of the mechanisms responsible for their premature release from the bone marrow. Our ability to detect such an adhesion defect in our cell line model is most likely due to the selection of clones in which Bcr-Abl is not overexpressed to an excessive degree. Taken together these data suggest that subtle differences in expression of Bcr-Abl can profoundly affect cellular properties.

Poster 3:

Creation of a locally inducible primary tumor model expressing HPV16 E6/E7 oncogenes

Gabriele B. Beck-Engeser, Michael T. Spiotto, Karin Schreiber, Hans Schreiber

E6/E7 oncogenes of high-risk human papilloma virus subtypes (HPV16+18) play an essential role in the carcinogenesis of certain types of cancers. These tumors pose important problems: active immunization against E7 appears to be effective in clearing patients of infectious viruses but it may not clear patients of those cells which integrated HPV16 E6/E7 sequences (1). Unfortunately, these are the cells from which cancer originates since integration is required for invasive cancer to develop (2). The possible failure of active immunization to eliminate cells with integrated viral sequences suggests that a model is needed in which novel approaches for eliminating cells with integrated viral sequences can be readily explored. For this goal we are developing a Tamoxifen-inducible Cre-lox mouse tumor model, that will have the following advantages: (i) genetic design that allows us to predict the relative levels of E6/E7 expression before the gene is actually expressed, (ii) induction of the E6/E7 gene in the adult mouse only after application of Tamoxifen, so that there should be no neonatal or peripheral tolerance to E6/E7 prior to induction, and (iii) expression of the E6/E7 oncogene under the control of a keratinocyte specific K14 promoter in only a finite number of keratinocytes in a small circumscribed area using topical application of tamoxifen (3) which will cause a local green to white color change. The latter makes complete elimination of the E6/E7 positive cell population, and therefore cure, possible. Studying these mice allows us to answer the critical question of whether, and under which conditions, E6/E7-specific T cells can prevent or cure HPV E6/E7-induced primary (non-transplanted) tumors. To create this model we have generated a transgenic mouse line possessing the inducible HPV16 E6/E7 gene, whose transcription has been interrupted by a floxed EGFP gene. These mice were bred to transgenic mice expressing Cre and a fusion protein between Cre recombinase and the Tamoxifen responsive hormon-binding domain of the estrogen receptor (CreERtam) (3). Upon topical Tamoxifen application Cre-ERtam protein is activated and recombines the loxP sites and excises the inhibitory EGFP sequences thereby allowing E6/E7 gene expression. Keratinocytes were isolated from areas of the mouse back skin after the induction of HPV16 E6/E7 expression. The cells were cultured and subjected to ^{51}Cr release assays in order to determine the cytolytic activity of E6/E7 specific cytotoxic T cells.

Poster 4:

Deregulated platelet-derived growth factor receptors in chronic myeloproliferative disorders

E. Joanna Baxter, N.C.P. Cross

The myeloproliferative disorders encompass a wide clinical spectrum of diseases, the most common being CML, which is characterised by the presence of a t(9;22) translocation resulting in the fusion of BCR to ABL. However up to 10 % of cases with a clinical and morphological phenotype that resembles CML are BCR-ABL negative. We have analysed several cases with chromosomal translocations of 5q31-33 and determined that many disrupt the receptor tyrosine kinase PDGFRB. We also studied two patients with atypical CML and a t(4;22)(q12;q11). Using FISH we demonstrated that BCR was rearranged but RT-PCR for BCR-ABL was negative. Chromosome 4q12 contains three receptor tyrosine kinase-encoding genes, c-KIT, KDR and PDGFRA that were obvious candidates for disruption by the t(4;22). We employed a multiplex RT-PCR strategy to amplify any possible fusions between BCR and the tyrosine kinase-encoding exons of each of these genes. Sequencing showed a fusion between BCR and PDGFRA was present in both patients. The first was between BCR exon 7 and PDGFRA exon 12 and the other between BCR exon 12 and PDGFRA exon 12, although unusually the PDGFRA breakpoint were at different positions mid-exon in each case with several bp of BCR intronic material retained in the fusion mRNAs. Gene fusions involving PDGFRA have not previously been reported in either leukaemia or solid tumours but very recently a novel cytogenetically cryptic fusion between FIP1L1 and PDGFRA has been identified in a subset of patients with hypereosinophilic syndrome (HES). To determine whether this fusion occurs in other eosinophilic MPDs we used RT-PCR on patient RNA or bubble PCR on patient DNA. FIP1L1-PDGFRA was found in 2/16 patients with HES and 1/12 with atypical CML. As seen in the t(4;22), the PDGFRA breakpoints cluster tightly within exon 12, disrupting the sequence encoding a putative WW-like protein-protein interaction domain that may negatively regulate PDGFRA activity. We conclude that disruption and deregulation of PDGFRs is a significant cause of MPDs. Clinically the finding of a PDGFR gene fusion is significant as patients with these rearrangements are responsive to small molecule tyrosine kinase inhibitor imatinib mesylate (Glivec).

Poster 5:

Mouse Models for AML1-ETO Transformation and Identification of Cooperating Mutations

Martin Forster, Maike Schwieger, Meike Fischer, Ivan Horak, Jürgen Löhler, Carol Stocking

Molecular Pathology, Heinrich-Pette-Institut, Hamburg, Germany

The characterization of chromosome translocations found in distinct classes of AMLs have identified several chimeric oncoproteins important in the establishment of the leukemia clone. Importantly, however, secondary mutations are normally required for the full transition to an acute leukemia. We have embarked on studies to develop mouse models where these secondary mutations can be identified. For this we have chosen a subset of leukemias that carry the t(8;21) translocation, which juxtaposes the *RUNX1* gene with the *CBFT1* gene, generating the AML1-ETO fusion protein. Using retroviral vectors to transduce AML1-ETO into murine bone marrow cells and retransplantation, we have been able to demonstrate that expression of AML1-ETO in bone marrow cells stimulates myelopoiesis at the expense of erythropoiesis and impairs maturation of both myeloid and lymphoid lineages *in vivo*. Despite the observed disrupted myelopoiesis, the bone marrow environment is restrictive for the expansion of these differentiation impaired cells. We thus tested the effect of AML1-ETO expression in the ICSBP$^{-/-}$ genetic backbone. ICSBP is a member of the interferon regulatory factor family and has been shown to act as a tumor suppressor in the myeloid compartment. Previous studies have shown that it is downregulated in 66 % of AMLs. In the ICSBP$^{-/-}$ background, AML1-ETO expression induced myeloblast transformation, as demonstrated by >25 % myeloblasts in the bone marrow and the development of localized granulocytic sarcoma-like tumors, clearly demonstrating the ICSBP deficiency cooperates with AML1-ETO in transformation. After a long latency, transition to a full leukemic phenotype was observed. To identify additional mutations that cooperate with ICSBP$^{-/-}$ and AML1-ETO in leukemogenesis, we have performed studies using murine leukemia retrovirus (MuLV) as insertional mutagens. In pilot studies in the ICSBP$^{-/-}$ backbone, we could demonstrate that the incidence, latency, and lineage-specificity of leukemia induction by MuLV is greatly altered as compared to that in ICSBP$^{+/+}$ mice. Analysis of retroviral integration sites have confirmed activation of genes previously implicated in myeloid leukemias (e.g. HoxA7 and HoxA10). In addition, we have identified three genomic loci, in which retroviral integration is detected in multiple tumors, as determined by Southern blot analysis. These loci have not been previously associated with human leukemia nor in mouse models. Genes deregulated by the retroviral integration are currently being characterized. Our results underline the power of retroviruses for establishing effective models for identifying the molecular mechanisms of leukemogenesis.

This work was supported by funds of the Deutsche Forschungsgemeinschaft and the José Carreras Leukämie-Stiftung .

Poster 6:

The comparison between the blood serum protein analysis of Pike perches and tumor bearing and tumor free Xiphophorus

Asiful Islam

The comparative blood serum protein analysis of the 2 species of Pike perches (Stizostedion lucioperca and St. volgense) and tumor bearing and tumor free Xiphophorus fishes are investigated by using the method of native polyacrylamide gel electrophoresis. In the experiments, it is revealed that the protein bands, which are composed of albumins, transferrins, globulins and lipoproteids have shown the diversified differences and polymorphism between the two species of Pike perches and normal (tf) and tumor bearing (tb) Xiphophorus fishes. The different protein bands of the Stizostedion species have the polymorphism with the globulin and transferrins fractions. The St. volgense has the higher peaks of transferrins than the other species of Pike perches but they are from the same age group. Transferrin is responsible for the transportation of the metabolic products and the defense system of the body. But in comparison with Xiphophorus, it reveals that the warm water fishes have the higher fragments of protein bands than the cold water fishes. The comparative serum proteins analysis of tf and tb Xiphophorus shows that the globulin fractions are depressed in the normal but more declining in the acute and tumor bearing melanoma formed fishes. The tumor free fishes have the up rising peaks of globulin and transferrins in comparison with the tb species. Besides, the albumin peaks of both the tf and tb fishes show more or less similar peaks with the molecular weight of 65 kD. In tumor bearing fishes, the lipoproteid peaks are higher and more variable than in the tumor free Xiphophorus.

Poster 7:

Expression of a Mutated Form of the p85alpha Regulatory Subunit of Phosphatidylinositol 3-Kinase in a Hodgkin's Lymphoma Derived Cell Line (CO)

Manfred Jücker, Kirstin Südel, Stefan Horn, Wiebke Wegner, Walter Fiedler, Ricardo A. Feldman

Phosphatidylinositol (PI) 3-kinase plays an important role in a variety of biological processes including proliferation and apoptosis. PI3-kinase is a heterodimer consisting of an 85 kDa adapter protein (p85a) containing one SH3 domain and two SH2 domains and a 110 kDa catalytic subunit (p110). Recently an oncogenic form of p85 named p65-PI3K lacking the C-terminal SH2 domain has been cloned from an irradiation-induced murine thymic lymphoma and transgenic mice expressing p65-PI3K in T lymphocytes develop a lymphoproliferative disorder. Here we describe the cloning of a C-terminal truncated form of p85a expressed in a human lymphoma cell line (CO) with a T cell phenotype derived from a patient with Hodgkin's disease. As a result of a frame-shift mutation at amino acid 636, p76a is lacking most of the C-terminal SH2 domain but contains the inter-SH2 domain and is associated with an active form of PI3-Kinase. A PI3-kinase-dependent constitutive activation of Akt was detected in CO cells which was only partially reduced after serum starvation. Treatment of CO cells with the PI3-kinase inhibitor Wortmannin resulted in a concentration-dependent inhibition of cell proliferation associated with an increased number of apoptotic cells.
This is the first detection of a mutated form of the p85 subunit of PI3-kinase in human hematopoietic cells further underlining a potential role of PI3-kinase/Akt signalling in human leukemogenesis.

Poster 8:

The role of RUNX-1 as a dominant acting oncogene

Anna Kilbey, S. Wotton, K. Blyth, L. Hanlon, E. Cameron, J. C. Neil

The mammalian Runx genes comprise three known family members (Runx 1-3) that encode the α subunit of the heterodimeric core binding transcription factor. The β subunit is encoded by a single gene CBFβ that is crucial for the proper functioning of all Runx proteins. Homozygous disruption of either CBFβ or Runx 1 (AML1, CBFA2) in mice produces an almost identical phenotype characterised by a complete lack of definitive haematopoiesis. The identification of Runx binding sites in multiple haematopoietic specific genes and the expression of Runx 1 in both myeloid and lymphoid lineages further support a role for Runx 1 in haematopoietic development. Indeed, RUNX 1 is the most frequent target of chromosomal rearrangements in human leukaemias resulting in the expression of abnormal fusion proteins that inhibit normal RUNX 1 function. This, and the observation that RUNX 1 haploinsufficiency predisposes to leukaemia has led to the conclusion that RUNX 1 loss-of-function is a key event in the disease. Similar conclusions have been drawn regarding the role of RUNX 3 in gastric cancer[1] and RUNX 2 in the bone disease Cleidocranial Dysplasia[2]. Interestingly, we have recently shown by retroviral tagging experiments that Runx 1, like Runx 2 and Runx 3 can function as a dominant oncogene in T-cell lymphomas of mice[3-5]. Promoter insertion was found to induce Runx 1 over expression without affecting the integrity of the coding sequences. This observation led us to investigate the alternative hypothesis that Runx 1 can act as a dominant oncogene in certain cellular contexts. I will describe the *in vitro* and *in vivo* systems currently in use in our lab to address the oncogenicity of Runx 1 and the effects of Runx 1 on cellular proliferation, differentiation and survival.

1. Q.L. Li et al., Cell 109, 113 (2002).
2. T. Yoshida et al., Am. J. Hum. Genet. 71, 000 (2002).
3. S. Wotton et al., Cancer Research 62, 7181 (2002).
4. M. Stewart et al., Proc. Natl. Acad. Sci. USA 94, 8646 (1997).
5. M. Stewart et al., J. Virol. 76, 4364 (2002).

Poster 9:

Reversal of commitment to the B cell lineage in acute lymphoblastic leukemia harboring a BCR-ABL1 gene rearrangement

Florian Klein, Varun Singh Barath, Niklas Feldhahn, Sanggyu Lee, Guolin Zhou, San Ming Wang, Janet D. Rowley, Svetlana Harder, Reiner Siebert, Martin Krönke, Markus Müschen

Pre-B cells (PBC) have been identified as the precursor of B-ALLs harboring a BCR-ABL1 fusion gene, which is generated by a t(9; 22), termed Philadelphia Chromosome (Ph). Normal PBCs are subject to negative selection within the bone marrow and are destined to die by apoptosis unless they are rescued by survival signals through the pre-B cell receptor (pBCR). To define growth requirements of Ph+ B-ALL clones, we generated and compared genome-wide gene expression profiles of CD34+ hematopoietic progenitor cells (HSC), PBCs and two cases of Ph+ B-ALL.

To analyze global gene expression profiles of t(9;22)+ leukemia cells and their normal precursors, we chose the SAGE (serial analysis of gene expression) technique. As opposed to alternative approaches, SAGE provides quantitative gene expression data at a genome-wide level, i.e. also including expressed genes, which have not previously been identified. A total of 277,000 SAGE-tags were sequenced and matched to the reference data base UniGene, which identifies the expressed genes from a 14 bp tag sequence.

About 80 percent of the genes initially expressed at the HSC stage were silenced in Ph+ leukemia. Unexpectedly, these genes include virtually all determinants of the B cell lineage. Loss of PBC identity involves constituents of the pBCR (VpreB, l5, IgH Cm, Iga, Igb) and the V(D)J-recombination machinery (RAG1, RAG2, TdT), proximal protein tyrosine kinases such as BTK and BLK and key transcription factors, which are known to initiate and maintain commitment to the B cell lineage, namely E2A, EBF and PAX5. Given the critical role of pBCR-mediated signals for the survival of PBCs, the question arises, how Ph+ leukemia cells lacking the expression of pBCR-related genes can nonetheless establish a highly proliferating clone. Indeed, expression levels of NF-kB and other activation-induced nuclear molecules are similar in t(9;22)+ leukemia as in their normal counterpart.

These findings suggest that a transcriptionally silenced pBCR signaling complex in BCR-ABL1+ leukemia cells can be effectively replaced by alternative survival signals, which most likely arise from the oncogenic ABL1 tyrosine kinase. Specific silencing of genes, whose expression was induced during the transition from the HSC to the PBC stage of development demonstrates that Ph+ leukemia cells have phenotypically reversed their commitment to the B cell lineage.

Poster 10:

Depletion of AML1/MTG8 by specific siRNAs enables induction of myeloid differentiation in t(8;21) positive Kasumi-1 cells

Jürgen Krauter, Bettina Drescher, Olaf Heidenreich, Kerstin Görlich, Arnold Ganser, Gerhard Heil

The reciprocal translocation t(8;21)(q22;q22) in acute myeloblastic leukemia (AML) fuses the N-terminal part of the AML1-gene on chromosome 21 to the nearly complete MTG8-gene on chromosome 8 resulting in a chimeric AML1/MTG8 fusion mRNA and protein. The AML1-gene encodes for a transcription factor essential for normal fetal liver hematopoiesis whereas the function of MTG8 is still largely unknown. AML1/MTG8 has been shown to interfere with TGFbeta1- and vitaminD3-signalling. To further elucidate these mechanisms, we asked if downregulation of AML1/MTG8 by RNA interference enhances the sensitivity of t(8;21) positive Kasumi-1 cells to TGFbeta1/vitaminD3 induced differentiation. Kasumi-1 cells were electroporated with 200nM of siRNA against AML1/MTG8 (siAM) or an irrevelant control (siGL2) and incubated for 48h. At that time point, siAM led to an approximately 70 % downregulation of AML1/MTG8 mRNA and protein as detected by real time PCR and Western blotting whereas the expression of wild-type AML1b was not affected. Thereafter, the cells were stimulated with TGFbeta1 (1nm) and vitaminD3 (100nM). After further 48h of incubation, the cells were analyzed for the expression of the differentiation-associated myeloid transcription factors C/EBPalpha, beta and epsilon by quantitative real time PCR as well as for surface antigen expression. Cells without TGFbeta1/vitaminD3 served as a control. siAM or TGFbeta1/vitaminD3 alone enhanced C/EBPalpha expression about threefold. The combination of both led to a 16-fold increase in C/EBPalpha expression. TGFbeta1/vitaminD3 alone induced C/EBPbeta 11-fold whereas siAM alone had no major effect. Treatment with siAM followed by TGFbeta1/vitaminD3 led to a 31-fold upregulation of C/EBPbeta mRNA. Regarding C/EBPepsilon expression, neither TGFbeta/vitaminD3 nor siAM alone had a major effect, whereas the combination induced C/EBPepsilon mRNA about 6-fold. In addition, the combination of TGFbeta1/vitaminD3 and siAM enhanced the surface expression of CD11b and CD14 on the Kasumi-1 cells. Moreover, Kasumi-1 cells treated with TGFbeta1/vitaminD3 and siAM showed increased expression of the M-CSF- and G-CSF-receptor which are known targets of C/EBPalpha. Taken together, evidence was found that AML1/MTG8 inhibits TGFbeta1/vitaminD3 dependent gene regulation in Kasumi-1 cells. The depletion of AML1/MTG8 by RNA interference enables the induction of transcription factors involved in myeloid differentiation. The combination of AML1/MTG8 downregulation with differentiating agents in t(8;21) positive leukemic cells might be a future option to overcome the block of differentiation in this distinct subtype of AML.

Poster 11:

Telomerase is limiting in acute myeloid leukemia

Alexander Röth, Suzanne Vercauteren, Heather J. Sutherland, Peter M Lansdorp

Telomere regulation plays an important role in cellular proliferation and senescence in normal cells as well as in malignant cells. To test the role of telomerase in acute myeloid leukemia (AML), we expressed the hTERT gene, a dominant-negative DN-hTERT (D868A, D869A) gene or a green fluorescence protein (GFP) gene in K562 leukemia cell line cells as well as primary AML cells from different patients using retroviral vectors. Cells transduced with hTERT exhibited elevated levels of telomerase activity compared to GFP controls whereas cells expressing DN-hTERT had decreased telomerase activity. K562 populations transduced with DN-hTERT showed reduced clonogenicity, telomere dysfunction and increased numbers of apoptotic cells compared to GFP or hTERT-transduced cells. Two of four clones transduced with DN-hTERT died after 30 and 53 population doublings (PDs), respectively. Transduced AML cells were tested in primary colony forming unit (CFU) and suspension culture assays. Relative to DN-hTERT and GFP transduced controls, AML cells transfected with hTERT produced more CFU for longer periods and showed higher engraftment after transplantation into sublethally irradiated β2-m-/-NOD/SCID mice. We conclude that telomerase levels are limiting in primary AML progenitor cells. Our data warrant further studies of the therapeutic use of telomerase inhibitors in AML.

Poster 12:

SU11248 inhibits the activation and transforming ability of AML-typical Flt3 mutations

Joachim Schwäble, Carsten Müller-Tidow, Carsten Hullermann, Bülent Sargin, Michael Mohr, Christian Brandts, Anne-Marie O'Farrell, Wolfgang E. Berdel and Hubert Serve

Activating mutations in the receptor tyrosine kinase Flt3 are found in about 30 % of AML cases and induce malignant transformation of myeloid cells. SU11248 is an oral selective inhibitor, which targets FLT3, and other class III and V split kinase domain receptor tyrosine kinases (RTK). We compared the effects of the tyrosine kinase inhibitor SU11248 on the activation and biological functions of wildtype and mutationally activated Flt3 (Flt3-ITD) using a 32D cell model system. SU11248 dose-dependently inhibited autophosphorylation of both ligand-stimulated Flt3 and Flt3-ITD. SU11248 also inhibited FLT3-ITD mediated STAT5 activation, a pathway implicated in FLT3-ITD oncogenesis. Expression of sixteen FLT3-ITD target genes was investigated and SU11248 antagonized Flt3-ITD-specific effects in all cases. Furthermore, incubation of 32D cells stably expressing Flt3-ITD with SU11248 inhibited their autonomous proliferation and radiation resistance. In conclusion, SU11248 is an interesting small molecule with potential therapeutic application in Flt3-driven AML.

Poster 13:

Expression of AML1-ETO in human CD34+ cells blocks granulocytic differentiation and increases self-renewal

Alex Tonks, Lorna Pearn, Amanda J Tonks, Terry Hoy, Janet Fisher, James R Downing, Alan K Burnett and Richard L Darley

The t(8;21) translocation that encodes the AML1-ETO fusion protein (now known as RUNX1-CBF2T1), is one of the most frequent translocations in acute myeloid leukaemia. Though its role in leukaemogenesis is unclear, the fusion gene is believed to dysregulate the expression of genes that are important for normal differentiation and proliferation of haemopoietic progenitors. We have previously shown that the AML1-ETO fusion protein promotes the long-term self-renewal of human erythroid progenitors (Blood 101:624-632, 2003). Here, we describe the effect of AML1-ETO on granulocyte and monocyte development. Expression of AML1-ETO in human CD34+ cells was achieved by retroviral infection of these cells using a vector co-expressing green fluorescent protein (GFP). The effect of AML1-ETO on myeloid growth and development was initially studied in bulk liquid culture, in the presence of IL-3, SCF and G/GM-CSF. Exogenous expression of AML1-ETO in these cells had an initial inhibitory effect on myeloid growth. Using a panel of lineage markers we determined that the growth of both monocytic (GFP+, CD13+, M-CSF-R+) and granulocytic lineages (GFP+, CD13+, M-CSF-R-) was transiently growth inhibited over the first 6 days of culture, when compared with control cells expressing GFP alone ($P<0.01$). However, despite this initial suppression of growth, AML1-ETO expressing cells continued to proliferate for more than 80 days, resulting in a 5×10^3-fold expansion over that observed for control cells (which stopped growing around day 28). In addition, expression of AML1-ETO was able to suppress granulocytic colony formation by 45 ± 28 % when compared to control cultures expressing GFP alone. In contrast, there was no observed decrease in monocytic colony forming efficiency in cells expressing AML1-ETO. Morphologically, granulocyte terminal differentiation in AML1-ETO expressing cells was inhibited by 83 ± 5 % ($P<0.01$) giving rise to a build-up of early to intermediate granulocytes which maintained this appearance throughout the prolonged period of expansion. Phenotypically, these cells were GFP+, CD13+, M-CSF-R- and showed developmental irregularities such as the failure to lose expression of CD34+ or significantly upregulate CD11b in the same manner as control cells ($P<0.01$ at day 6). On the other hand, there was no significant difference in CD14 expression in monocytic cells expressing AML1-ETO (GFP+, CD13+, M-CSF-R+). Taken together, these results suggest that expression of AML1-ETO suppressed the differentiation of granulocytic cells and allowed extensive self-renewal to take place supporting a causal role for t(8;21) translocations in leukaemogenesis.

Poster 14:

Disruption of the C/EBPalpha gene leads to an immature phenotype of a bcr/abl-induced myeloproliferative disease in a murine transplant model

Katharina Wagner, Pu Zhang, Daniel G Tenen

The genetic hallmark of chronic myeloid leukemia is the chromosomal translocation t(9;22) which results in the chimeric bcr/abl gene. Bcr/abl codes for a constitutively active tyrosine kinase. Expression of bcr/abl leads to an increased proliferation of myeloid progenitors and the accumulation of mature myeloid cells in bone marrow and peripheral blood of the patients ("chronic phase"). However, during the course of the disease, CML patients acquire a block in differentiation. Ultimately, the phenotype resembles acute leukemia ("blast crisis") with a very poor prognosis. The mechanisms involved in and crucial for progression from chronic phase to blast crisis are poorly understood. Recently, downregulation of C/EBPalpha by bcr/abl has been implicated in the transition from chronic phase to myeloid blast crisis (Perrotti et al, Nature Genet. 30:48, 2002). C/EBPalpha is a transcription factor essential for the normal differentiation of myeloid progenitors. We therefore asked whether the absence of C/EBPalpha is sufficient to alter a murine retroviral model of CML into a model of blast crisis. We transduced C/EBPalpha -/- mouse fetal liver cells with a retrovirus encoding bcr/abl-IRES GFP. Subsequently, the cells were transplanted into sublethally irradiated C57B6 mice. Mice transplanted with C/EBPalpha +/+ or +/- fetal liver cells expressing bcr/abl developed a lethal CML like disease. This was characterized by a highly elevated white blood cell count (WBC) with >80 % mature granulocytes, massive splenomegaly and hepatic infiltration. FACS analysis of the spleen showed infiltration by mature myeloid cells characterized by expression of Gr1 and Mac1. These cells expressed bcr/abl mRNA and protein. In mice transplanted with C/EBPalpha -/- cells expressing bcr/abl immature cells were found in the peripheral blood. In addition, they developed splenic as well as hepatic infiltration. In contrast to the controls, these mice had predominantly immature blast like organ infiltrates that expressed neither Gr1 nor Mac1.
In conclusion, the CML-like myeloproliferative disorder induced by bcr/abl in a wild type background has an immature phenotype resembling acute leukemia in a C/EBPalpha knockout background.

Poster 15:

Molecular cytogenetics of Mantle cell lymphoma with leukaemia: A comparison with CD5 positive mature B cell leukaemia (unclassified).

Nilima Parry-Jones, E. Matutes, V. Brito-Babapulle, R. Morilla, D. Catovsky

We studied by FISH peripheral blood samples from 58 cases of mantle cell lymphoma (MCL) with leukaemia, and 23 cases of CD5 positive, leukaemic B cell proliferation, unclassifiable by current criteria. Typical CLL was excluded on the basis of immunophenotype and morphology. All MCL cases had t(11;14) by FISH and/or conventional cytogenetics. All unclassified cases were negative for the translocation by FISH analysis.

Clinical and haematological features are summarised below:

	MCL	'unclassified'
Median age	65	61
M:F ratio	2.6:1	3:1
Splenomegaly	79 %	41 %
Hepatomegaly	15 %	0 %
Lymphadenopathy	48 %	47 %
Median Hb (g/dl)	11	13.2
Median lymphocytes (x 10^9/l)	16.8	21.8
Median plt count (x 10^9/l)	127	225

Among the MCL cases, 7 developed CNS disease, 3 had gastrointestinal disease and 3 had pleural involvement. CNS disease occurred in one unclassified case. Morphology in the unclassified group was indistinguishable from MCL, with medium sized cells with stippled chromatin and irregular nuclei, often with indentations. Among the MCL patients, 82 % had typical morphology, 14 % were blastoid and 4 % had small cell morphology. 10 % of the unclassified group had blastoid features. All cases in both groups were CD5 positive by flow cytometry and had low 'CLL scores' of 1 or 2 out of 5. (Typical CLL normally scores 4-5, and MCL 1-2). 15 % of MCL cases and 30 % of unclassified cases expressed CD23.

FISH results:

	MCL cases	'unclassified cases'	
+12	7 %	39 % #	
del 6q21	12 %	8 %	
del 11q23	27 %	7 %	
del p53	40 %	13 % +	
del 13q14: (3 loci studied)*			*all within frequently deleted region reported in CLL
D13S25	43 %	30 %	
D13S319	38 %	17 %	# p<0.01 (Chi-Square Test)
RB	24 %	17 %	+ p< 0.025

We conclude that while the two groups may be difficult to distinguish on morphological grounds, there are significant differences in the molecular cytogenetics. Trisomy 12 is rare in MCL, but occurs in more than a third of unclassified cases, a frequency comparable to that seen in CLL, particularly cases with increased prolymphocytes or atypical morphology. Deletions of 11q23 and p53 are more prevalent in MCL cases, and this group were more likely to have splenomegaly and thrombocytopenia, although the frequency of lymphadenopathy was similar between the groups. These differences may have prognostic importance, which we will evaluate further.

Cell cycle and apoptosis

Poster 16:

FoCA1 a novel cyclinA1 interating protein inhibits G1/S cell cycle progression

Shuchi Agrawal, Sven Diederichs, Wolfgang E. Berdel, Hubert Serve, Carsten Müller-Tidow.

CyclinA1 is tissue specifically expressed in testis, hematopoietic cells and in leukemic blasts. Its over-expression induces acute leukemia in mice. To analyze the functions of cyclinA1 in more detail, we performed a yeast triple hybrid screening experiment to identify proteins that interact with cyclin A1 in the presence of cdk-2. Five clones of a so far unknown protein were identified and the protein was named FoCA1 (Friend of Cyclin A1). The entire cDNA was cloned by 5′ RACE and was shown to consist of 1225 bp that encode a 221 amino acid protein. To analyze the functions of FoCA1, we performed cell cycle analyses in NIH3T3 cells after transient transfections. Briefly, cells were serum starved for 24 hr in order to enrich cells in G1 phase. Subsequent to the release by serum addition, cells were synchronized in G2 phase by addition of nocodazol. This treatment resulted in 90 % of cells in G2 phase after 24 hr of nocodazole exposure. Following nocodazole treatment, cells were transfected with FOCA1 or empty expression vector using nucleofector technology (AMAXA). Subsequently to transfection (efficiency >90 %), cells were released from nocodazole block for 12 hr. Entry of cells into S phase was analyzed by BrdU incorporation assay. Cells were harvested and stained for BrdU with anti BrdU-FITC along with Propidium iodide DNA staining. We observed that FoCA1 expressing cells showed significantly reduced DNA synthesis compared to control cells (vector alone) as shown, by the number of BrdU positive green cells. When we calculated the percentages of cells at different cell cycle check points, we found a difference of 11 % at G1/S transition between control and FoCA1 cells. Among the control cells, 39 % were BrdU positive, whereas only 28 % of the FoCA1 expressing cells incorporated BrdU. Interestingly, there was no difference observed in the cells at G2/M transition. These findings indicated that FOCA1 did neither inhibit G2/M transition nor mitosis. However, FoCA1 inhibited cell cycle progression at G1/S transition. Although the precise cellular functions of FoCA1 remain to be elucidated, we have demonstrated that its antiproliferative effect is mediated via modulation of the G1/S check point.

Poster 17:

FoCA1 (Friend of Cyclin A1) suppresses colony growth and proliferation, is induced upon cell cycle arrest and is absent in malignant diseases

Sven Diederichs, Shuchi Agrawal, Wolfgang E. Berdel, Hubert Serve, Carsten Müller-Tidow

Cyclin A1 is highly expressed in acute myeloid leukemic blasts. Cyclin A1 overexpression results in abnormal myelopoiesis and contributes to leukemogenesis in mice. From a yeast-triple-hybrid-system, we cloned FoCA1 (Friend of Cyclin A1, 221 aa) and its murine homologue (mFoCA1, 229 aa) as a novel interacting protein of Cyclin A1. FoCA1 bound to Cyclin A1 in vivo and in vitro and was phosphorylated by the Cyclin A1/CDK2 complex. Neither the human nor the murine FoCA1 showed any significant sequence homology to any other known protein. Using quantitative RT-PCR, highest levels of FoCA1 expression were found in the testis similar to the tissue-specific expression pattern of Cyclin A1. Intermediate expression was observed in ovary, pancreas, spleen, and lung. The high expression in testis was verified by immunohistochemistry. We also analyzed FoCA1 mRNA expression in 304 tumor samples, predominantly AML (n=82), ALL (n=21), NSCLC (n=63), and brain tumors (n=35), but also including CML, CLL, myeloma, and solid tumors from various origins. In all tumor samples, FoCA1 expression was notably absent or detected at very low levels. Direct comparison of normal and malignant testis, ovary or lung tissue showed dramatically lower FoCA1 expression in tumor samples compared to normal tissue. NIH3T3 fibroblasts were used to analyze FoCA1 expression throughout the cell cycle. Arresting NIH3T3 cells in G1 phase by serum starvation induced FoCA1 expression. After release of the cell cycle arrest, FoCA1 expression decreased rapidly. Cell cycle arrest with aphidicolin or colcemide showed the same effect. Starvation or arrest of primary murine embryonic fibroblasts led to increased FoCA1 expression after serum starvation. For colony formation assays, HeLa, HuTu80 or 32D cells were transfected with FoCA1. FoCA1 expression reduced the number of formed colonies by 40 %. In a stable cell line inducible expressing FoCA1, proliferation was reduced upon induction of FoCA1 as shown by [3H]thymidine incorporation. In summary, expression of FoCA1 was suppressed in malignant diseases and its expression was linked to cell cycle arrest. FoCA1 inhibited colony formation of different tumor cell lines and reduced proliferation. Taken together, our data suggest a growth suppressive function for FoCA1. Further studies will be performed to elucidate the role of the interaction between the leukemia-specific oncogene Cyclin A1 and the novel growth suppressor FoCA1 in physiological and pathological conditions such as in tumorigenesis.

Poster 18:

Peroxisome proliferator-activated receptor gamma ligands induce growth inhibition and apoptosis of human acute lymphocytic leukemia

Chuanbing Zang, Hongyu Liu, Maximilian G. Posch, Martin H. Fenner, Maries Waechter, Martin Ruthardt, Kurt Possinger, H. Phillip Koeffler, Elena Elstner

This study examined the expression and structural intactness of peroxisome proliferator activated receptor gamma (PPAR-gamma) in human acute lymphocytic leukemia (ALL) cells and determined the effect of PPAR-gamma ligands on growth and apoptosis of these cells. We noted that all lymphocytic leukemia cell lines expressed PPAR-gamma, and no PPAR-gamma mutations were found in these cell lines as indicated by SSCP analysis. Treatment of these cells with the PPAR-gamma ligands Pioglitazone (PGZ) and 15-deoxy-delta (12,14)-prostaglandin J2 resulted in growth inhibition in a dose-dependent manner. However, this effect appeared to be PPAR-gamma-independent since a PPARgamma antagonist could not reverse this effect. The inhibition of proliferation was associated with a G1 to S cell cycle arrest. This G1 blockade was associated with upregulation of the cyclin dependent kinase (CDK) inhibitor (CDKI) p27kip1 and downregulation of cyclin D1. Furthermore, five out of nine cell lines underwent apoptosis after culture with the PPAR-gamma ligands, as measured by both terminal deoxynucleotidyl-transferase-mediated UTP nick end labelling (TUNEL) assay and cell-death ELISA. This effect was partially caspase-dependent because a pan-caspase inhibitor partially reversed this effect. In order to know more details about the mechanisms underlying the PPAR-gamma ligand induced apoptosis, we performed microarray analysis of untreated and PGZ treated BV-173 cells by using Affymetrix U133A chips. Our results indicated that altogether 588 genes were upregulated and 286 were downregulated at least greater than 2 fold by PGZ as compared to untreated control. Among these genes 15 apoptotic related genes were detected suggesting the possible roles of these genes in the PGZ-induced apoptosis in human ALL cells. Verification of these results and further determination of the roles of these genes in human ALL are in progress in our laboratory. In conclusion, our results suggest that PPAR-gamma ligands may offer a new therapeutic approach to aid in the treatment of ALL.

Poster 19:

Cyclin A1 is induced by p53 following DNA damage and modulates DNA repair by interacting with Ku proteins

Carsten Müller-Tidow, Ji Ping, Sven Diederichs, Jenny Potratz, Wolfgang E. Berdel, Hubert Serve

Cyclin A1 is a second A-type cyclin that is overexpressed in acute myeloid leukemia. In a triple hybrid screen that used cyclin A1/cdk2 as a bait, the Ku70 repair protein was identified as a specific interacting protein. Cyclin A1/cdk2 interacted with Ku70 in vitro and in vivo and cyclin A1/cdk2 complexes phosphorylated Ku70 in vitro. Subsequent experiments demonstrated that cyclin A1-/- cells showed reduced DNA double strand break repair. Cyclin A1 activity in DNA repair was dose dependent, since higher levels of cyclin A1 reduced DNA repair. Further experiments elucidated that the physiologically tissue specific cyclin A1 showed widespread expression in mice following γ-irradiation. Cyclin A1 induction occurred on the transcriptional level and was mediated by p53. The p53 tumor suppressor activated the human cyclin A1 promoter by enhancing Sp1 activity at two GC boxes located at -90 to -110. Cyclin A1/cdk2 complexes interacted with p53 and phosphorylated p53 indicating a possible feedback loop. Taken together, cyclin A1/cdk2 modulate DNA double strand repair and possibly p53 function following γ-irradiation. These findings indicate a novel function for cyclin A1/cdk2 in DNA repair and possibly genomic stability.

Poster 20:

Treosulfan induced apoptosis in AML cells is accompanied by translocation of PKC delta and enhanced by Bryostatin-1

Ralf Schmidmaier, Oellerich M, Baumgart J, Emmerich B, Meinhardt G

Acute myeloid leukemia (AML) is a rapidly progressive disease which remains fatal in the majority of patients despite recent advances in the understanding of the underlying biology. Searching for new therapeutic options in the treatment of AML we evaluated the antileukemic effect of the alkylating agent treosulfan in AML cells by MTT assay, propidium iodide incorporation and annexin V binding assays. In U937 cells, 10 and 30 µM treosulfan reduced viability by more than 70 % and 90 %, respectively. Similar results were obtained in HL-60 and THP-1 cells. Furthermore, TUR cells, previously characterized as apoptosis- and differentiation-resistant subclone of U937 cells, demonstrated comparable sensitivity to treosulfan. Primary leukemia cells obtained from five consecutive patients with AML displayed similar sensitivity to treosulfan. The LC90 was approximately 100µM, which is several fold below the clinically achievable plasma levels. Treosulfan induced cell death was associated with cellular events indicating apoptosis such as breakdown of the mitochondrial transmembrane potential, the proteolytic activation of caspase 3 or the appearance of a subG1 DNA peak. As protein kinase C (PKC) is known to play a role in the regulation of diverse cellular functions such as apoptosis, several PKC modulators were evaluated in conjunction with treosulfan. In coculture experiments, synergistic antileukemic effects using the PKC activators bryostatin-1 and TPA were observed in all cell lines and patient samples tested. Treosulfan induced apoptosis was substantially reduced by coincubation with the PKC inhibitor GF109203X. Furthermore, long-term preincubation of U937 or TUR cells with PKC activators like bryostatin-1 or TPA, which are known to downregulate PKC protein levels, likewise inhibited treosulfan induced apoptosis. Immunoblots with cytosolic and membrane extracts revealed membrane translocation of PKC delta indicating activation of this enzyme upon treosulfan exposure. In conclusion, our data provide evidence for a strong antileukemic effect of treosulfan at clinically achievable dosis both in cell lines and AML blasts from patients. Furthermore, the proapoptotic effect of treosulfan is mediated at least in part by activation of PKC isoforms and can be augmented by co-incubation with bryostatin-1.

Multipotent stem cells

Poster 21:

Hair follicle dermal cells as a novel source of haematopoietic cells

Lyle Armstrong, Majlinda Lako, Nicholas Hole, Colin Jahoda

Skin and hair follicle stem cell biology is the focus of increasing interest, not least because the adult hair follicle has well defined dermal and epithelial populations that display distinct developmental properties. Recent evidence suggests that a number of adult cell populations have much broader stem cell capabilities than previously thought. To examine whether this applied to the hair follicle and with a view to developing the follicle as a stem cell model we investigated whether adult hair follicles were capable of demonstrating haematopoietic stem cell (HSC) activity. To investigate haematopoietic activity in hair follicles we first used in vitro haematopoietic colony assays. This demonstrated that rodent hair follicle end bulbs as well as micro dissected dermal papilla and dermal sheath cells actively produced cells of erythroid and myeloid lineages, but that follicle epithelial cells did not. As a more stringent test, we transplanted cultured dermal papilla or dermal sheath cells from transgenically marked donor mice into lethally irradiated recipient mice and observed multi-lineage haematopoietic reconstitution when assayed at intervals up to one year. Colony assays from bone marrow of primary recipients revealed that over 70 % of clonogenic precursors were derived from donor hair follicle cells. When bone marrow from primary mice was harvested and used to repopulate secondary myeloablated recipients, multi-lineage haematopoietic engraftment was observed. Our data shows that dermal but not epidermal compartments of the adult hair follicle have much broader stem cell activities than previously described. Although the treatment for many forms of blood disorder, such as leukaemia, often requires transplantation of haematopoietic stem cells (HSC), their availability can be rate limiting. Given its easy accessibility, our identification of the hair follicle as a source of extramedullary haematopoietic stem cell activity makes it an attractive potential source for blood stem cell therapeutics, and highlights its value as a model system in adult stem cell biology.

Poster 22:

ES-cell-derived erythroid progenitors: long-term mass culture and terminal differentiation in vitro and in vivo

Sebastian Carotta, Sandra Pilat, Andreas Mairhofer, Uwe Schmidt, Peter Steinleir, Hartmut Beug

In vitro hematopoietic differentiation of mouse embryonic stem cells has been demonstrated to be extremely useful for the analysis of early embryonic lethal mutations. Thus, several methods have been described for the generation and cultivation of distinct hematopoietic cell types from differentiating ES cells. However, a major obstacle to the subsequent analysis (e.g. gene expression analysis) of the desired cell type generated by these methods is the presence of contaminating cells of other lineages. We therefore developed a three-step strategy to expand pure, homogenous populations of erythroid progenitors from differentiating mouse ES cells (ES-EPs). Briefly, (1) ES cells were differentiated into embryoid bodies (EBs), differentiation then was stopped, EBs dissociated into single cell suspension and (2) cultivated in serum-free medium plus recombinant cytokines. A continuously growing erythroid culture was obtained which finally (3) could be induced to terminally differentiate into mature red blood cells in vitro. In this work we show that ES-EPs represent adult, definitive erythroid progenitors that have similar properties as fetal liver derived erythroblasts (FL-EPs), although the in vitro life span of ES-EPs is dramatically prolonged compared to FL-EPs. Due to the long life span, ES cell derived erythroid progenitors can be generated in virtually unlimited numbers, facilitating biochemical and molecular analysis. In addition, ES-EPs could be differentiated into mature, enucleated red blood cells, in vitro as well as after transplantation into mice, in vivo. Furthermore, ES-EPs did not lead to any signs of malignancy in mice within the observation period of one-year after transplantation. Using our system, we were able to analyse the embryonically lethal Flk-1-/- mutation in a fast and detailed manner in vitro. Moreover, this in vitro method may be also useful for generating human erythroid progenitors suitable for therapeutic purposes.

Poster 23:

Neuronal differentiation of mouse mesenchymal stem cells

Eric Eisermann, Stefan Linke, Udo Bartsch, Melitta Schachner, Gisbert Richard, Axel Zander, Claudia Lange

Since their first description by Friedenstein in 1968, mesenchymal stem cells (MSCs) have been demonstrated to display an unexpected potential to differentiate into a variety of different cell types. Today, in vitro differentiation of MSC into cells of adipo-, osteo- and chondrogenic lineages serves as a standard to characterize the multipotentiality of MSC cultured under defined conditions. Human and rat MSCs, but not mouse MSCs, have also been demonstrated to differentiate into neuronal cell types. We established the growth conditions for mouse MSCs by testing fetal calf sera from different sources. With the optimal serum mice MSCs could be propagated for more than 20 passages. FACS-analysis of cultured mouse MSCs from passage 17 revealed expression of Sca-1, CD90, CD59 and CD105 but not CD34, CD117 and CD45. In standard differentiation assays mouse MSCs showed the potential to differentiate into adipocytes, osteoblasts and chondroblasts.
Additionally, MSCs differentiated into cells with a neuronal phenotype after induction with dimethylsulfoxid and butylhydroxyanisol (Woodbury et al., 2000). Already 4 hours after induction the majority of cells displayed a neuronal morphology and a faint positivity for ß-tubulin III and neurofilament. Induction with isobutylmethylxanthine (modified from Deng et al., 2001) led to a neuronal morphology of less than 1 % of cells after 24 hours. These cells were strongly positive for ß-tubulin III and neurofilament, and could be maintained for at least 30 days in culture. Taken together we demonstrated differentiation of mouse MSCs into cells with neuronal morphology and expression of neuronal markers. We are currently investigating the fate of mouse MSCs after transplantation into neural tissues in vivo.

Poster 24:

Isolation of Brachyury expressing cells from in vitro differentiating ES cell cultures using a gene\"knock-in\" approach: towards the identification of the elusive (pre-)hemangioblast

Hans Jörg Fehling, Georges Lacaud, Atsushi Kubo, Marion Kennedy, Scott Robertson, Gordon Keller, Valerie Kouskoff

The hematopoietic and endothelial lineages derive from mesoderm and are thought to develop through the maturation of a common progenitor, a cell known as the hemangioblast. A progenitor with characteristic of the hemangioblast, referred to as the blast colony-forming cell (BL-CFC) and representing the earliest stages of hematopoietic and endothelial development has been identified in embryoid bodies (EBs) generated from embryonic stem (ES) cells differentiated in culture. To further investigate the developmental processes that lead to the formation of this bi-potential progenitor, we generated an ES cell line with the green fluorescent protein (GFP) cDNA targeted to the Brachyury locus allowing us to track the formation of mesoderm and its commitment to the hematopoietic program. Expression analysis and cell sorting studies demonstrated that mesodermal populations could be easily and accurately identified and isolated on the basis of GFP expression following ES cell differentiation. Co-expression of GFP with the receptor tyrosine kinase Flk-1 during a time course of ES cell differentiation revealed the emergence of 3 distinct cell populations, GFP-Flk-1-, GFP+Flk-1- and GFP+Flk-1+, the latter of which contained the BL-CFC. Further characterization of these populations supports the interpretation that they represent a developmental progression ranging from the pre-mesoderm (GFP-Flk-1-) stage to pre-hemangioblast mesoderm (GFP+Flk-1-) to the hemangioblast (GFP+Flk-1+).

Poster 25:

Myogenic differentiation of rat mesenchymal stem cells

Kay Jaquet, Korff Krause, Axel R. Zander, Claudia Lange

The cardiac muscle lacks the ability to regenerate after ischemic injury. However, bone marrow derived cells have been found to migrate into injured heart tissue and adopt myogenic characteristics. Mesenchymal stem cells (MSCs) are a candidate population of adult stem cells with differentiation properties to repair ischemic heart tissue. We generated rat MSCs and characterized their antigen profile and in vitro differentiation potential. Rat MSCs grown for >20 passages are positive for CD59 and CD90 and negative for CD45. They can be induced to differentiate into adipocytes, osteo- and chondroblasts. The same results were found in non-clonal and clonal rat MSCs. Additionally, clonal GFP-transduced rat MSCs develop a myogenic phenotype when treated with 5-azacytidine. The number of rMSCs expressing smooth muscle actin (SMA) could be enhanced up to 85 % compared to less than 10 % in non-stimulated controls. To a lesser extend, (40 %) stimulated rMSCs were positive for troponin T as well. Other myogenic markers (alpha-actinin, alpha-SR-1, desmin) as well as the endothelial cell specific mAb CD31 stained negative.

Conclusions: 1. In-vitro stimulation of rMSCs induces a myogenic phenotype in up to 85 % of the cells; 2. Spontaneously contracting myocytes could not be generated via azacytidine treatment in-vitro. Currently, clonal GFP-transduced rat MSCs are transplanted into rat heart myocardium to investigate their differentiation capacity in-vivo.

Poster 26:

Gene expression profile of human mesenchymal stem cells in comparison to skin fibroblasts employing cDNA microarray analysis of 9600 genes

Larissa Kuklick, Oliver Hartmann, Dagmar Schwell, Ulrich Boudriot, Markus Ritter, Andreas Neubauer, Cornelia Brendel

Broad differentiation capacity has been described for mesenchymal stem cells (MSC) from human bone marrow previously. We were interested to identify genes responsible for the immature state and pluripotency of this cell type. Using the cDNA microarray technique we analyzed expression of 9200 genes in MSC hybridized versus skin fibrocytes, which are the mature counterparts. We compared the expression profile of MSC cDNA obtained from bone marrow of four healthy donors with cDNA from one primary human skin cell line as control. The identity of all relevant genes was confirmed by direct sequencing and representative genes array expression was compared to quantitative PCR analysis. About 80 genes were expressed differently more than three-fold in MSC compared to mature skin fibroblasts. The undifferentiated human MSC were found to express a number of genes important for embryogenesis such as distal-less homeobox 5, elastin and LIM protein. Some mesenchymal lineage genes were down-regulated in undifferentiated MSC in comparison to skin cells. Interestingly, some genes involved in differentiation processes, like secreted frizzled-related protein 4 and inhibitor of DNA binding 3 are up-regulated in MSC as well. In order to prove that the MSC, we generated in vitro, have pluripotent plasticity, they were differentiated into osteoblasts, adipocytes and chondrocytes in vitro. In contrast, the skin cells did not harbour these differentiation abilities. We conclude that cDNA microarray analysis is a useful technique that can help to understand gene regulation processes in stem cell maintenance as well as induction of differentiation.

Poster 27:

Liver stem cells from bone marrow: liver-specific gene expression in CD34-positive human hematopoietic stem cells during long-term culture

Michael V. Lioznov, Henning C. Fiegel, Claudia Lange, Boris Fehse, Dietrich Kluth, Axel R. Zander

Background: The potential of adult bone marrow stem cells to differentiate into non-hematopoietic tissues (e.g. endothelial cells, skeletal muscle, neuronal cell types) was revealed in the past years by several groups. Liver cells originating from bone marrow were first observed in patients after bone marrow transplantation for hematological disorders. Animal studies revealed that hepatocytes and liver stem cells (oval cells) might originate from bone marrow under certain conditions. Therefore, in this study we investigated the potential of sorted CD34-positive adult human bone marrow stem cells (CD34+) to differentiate under the specified culture conditions into hepatic-lineage cells in vitro.

Methods: Bone marrow was harvested from healthy donors for allogeneic bone marrow transplantation. CD34+ bone marrow cells were enriched by positive magnetic cell sorting under the control of fluorescence activated cell sorting analysis. Cells were cultured in a defined medium containing hepatocyte growth factor, stem cell factor, 10 % fetal bovine serum, 10 % horse serum, Insulin and Dexamethason. Cells were seeded on collagen-I-matrix at a high density ($2x10^6$ cells/ml), and were observed for 35 days. Expression of albumin and cytokeratin19 (CK-19) mRNA was assessed by RT-PCR.

Results: Cell number of CD34-positive cells increased during the culture period, whereas CD34-negative cells (CD34-) diminished. Cultured CD34+ cells became positive for albumin mRNA or CK-19 mRNA after day 28 of culture, as detected by RT-PCR. Cultures of CD34- bone marrow cells showed no signal for albumin-mRNA at all time points.

Conclusions: Our in vitro data indicate for the first time, that human hematopoietic stem cells may be potential liver stem cells. Therefore, the use of such cells for new cell-based therapies for liver diseases is worth studying. Further studies must define the in vivo potential and conditions for differentiation of bone marrow into liver.

Poster 28:

Targeted insertion of a Cre-inducible Red Fluorescent Protein (RFP) into the ROSA26 gene locus of ES cells

Herve Luche, Odile Weber, Roger Tsien, Hans-Jörg Fehling

We are currently generating a new reporter mouse strain in which a Red Fluorescent Protein (RFP) is expressed from the ubiquitously transcribed ROSA26 gene locus following Cre-mediated excision of a transcriptional "stopper\" element. These mice will be useful for studies involving cell fate mapping as well as for monitoring the expression of Cre transgenes in combination with additional fluorescent marker genes such as EGFP, EYFP or ECFP. In line with reports from other laboratories, all our previous attempts to generate targeted ES cells expressing commercially available variants of RFP have failed, possibly due to toxicity of the respective RFP proteins in ES cells. Thanks to a novel dimeric RFP variant (tdRFP) engineered in R. Tsien's laboratory (San Diego), we now succeeded in isolating several ES clones exhibiting strong red fluorescence with an unprecedented spectral separation from cell autofluorescence. This is the first report of a red fluorescent protein being stably expressed in ES cells. Generation of the corresponding mouse line is underway.

Poster 29:

Molecular analysis of AGM derived stromal cells

Kenji Nagao, Takayuki Ohta, Satoru Mizutani, Toshio Heike, Tatsutoshi Nakahata, Mitsuo Nishikawa

The microenvironment of the aorta-gonad-mesonephros (AGM) region plays an important role in the hematopoietic development and the hematopoietic stem cell (HSC) self-renewal in the early embryo. We have established a stromal cell line, AGM-S3, from the AGM region of 10.5 dpc mouse embryo. AGM-S3 has prominent effect on HSCs, (i) the ability to maintain HSC activity for long term, (ii) AGM-S3 could generate definitive HSCs from cells derived from yolk sac or paraaortic splanchnopleures when cocultured with AGM-S3 cells, (iii) HSCs self-renew on AGM-S3 in the presence of cytokines, and it is considered that molecular analysis of AGM-S3 provides us better understanding of hematopoiesis supportive niche. We obtained subclones, S3-A9 and S3-A7, a hematopoiesis supportive cell line and non-supportive one from AGM-S3 respectively. We have undertaken a comprehensive molecular screen to identify candidate molecules that can support maintain HSCs based on the hypothesis that such molecules are expressed higher in S3-A9 than in S3-A7. We have analyzed cDNA libraries of S3-A9 and S3-A7 by a unique gene profiling technique for differential expression, Sequencing By Hybridization (SBH) method and identified 274 genes that are preferentially expressed in S3-A9. S3-A9 expresses SCF, SDF-1 alpha and other molecules that are implicated in the regulation of stem cells. However they do not confer the HSC supportive activity to non-supportive stromal cells. Among the other genes we found a molecule, #2728, a type I transmembrane protein. In the presence of some cytokines stromal cells expressed #2728 maintain HSCs able to competitively repopulate the bone marrow of lethally irradiated mice efficiently. It suggests that #2728 might be involved in sustaining the microenvironment capable of supporting HSCs.

Poster 30:

Functional analysis of stromal cell lines derived from non-adherent fraction of long-term bone marrow cultures of TNF knockout mice.

Irina N. Nifontova, Nina J. Drize

Mesenchymal stem cells forming adherent stromal cell lines were revealed in suspension fraction (SF) of 70+ weeks old long-term bone marrow culture (LTBMC) of TNF knockout mice. The proportion of CD45+ cells in such old cultures decreased significantly, while the cellularity of SF increased that was accompanied the first successful generation of cell lines. Seventy-five cell lines from SF of LTBMC of different age were established. The clonal efficiency of SF cells was low (14 %) and at least 100 cells per well of 96-well plate were needed for successful cloning. The efficiency of cell lines establishment increased up to 100 % in the media supplemented with dexamethasone, ITS and ascorbic acid. Cell lines from the culture with unusually high percent of CD45+cells were generated only after addition of 10 % mix of mediums conditioned with WEHI-3B and L929. All lines were split 1:2, 1:3 15-40 times. There was an aneuploidy detected (37-79 chromosomes) in most of the cell lines. Almost all of them have fibroblast-like morphology and express collagens type II and IV; none of tested express PECAM-1, vWFVIII and other markers of endothelial cells. Both non-irradiated and 40 Gy irradiated cell lines could maintain hematopoiesis (CFU-S, CFU-GM and CAFC production) for 10 weeks. Some of the cell lines were able to maintain growth without differentiation of murine ES cells. All cell lines produce hematopoietic growth factors – their supernatants stimulate growth of CFU-GM in semisolid media. RT-PCR analysis revealed that the most lines tested express SDF1 and c-kit. The expression level of LIF and LIF-receptor in some lines was essentially higher than in fresh bone marrow cells. The data suggests that stromal cell lines of mesenchymal stem cells origin capable to maintain multilineage hematopoiesis and ES cells in non-differentiated status could be revealed in SF of LTBMC from TNF knockout mice after 70 weeks of culture.

Poster 31:

Mobilization of hematopoietic stem cells (HSC) prior to induction of ischemic acute renal failure (ARF) in mice does not improve but significantly worsens outcome

Florian Tögel, Zhuma Hu, Robert L. Baranowski, and Christof Westenfelder.

Clinical ARF from all causes remains a common complication associated with high, and largely unchanged morbidity and mortality. Effective new therapies for its prevention and treatment are needed. HSCs are recognized to possess a high level of plasiticity, enabling their transdifferentiation into a large number of distinct cell types. Normally, the number of HSCs in the circulation is extremely low. It was recently reported [Orlic, PNAS 2001] that mobilization of HSCs with Stem Cell Factor (SCF) and Granulocyte-Colony Stimulating Factor (G-CSF) prior to the induction of experimental myocardial infarction in mice resulted in improved function and tissue repair, beneficial effects suggested to be mediated by mobilized HSCs. The aim of the present study was, therefore, to test whether HSC mobilization (Cytoxan 200 mg/kg, followed by G-CSF, 125 µg/kg b. wt., given in two daily doses x 4 days) prior to the induction of ARF (60 min bilateral renal pedicle clamp on day 6 after cytoxan or vehicle) in FVB mice would improve outcome. Control groups consisted of animals (1) with ARF without HSC mobilization, (2) subjected to sham surgery, (3) subjected to HSC mobilization only, and (4) rendered neutropenic prior to induction of ARF. ARF in control group (1) caused a rise in serum creatinine (SCr) to 1.6 mg/dL on day 2 and 55 % mortality on day 3. The peak of circulating HSCs following mobilization, assessed by colony assay (CFU-C), occurred on day 6, and white cell counts increased significantly to 70,000/µl with a shift from lymphocyte-predominant to granulocyte-predominant pattern. HSC mobilization prior to induction of ARF caused a significantly greater, vs. control group (1), increase in SCr levels to 3.4 mg/dL on day 2, and mortality of 83 % on day 3. Renal function in the other control groups (2) and (3) remained unchanged and there was no mortality. Renal histology in the ARF/HSC-mobilized animals showed massive infiltrates of granulocytes and a few ED1 positive macrophages, surrounding primarily necrotic S3 segments, and severe medullary congestion. Neutropenic ARF animals, control group (4), developed a modest but more prolonged rise in SCr, a mortality of 8 %, and severe histological damage. Conclusion: HSC mobilization-associated leukocytosis greatly aggravates the severity of ARF, while neutropenia delays recovery in ARF. Strategies that increase the delivery of HSCs without associated leukocytosis will have to be tested as to their renoprotective potential in ARF.

Hematopoiesis

Poster 32:

CD133 and priming of hematopoietic stem cells by cellular interactions

Sandra Gottschling, Volker Eckstein, Katrin Miesala, Michael Punzel and Anthony D. Ho

Summary: Interactions between stem cells and their microenvironment play an essential role in determining their differentiation pathway. The exact mechanism of this process is poorly understood. CD133, a stem cell marker with still unknown function, is thought to play a role in cell contact mediated cell determination, particularly because of its preferential localization on the tips of podia. We therefore investigated CD133+ and CD133- cells for podia formation, adhesion kinetics and hematopoietic potency in dependence of cellular interactions.

Methods: CD34+/133+ and CD34+/133- cells from cord blood and G-CSF mobilized peripheral blood were isolated by Ficoll-Histopaque Centrifugation, MACS enrichment and FACS analysis. Cells were stained with the fluorescent dye PKH-26 and seeded in fibronectin- or BSA-coated cell culture plates in LTBMC medium +/- 10 ng/ml SCF and SDF for 48 h. 200 Cells were scored for podia formation by fluorescence microscopy each 12h. For cellular adhesion 100 000 CD133+ cells were allowed to adhere 30min. to12h to the stem cell supporting murine stromal cell line AFT024. Non adherent cells were removed by four standardized washes. Adherent cells were obtained by brief trypsinization. The number of adherent/non adherent cells was determined related to an input control by FACS analysis using 100.000 PKH-26 stained U266 cells instead of beads. CFC was performed using standard protocol. Briefly cells were allowed to adhere for 4h to AFT024 and a murine neuronal feeder layer. The non adherent fraction was removed by four standardized washes. The adherent fraction was overlayed with colonogenic methycellulose and scored after 10 days for the presence of colonies.

Results: During the whole observation period CD133+ cells showed a 18 % more frequent podia formation than CD133- cells. Podia formation occurred after 2h and remained stable for 48h. The podia formation was independent of used culture plate coating and culture medium. Morphological differences of podia types between both populations did not occur. Because of better podia formation further experiments were firstly performed with CD133+ cells. CD133+ cells showed an adhesion of 16 % ± 10 % after 30min. and 41 % ± 13 % after 2h that remained unaltered for 12h. If CD133+ cells were allowed to adhere to different feeder layers (stromal, neuronal), the adherent fractions showed no difference in their ability to form colonies.

Discussion: Our results show an advantage of CD133+ expressing cells in podia formation. This corresponds with observations of D. Corbeil et al., who found that CD133 is a component of cell membrane domains that contribute to podia formation. We further could show that optimal adhesion of CD133+ cells to AFT024 occurs after 2h and remains stable for up to 12h. Interestingly the hematopoietic potency of optimal adherent CD133+ cells seems to be independent of the type of feeder layer used. Additional experiments with different feeder layers and conditions are necessary.

Poster 33:

Essential role of an -14 kb upstream regulatory element for PU.1 gene regulation and hematopoiesis in vivo

Frank Rosenbauer, Katharina Wagner, Pu Zhang, Steven Fiering, and Daniel G. Tenen

The Ets transcription factor PU.1 is a master regulator in hematopoiesis. Mice with a null-mutation of the PU.1 gene die shortly after birth, and lack mature myeloid cells and B-lymphocytes. We have previously identified 3 clusters of DNaseI hypersensitive (HS) sites within the murine PU.1 locus. While the proximal PU.1 promoter alone failed to drive a reporter gene in mice, the combination of the -14kb HS (cluster 1) plus the promoter is sufficient for lineage specific transgenic gene expression. Here, we have analyzed the endogenous role of the -14kb HS using homologous recombination in mice and transgenic rescue approaches. -14kb HS-/-neo mice (HS replaced by neomycin) were viable but developed severe hematopoietic defects due to a 70 % downregulation of the PU.1 gene expression. In contrast, -14kb HS+/+neo mice (neomycin directly upstream of HS) are normal, thus an effect of the neomycin resistance cassette is very unlikely in these animals. The deletion of the -14kb HS led to a 3 to 5-fold increase in c-kit+ progenitor cells, and an almost completely blocked macrophage and B-lymphocyte development. Most interestingly, the granulocyte numbers were profoundly increased over time, to > 65 % in the spleen. Next, we show that transgenic PU.1 expression driven from the -14kb HS/promoter cassette was sufficient to rescue up to 15 % of the myeloid compartment in PU.1 null-mice, but did not effect B-cell development. Together, we present evidence for an essential role of the -14kb HS in the regulation of the PU.1 expression and normal hematopoiesis in vivo. In addition, the -14kb HS-/-neo \"PU.1 knock down\" mice are an important model to study the in vivo function of PU.1 in adult hematopoiesis.

Poster 34:

The role of Wnt/beta-catenin signaling in hematopoietic differentiation

Lara Tickenbrock, Carsten Müller-Tidow, Hubert Serve

The Wnt-signalling pathway regulates processes in embryonic development, cell migration, cell polarity and cell proliferation. It also plays an important role in the development of many different human cancers. One general characteristic feature of these functions is the regulation of cell fate decisions, namely the balance between differentiating, proliferating and survival signals. Tight regulation of cytosolic levels of the oncoprotein beta-catenin determines the activity of this pathway. beta-catenin stability is mainly regulated by phosphorylation through the serin/threonine kinase GSK3ß, which targets the protein to proteasomal degradation. Activation of the Wnt signalling cascade inhibits the activity of GSK3ß and thus increases cytosolic beta-catenin levels. The protein then enters the nucleus and acts as a transcriptional coactivator of TCF/LEF-family members. These transcription factors induce Wnt-target genes, for example c-myc or cyclin D1. In previous work, we found that AML-associated translocation products may induce the Wnt-signaling pathway. So far, only very preliminary data on the functional role of Wnt in hematopoiesis are available.

Here, we analyzed the effects of the Wnt/beta-catenin signaling pathway on the differentiation of AML cell lines. To activate the Wnt/beta-catenin pathway, we incubated the cells in LiCl that inhibits GSK3ß activity and thus specifically induces beta-catenin stability. Western blot analyses revealed that LiCl readily induced cytosolic levels of beta-catenin in the cytosol of the AML cell lines NB4, HL60 and U937. Whereas beta-catenin was translocated into the nucleus of NB4 and HL60, no increased levels of beta-catenin could be found in U937 nuclei after LiCl treatment.

Treatment of NB4 cells with combinations of ATRA and LiCl revealed synergistic actions of the two agents on granulocytic differentiation. Before incubation with ATRA for up to 48 hrs, cells were pretreated with or without LiCl for 24 hrs. The degree of differentiation was analyzed by immunofluorescence for granulocytic markers. LiCl markedly increased the sensitivity of NB4 cells to ATRA treatment. At lower concentrations of LiCl (5mM and 10mM), combined with ATRA doses of 10^{-7} M of 10^{-8} M of ATRA led to a higher percentage of granulocytes after 24 hrs. At higher doses of LiCl (20mM), even very low concentrations of ATRA (10^{-9} M) resulted in an increased number of differentiated cells after 48 hrs in comparison to the control cells treated with ATRA only. Immunoblot analysis also revealed, that increasing beta-catenin levels by the treatment of LiCl resulted in an increased RARalpha protein level. Taken together, activation of the Wnt/beta-catenin pathway sensitizes APL blasts to ATRA treatment. This might be by the result of direct induction of its receptor RARalpha. The importance of the Wnt/beta-catenin signaling pathway in other hematopoietic differentiation models is subject to further studies that will be presented.

Knowledge about the precise role of the Wnt-signaling pathway during hematopoietic differentiation could help to therapeutically target its function in order to overcome leukemia-induced block in myeloid differentiation.

Poster 35:

Telomerase activity in serially transplanted bone marrow cells from thymectomized mice

Tamara V. Todria, Axel Zander

Purpose: In order to investigate whether telomerase might play a role in the maintain of donor-derived bone marrow (BM) cell division capacity in vivo, we analysed telomerase activity (TA) in BM cells from normal and adult thymectomized (A.Tx) mice in term of steady state hematopoiesis and during serial transplantation. Comparative analysis with cell cycle status was done also.

Objectives: BM from neonatally and A.Tx mice has an impaired radioprotective effect upon inoculation into lethally irradiated recipients due to decrease of HSC proliferative potential.

Methods: Four weeks old female mice were thymectomized and 12 months later they were used as a donor of BM cells for reconstitution of lethally irradiated recipients. BM from primary recipients were used for obtaining the secondary hosts 9 months later. Normal mice were the same age as A.Tx mice. BM from primary and secondary radiation chimeras was investigated 9 and 12 months after restoration of hematopoiesis, accordingly. TA was determined by PCR-ELISA method. Cell cycle status for primary recipients was analysed by flow cytometry on FACScan.

Results: Low level of TA (2–5 %) was shown in BM of normal mice in steady state hematopoiesis and for both primary and secondary recipients reconstituted with the same BM. Age-dependent correlation of TA was not observed. However, telomerase is expressed much more in mice after physiological stress (after thymectomy). It was observed 3 fold higher TA from A.Tx donors (12 mo mice) and in 9 mo chimeras, restored with A.Tx BM. There was a remarkable decrease of TA (less than 1 %) in donor-derived cells after second round of transplantation. The fraction of cycling cells in BM from A.Tx donor mice was slightly decreased as compared to normal mice. Cycling activity of transplanted BM pool during hematopoietic reconstitution in primary recipient mice was significantly increased in A.Tx donor-derived cells.

Conclusion: Together, these data suggest that under steady-state conditions (natural ageing) mouse haemopoietic cells are expressed telomerase over their life and the rate of TA is not strong associated with thymus function activity. However, the alteration of telomerase dynamics – its activation, are detected in BM cells with a nearly complete block in T cell development and in the donor-deriver cells from primary hosts restored with A.Tx mice BM. The dramatic reduction of telomerase expression on one hand and increase cycling activity of donor-derived cells from A.Tx mice after second round restoration on the other suggests a diminished repair mechanism for the long-term replicative capacity of BM cells. These new findings provide suggestion of unexpected effect of the influence of T-cell depletion in the telomerase regulation in mouse haemopoietic cells.

Poster 36:

Rescue of c-Kit-Null lethality by overexpression of erythropoietin.

Claudia Waskow, Hans-Reimer Rodewald

The receptor tyrosine kinase c-Kit and the erythropoietin receptor (EpoR) are crucial for definitive erythropoiesis. Outside erythropoiesis, c-Kit is expressed on hematopoietic progenitor cells for the lymphoid and myeloid lineage including stem cells. C-Kit also plays a role in the development of melanocytes, germ cells and interstitial cells of Cajal leading to white spotting (W-allele) sterility and intestinal disorders in mice carrying mutations in c-Kit. Analysis of adult c-Kit-Null mice (c-KitW/W) has been precluded by postnatal lethality presumably owing to severe anemia. Recently we obtained a first viable c-Kit-Null mouse by out-crossing WB-mice, the strain the W mutation is maintained on, to mice carrying genomic material from other mouse strains. While the molecular rescue mechanism in these mutants is not known, we noticed an improved hematocrit in Vickid mice strongly suggesting that, in fact, the anemia caused death of c-KitW/W mice. To further test this hypothesis, we introduced an erythropoietin (Epo) transgene into lethal c-KitW/W mice.
Interestingly, this strategy yielded the second viable c-KitW/W mouse line, termed Wepo (W rescued by Epo). Wepo mice still suffer from severe anemia and define the lowest level of red blood cell numbers compatible with adult life. Wepo mice may now serve to study the role of Epo in erythropoiesis in the presence and absence of c-Kit signaling in vivo.

Poster 37:

MDS and secondary leukemias in patients with congenital neutropenias

Cornelia Zeidler, Beate Schwinzer, G. Pracht, Karl Welte
for the European Branch of the SCNIR

The term congenital neutropenia (CN) has been used for a group of hematological disorders characterized by severe neutropenia with absolute neutrophil counts (ANC) below 0.5 x 10^9/L associated with increased susceptibility to bacterial infections. This group of diseases includes primary bone marrow failure syndromes with isolated neutropenias, and neutropenias associated with metabolic or immunological disorders or with a complex syndrome. To avoid confusion, we prefer using the term CN only for the most severe disorder among this group: Severe congenital neutropenia characterized by an early stage maturation arrest of myelopoiesis leading to bacterial infections from early infancy. This disease has originally been described as Kostmann syndrome with an autosomal recessive inheritance. However, recent pathogenetic investigations demonstrated that this clinical phenotype includes also autosomal dominant and sporadic cases. The underlying genetic defect is still unknown, but in a subgroup of patients different point mutations in the neutrophil elastase gene were reported. Long-term application of recombinant human granulocyte-colony stimulating factor (rHuG-CSF) is first choice treatment, whereas hematopoietic stem cell transplantation is still the only available option for patients refractory to rHuG-CSF treatment or leukemic transformation. Adverse events include mild splenomegaly, moderate thrombocytopenia, and osteoporosis. Malignant transformation into secondary MDS or leukemia in about 11 % of patients occurs only in the congenital neutropenia subtype compared to cyclic or idiopathic neutropenias. The development of additional genetic aberrations, e.g. G-CSF-receptor gene mutations, or monosomy 7, indicates an underlying genetic instability leading to an increased risk of malignant transformation. If and how rHuG-CSF treatment impacts on these adverse events remains unclear since there are no historical controls for comparison. In Europe 24 out of 346 patients with congenital neutropenia patients (7 %) with malignant transformation were reported, 4 patients were diagnosed with Shwachman-Diamond syndrome (4/67 SDS patients = 6 %), the other 20 patients belong to the unspecified group of CN (20/213 = 9,4 %). 17 of the 24 patients received hematopoietic stem cell transplantation. Of these 17 patients 7 patients (41 %) have been alive at the last contact.

Poster 38:

PA model system of in vitro osteoclast differentiation and function

Martin Fenner, P. Pelt, B. Ebert, K. Possinger and E. Elstner

Bone metastasis is a prominent feature of many solid tumors including breast and prostate cancer. The bone destruction seen in breast cancer is mediated mainly by osteoclast activation rather than the direct effect of tumor cells. Receptor activator of NF-Kb ligand (RANKL) and macrophage colony stimulating factor (M-CSF) are sufficient to differentiate monocytes from peripheral blood into functional osteoclasts. Here we describe a model system where we study osteoclast differentiation and function in vitro using human monocytes and the mouse cell line RAW 264.7. Osteoclast differentiation can be detected by the appearance of multinuclear TRAP-positive cells in the cell culture and by quantitative RT-PCR for the osteoclast-specific markers Acp5, Ctsk and Atp6i. RNA Microarray analysis can be used to study the gene expression profile of these cells systematically. Osteoclast function is measured by von Kossa staining of cells grown on dentin slices or artificial bone matrix. This model system can be used to study the effects of pharmacological substances on osteoclasts and for coculture experiments with breast cancer cells.

Clinical bone marrow transplantation

Poster 39:

Regulatory polymorphism of MMP-1, PAI-1 and MDR-1 may influence the clinical outcomes in hematopoietic stem cell transplantation

Alexei B. Chukhlovin, Tatyana S. Zabelina, Ludmilla S. Zoubarovskaya, K. V. Bogdanov, Nicolaus Kröger, Boris V. Afanasiev, Axel R. Zander, Boris Fehse.

Since last decade, a variety of common mutations was discovered that affect regulatory regions of physiologically active genes, thus causing altered synthesis of specific gene products. Several workers have shown some correlations between the cytokine allele variants and frequency of acute graft-versus-host disease (aGvHD) in allo-HSCT.

Objectives: The aim of present study was to assess the possible role of some "non-immune" genes in the development of aGvHD and other potentially fatal HSCT complications. Patients and methods. The study involved 140 consecutive oncohematological patients and their donors (71, familial and 69, unrelated HSCTs) observed at the BMT clinics of Hamburg and St.Petersburg. Regulatory variants of plasminogen activator inhibitor-1 (PAI-1-675 5G/4G variants), interstitial collagenase (matrix metalloproteinase, MMP-1 -16071G/2G alleles), multi-drug resistance glycoprotein-1 (MDR-1 C3435T mutation) were studied using the original allele-specific DNA PCR techniques.

Results: The studied allele pairs were in genetic equilibrium for the patients and control group. The frequency of PAI-1 promoter variants was similar among donors and recipients. Among 127 donor-recipient pairs, MMP-1 and PAI-1-based chimerism detection was feasible in 28% and 32% of cases, respectively. Single-step DNA-PCR was shown to be sensitive for gene markers assayed (detection of >0.1% recipient cells), thus allowing detection of recipient leukocyte DNA in the range of 0.1 to 10%. Meanwhile, the frequency of highly inducible MMP-1-1607 (2G/2G) genotype was significantly reduced in patients with aGvHD as compared to GvHD-free patients, whereas presence of MMP-1 2G/2G in donor cells did correlate with higher incidence of aGvHD ($p=0.02$). A combination of recipient 1G allele with donor 2G allele was significantly associated with increased aGvHD risk ($p<0.008$). Some interrelations between the MMP-1 genotype and severity of aGvHD were also analyzed. Grade I-II aGvHD was observed only in the patients with homo- or heterozygotes for 1G allele, whereas 2G/2G homozygosity tends to be a protective factor ($p=0.07$). Such interrelations were more pronounced in recipients who underwent non-myeloablative HSCT. Only one case of aGvHD (grade I) was detected among 2G/2G homozygotes (14%), as compared to 18/31 (58%) in the group of 1G carriers ($p=0.03$). Hyperinducible 2G/2G genotype of MMP-1 also tended to correlate with early posttransplant death caused, mainly, by fatal infections and/or severe aGvHD ($p=0.07$). Preliminary results of MDR-1 genotyping (C3435T polymorphism) were performed in a limited group of 38 patient/donor pairs, showing that the C variant (more active wild-type allele) in the patients tended to associate with post-transplant cytomegalovirus (CMV) infection ($r=0.25$; $p=0.06$). Likewise, the presence of hyperactive PAI-1 4G allele of donor origin does also correlate with CMV infection ($r=0.29$; $p=0.03$).

Conclusion: The 2G allele of MMP-1 gene is known to be highly inducible, thus, probably, providing higher MMP-1 production and increased invasive capacity of the cells carrying a hyperactive allele. DNA PCR of the regulatory PAI-1, MMP-1 and MDR-1 allelotypes allows to detect donor chimerism in ca. 50% of cases. In general, these results confirm an important role of donor/recipient discrepancy in MMP-1 gene alleles with respect to HSCT complications, aGvHD in first line. Probable involvement of

Poster 40:

Purification and genotyping of Langerhan's cells from skin biopsies for assessment of post-transplant chimaerism

Matthew Collin, Georgina Clark, Anne Dickinson, Derek Hart

In a transplant patient, antigen-presenting cells (APC) of recipient origin are gradually replaced by cells derived from the donor. It is anticipated that this transition has fundamental effects on graft versus host disease, graft versus leukaemia effects and immune reconstitution. Previous studies using immunofluorescence on tissue sections have indicated that host antigen-presenting cells persist for some time after transplantation. However, access to these cells is difficult and there is insufficient information relating to the effect of conditioning on APC, their rate of repopulation by donor cells and the relevance of both of these to GvHD risk, curative potential of transplant and immunocompetence. An improved method of genotyping tissue APC to determine post-transplant chimaerism is required to address this problem.

We have developed strategies to examine small numbers of LC present in clinical skin biopsies (4mm punch biopsy). Two techniques have been developed, both taking advantage of the migratory capacity of LC to achieve enrichment of the cells. A 4mm biopsy of skin contains approximately 8,000 LC. When an epidermal sheet is prepared, 85–95 % of these cells migrate from the skin over 48 hours and can be collected in the fluid phase of tissue culture. This population is 30–40 % LC with contaminating keratinocytes. Subsequent purification to >95 % can be achieved by FACsorting using a direct single tube/no wash method. This provides a small number (500–1000 cells) suitable for PCR/STR analysis of genotype. An alternative strategy has also been developed in which the migrated population of cells is adhered to a cytospin and probed with XY FISH for sex chromosome analysis. This method is suitable for analysis of sex-mismatched transplant patients and also serves to verify the first technique.

These methods are superior to immunofluorescence analysis of tissue sections as they are quicker and able to examine many more cells, whilst still using small biopsy specimens from patients. Recruitment to a clinical study to examine the role of LC chimaerism in GvHD is now underway.

Poster 41:

The absolute lymphocyte count (ALC) 1 month after unrelated hematopoietic stem cell transplantation as prognostic factor for clinical outcome

Vladimir Vavilov, Tatyana Zabelina, Nicolaus Kröger, Boris Fehse, Natalya Fehse, Helmut Renges, Alexander Pugachev, Natalya Michaylova, Boris Afanasyev, Axel Zander

The speed of ALC recovery after autologous and allogeneic haematopoietic stem cell transplantation (HSCT) is known as an important prognostic factor, which allows predicting of outcome for patients with different hematological and non-hematological malignancies.
We analyzed 163 adult patients who received allogeneic HSCT from unrelated donor (168 transplants) in BMT Centers of Hamburg (Germany) and St. Petersburg (Russia) in 1993 – 2003. 73 of them had CML, 44 - acute leukemia, 18 - multiple myeloma, 14 - MDS, and 13 - other hematological malignancies. Median follow-up was 211 days (21-2593). We took a cutoff of ALC 500 cells/µl at about 4 weeks after transplantation (mean point of study – 29 days, range 20-43).
The mean lymphocyte level on day 29 post transplant was 457 cells/µl (range 0-1980 cells). Superior overall and progression free survival by those of patients who reached ALC 500 cells/µl or more 1 month after transplantation were observed ($p<0.005$ and $p<0.01$, respectively). The estimated 4-years overall and disease-free survival was 66,8 % and 67,2 % for patients with rapid lymphocyte reconstitution versus 41,6 % and 32,6 % for patients with delayed lymphocyte reconstitution respectively. The mean ALC was also significantly higher in patients with reduced conditioning regimen intensity versus sequential regimen (576 cells/µl versus 404 cells/µl, $p=0.01$). ALC for patients who received myeloablative preparative regimen with lower ATG dose (45 mg/kg) versus 90 mg/kg ATG were 636 cells/µl versus 309 cells/µl with p-value of <0.0005. The comparing of lymphocyte reconstitution of patients depending on their CMV-seropositivity shows the significantly higher ALC in CMV-positive (anti-CMV IgG+) versus CMV-negative (anti-CMV IgG-) patients: 521 cells/µl versus 371 cells/µl, $p<0.05$. Absolute lymphocyte count at 4 weeks after unrelated allo-HSCT is an important prognostic factor affected with preparative regimen intensity, dose of ATG and CMV-status. The evaluation of ALC on day +28 allows prediction of the clinical outcome after transplantation.

Poster 42:

Evaluation of cytokine gene expression: a potentially new approach to prediction and monitoring of post-HSCT complications

Mikhail Zaraiski, Natalya Mikhaylova, Natalya Ivanova, Ludmilla Zubarovskaya, Boris Afanasiev.

Background: Development of major complications of allogenic HSCT – acute graft-versus-host-disease (aGvHD), immune suppression and infections – are very closely dependent on the activities of immune system. For this reason, a crucial role of certain cytokines in posttransplant immune disorders cannot be underestimated.

General purpose: Evaluation of IL-1beta, -2, -6, -10 mRNA expression levels as predictive markers for post-HSCT complications.

Materials and methods: We observed a group of thirteen hematological patients undergoing allogeneic unrelated HSCT. Total RNA was extracted by phenol-chloroform protocol from peripheral blood leukocytes, bone marrow cells, or skin bioptates (in aGvHD) before HSCT and at different terms (up to d+50) during post-HSCT period. RT-PCR-based protocols were performed for each interleukin, using sequence-specific primer sets. The relative quantities of electrophoresis-separated PCR products that corresponded to specific cytokine mRNA have been assessed by Gel-Pro Analyzer 3.1 software.

Results: IL-1beta, -2, -6, -10 mRNAs were not detectable for, at least, 7–10 days post-HSCT, independent of conditioning regimens applied. Recovery of cytokine mRNA production was observed with increase in peripheral leukocyte number. In general, elevated contents of IL-1beta, -2 and -6 mRNAs (5–7 times as compared to pre-HSCT levels) did significantly correlate with aGvHD occurrence and returned to basal levels upon immunosuppressive therapy (especially, with glucocorticoids). Interestingly, increased levels of IL-2, -6 and -10 correlated with emergence of viral infections (i.e., CMV and HSV type 1, 2). Upregulated cytokine gene expression was registered 3 to 5 days before the clinical manifestations of abovementioned complications.

Conclusions: Our preliminary findings indicate that monitoring of in vivo cytokine gene expression during post-HSCT period could be proposed as a new diagnostic approach to predict the early HSCT complications and, in particular, aGvHD.

Poster 43:

A comparison of early complications after allogeneic hematopoietic stem cell transplantation in children and adults.

Ludmilla S. Zubarovskaya, Ganapiev A.A., Golostchapov O.V., Boichenko E.G., Vitrischak A.A., Vladovskaya M.D., Pugachov A.A., Afanasyev B.V.

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is a treatment of choice for different hematological and oncological diseases in children and adults with long-term disease-free survival (DFS) of patients depending on the underlying disease and transplantation modalities.
This investigation analyzed data on 65 patients (age < 21, 44 pts vs. age > 21, 21 pts) who had undergone related or unrelated allo-HSCT between 1992 and 2002 with poor prognosis acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), myelodisplastic syndrome (MDS) and chronic myelogenous leukemia (CML) to evaluate impact of early complications on long-term DFS. Characteristics of the age < 21 years old patients (pts): AML- 12 (1 CR- 3, 2 CR - 7, relapse -2), MDS- 1, ALL- 23 (1 CR - 1, 2 CR - 10, 3-5 CR - 6, relapse/PIF - 6), CML-8 (1 CP- 5 , 2 CP-1, AP- 1 , BC - 1) and age > 21 years old pts: AML - 5 (1 CR- 2, 2 CR - 1, relapse-2), MDS- 2, ALL- 6 (1 CR - 1, 2 CR - 1, 3-5 CR - 2, relapse - 2), CML - 8 (1 CP-3, 2-3 CP-2, AP-2, BC -1). Cytogenetic assay was shown the following abnormalities: Ph (+) - 6 pts, 5q - 1 pts, 7q-1 pts, multiple -1 pts, inv-16 -1 pts. The main conditioning regimens were used by BuCy or BuCyVP-16. Prophylaxis of acute GVHD was performed by CSA + Mtx ± Prednison. Sourses of stem cells were BM for 36 pts vs PBSC for 28 pts.

Results: Ten-years overall survival (OS) of age < 21 years old pts with CML was 50,0 %, AML- 44,0 %, ALL- 23,0 % after related allo-HSCT. Our first experience after unrelated allo-HSCT revealed 1,5 years OS- 60% in age < 21 years old pts. The main early complications after allo-HSCT in pts with age < 21 vs. age > 21 were: TRM- 8 % vs. 15 %, infection - 32 % vs 18 %, acute GVHD - 32 % vs 48 %, VOD - 2 % vs 3 %, hemorrhagic cystitis 7 % vs. 0 %, non-engraftment - 2 % vs. 3 %. Unrelated allo-HSCT was increased the risk of developing CMV-infection (p=0,001), hemorrhagic cystitis (p=0,001) and brain complications (p=0,03) in age < 21 years old pts. Acute GVHD was one of the main reason of early death after allo-HSCT: in 18,5 % pts < 21 years old vs 35 % pts > 21 years old. Ten-years OS in age < 21 years old pts was 50 % without signs of acute GVHD, 40 % in pts with I-II stage and 2-years OS was 22 % in pts with III-IV stage. Univariate analysis revealed that CMV-infection was associated with increased risk of developing acute GVHD III-IV stage (RR=3,0, 95 %CI, 1,05-8,6, p=0,015) whereas acute GVHD III-IV stage increased risk of developing hemorrhagic cystitis (RR=7,7, 95 %CI, 2,75-16,1, p=0,023) after unrelated HSCT in age < 21 years old pts.
Information on CD34+ cell dose was available for 18 of the 44 pts < 21 years old. The median CD 34+ cell dose was 6,97 x 10^6/kg for BM and 12,62 x 10^6/kg for PBSC. The number of transplanted nuclear cells was correlated with CD34+ cells (p=0,033) and recovery of leukocytes (p=0,007) after allogeneic HSCT.
Thus, allo-HSCT is the treatment of choice for some patients with poor prognosis hematological diseases. The main early complications that put impact on long-term DFS after allo-HSCT are infection and acute GVHD.

Immunology/Immunobiology/ Cell biology

Poster 44:

Immunoglobulin gene conversion requires the activation induced deaminase (AID) gene.

Hiroshi Arakawa, Jessica Hauschild, Jean-Marie Buerstedde

Three phenotypically distinct processes – somatic hypermutation, gene conversion and switch recombination – remodel the functionally rearranged immunoglobulin (Ig) loci in B cells. Somatic hypermutation and switch recombination have recently been shown to depend on the activation induced deaminase (AID) gene product. Here we show that the disruption of the AID gene in the chicken B cell line DT40 completely blocks Ig gene conversion and that this block can be complemented by reintroduction of the AID cDNA. This demonstrates that the AID master gene controls all B cell specific modifications of vertebrate Ig genes.

Poster 45:

Novel stress-inducible gene Hi95 participates in defense against reactive oxygen species

Andrei Budanov[1], Anna Sablina[1,3], Ivan Glukhov[2], Peter M.Chumakov[1,2]

[1]Lerner Research Institute, The Cleveland Clinic Foundation, Cleveland, USA; [2]Engelhardt Institute of Molecular Biology, Moscow, Russia; [3]Cancer Research Center, Moscow, Russia.

Recently we identified the novel gene Hi95 that shows transcriptional upregulation in response to various stresses including prolonged hypoxia and DNA-damage. The gene belongs to poorly characterized sestrin family that includes p53-regulated PA26 gene. Transcriptional upregulation of the Hi95 gene is mediated through p53-dependent and p53-independent mechanisms. We found that subjection of cells to treatments known to increase intracellular ROS (hydrogen peroxide, UV irradiation etc.) results rapid translocation of Hi95 protein from cytoplasm to the nucleus. Inhibition of endogenous Hi95 gene expression by siRNA resulted significant increase in intracellular ROS levels monitored by DCF staining. Quite opposite, hyperexpression of the Hi95 protein using recombinant constructs resulted inhibition of ROS to almost undetectable levels. Inhibition of the Hi95 gene expression by siRNA in human primary fibroblasts WI38 resulted premature senescence visualized by staining to SA-beta-galactozidase and inhibition of cell divisions. We conclude that the Hi95 gene plays important role in regulation of intracellular ROS.

Poster 46:

The role of the inositol-5'-phosphatase SHIP for signaltransduction in hematopoietic cells

Lizet García-Palma, Anja Metzner, Stefan Horn, Elmar Endel, Wiebke Wegner, Michael V. Lioznov, Boris Fehse, Sonja Loges, Walter Fiedler, Thomas Streichert, Georg W. Mayr, Manfred Jücker

SHIP is a negative regulator of signaltransduction pathways in hematopoietic cells. The restoration of SHIP expression in a human leukemia cell line without detectable SHIP expression leads to the prolongation of the G1-Phase of the cell cycle and consequently to a partial inhibition of the proliferation of these cells. Microarray analyses were used to identify genes that are regulated by SHIP. They revealed a differential expression of 40 out of 33,000 (0.12 %) genes that are at least two fold up- or down-regulated after the induced expression of SHIP.
Our data implicate SHIP in the regulation of cell cycle progression and suggest that loss of SHIP expression in leukemia cells is associated with increased proliferation.

Poster 47:

TAG7/TAG-L –Mouse homologues of PGRP family in flies are more than peptidoglycan recognition proteins

Sergei Kiselev, Kibardin A., Mirkina I.

A novel family of the innate immunity recognition molecules was recently described for Drosophila and mammals. T phage lysozyme homology domain (also known as PGRP domain) allocated on the C-terminal part of the proteins was demonstrated to recognize peptidoglycan. Another characteristic feature of this family is a diversity of alternatively spliced mRNAs encoding for proteins with limited homology. Using mouse alternatively spliced forms of the tag-L gene (mouse homologue of Drosophila PGRP-L gene) we have demonstrated that T phage lysozyme domain is not essential for bacteria recognition, but bacteria recognition in all cases occurs via C-terminal portion of the proteins. Even more, all isoforms were able to recognize both Gram positive and negative bacteria. Using different cellular models we have demonstrated that mouse Tag-L proteins could be not only membrane bound but also secreted. Mouse tag7 gene (homologue of Drosophila PGRP-S), the first gene of the family described, has also a number of alternatively spliced forms, which encode for the same protein, but differentially regulated. We have also demonstrated that recombinant mouse Tag7 protein could induce chemotaxis of human monocytes.
Therefore, the spectrum of activities of the mouse Tag7/Tag-L proteins is wider as it was demonstrated for their Drosophila orthologues and suggests for them cytokine function in mammals.

Poster 48:

A role for G-CSF in functional differentiation of G-CSF receptor expressing T-cells

Wenji Piao, Jörg Lauber, Wiebke Hansen, Angela Schmitt-Thomsen, Bernd Hertenstein, Jan Buer, Arnold Ganser, Anke Franzke

Results from experimental models, in vitro studies, and clinical data indicate that G-CSF stimulation alters T-cell function and induces Th2 immune responses. The immune modulatory effect of G-CSF on T-cells results in an unexpected low incidence of acute graft-versus-host disease in peripheral stem cell transplantation. However, the underlying mechanism for the reduced (allo-) reactivity of T-cells upon G-CSF treatment is still unknown. In contrast to the general belief that G-CSF acts exclusively on T-cells via monocytes and dendritic cells, our results clearly show the expression of the G-CSF receptor in class I- and II-restricted T-cells at the single cell level both in vivo and in vitro. Kinetic studies demonstrate the induction and functional activity of the G-CSF receptor in T-cells upon G-CSF exposure. Expression profiling of T-cells from G-CSF treated stem cell donors allowed identification of several immune modulatory genes, which are regulated upon G-CSF administration in vivo (e.g., LFA1-a, ISGF3-g), and which are likely responsible for the reduced (allo-) reactivity. Most importantly, the induction of GATA-3, the master transcription factor for a Th2 immune response, could be demonstrated in T-cells upon G-CSF treatment in vivo accompanied by an increase of spontaneous interleukin-4 secretion. Hence, G-CSF is a strong immune regulator of T-cells and a promising therapeutic tool in acute graft-versus-host disease as well as in conditions associated with Th1/Th2 imbalance, such as bone marrow failure syndromes and autoimmune diseases.

Poster 49:

Artifical antigen presenting cells for tolerance induction in BMT

J. Ulrich, F. Spillmann, Birgit Vogt, Ahmed Sheriff

GvHD in BMT can be reduced using recipient-derived immature or costimulation reduced dendritic cells (DC) and donor derived T cells. Regulatory T cells produced by this method prevent GvHD, while still preserving GvL reactions. However, this setting bears the risk of recipient-derived dendritic cells presenting leukaemic antigen in a tolerogenic setting and thereby tolerizing the donor T cells to these antigens. Therefore, we used an alternative strategy by creating artificial antigen presenting cells (aAPC). Immature APC are characterised by the expression of MHCII molecules and few costimulatory molecules whereas in mature activated APC expression of costimulatory molecules is upregulated. In our system, we generated aAPCs by inducing expression from the MHCII locus. This was achieved by transfecting CIITA into fibroblasts using the C3H derived L929 cell line as a model. Coculture of this aAPC with allogenic Balb/C T cells lead to reduced T cell proliferation after second encounter with C3H cells (compared to T cells cultured on DC). We could also show the generation of CD25+ CTLA4 expressing T cells, which are assumed to be of a regulatory phenotype.

Gene therapy

Poster 50:
Lentiviral-based vectors for gene silencing

S. Chumakov, V. Orlova

RNA-interference represents a powerful method for gene silencing. However, introduction of small interfering RNA by direct transfection suffers numerous limitations and is prone to artifacts related to transfection itself. An alternative approach involves ectopic expression of small hairpin RNA by means of recombinant constructs. We designed and tested several lentiviral constructs carrying RNAi-expressing cassettes from RNA-polymerase III specific promotor of H1RNA. The cassette was placed to either within 3'-LTR, or between two LTR's of the lentiviral constructs. The titers of lentiviral stocks allowed infection of 100 % of cells in culture even without concentration of the virus. The degree of inhibition of several target genes monitored by Western and Northern blotting was more then 95 %. To optimize the layout of the hairpin RNAi transcript we tested different lengths of sequences complementary to mRNA (from 19 to 29 nucleotides). In general, there was no significant influence of the RNAi length on the efficiency of inhibition. In addition, we compared several types of loops separating self-complementary sequences in the small hairpin transcript. The size of the loop between 8 and 16 nucleotides did not affect the efficiency of inhibition, however certain sequences within the loop contributed to the effect. The developed lentiviral RNAi-delivery vectors have been tested in a number of different cell lines with reproducible success. The potentials of this system for gene therapy applications will be discussed.

Poster 51:

Life-long persistance of non-integrated lentiviral vector

Maxim Ershler, Alexey Terskikh, Nina Drize, Irina Nifontova, Irving Weissman, Joseph Chertkov

We analyzed the clonal progeny of lentivirally-marked hematopoietic stem cells (HSC) over the lifetime of a mouse. Lethally irradiated females were reconstituted with 300 and 3000 of lentivirally transduced male double sorted (Lin-Sca1+c-kit+Thy-1.1low) hematopoietic stem cells (HSC). The self-inactivating (SIN) HIV-based vector (p156RRLsinPPTCMVGFPPRE) was used. The multilineage reconstitution was confirmed in peripheral blood by flow cytometry at 2 month after the transplantation. Seven to 16 months later bone marrow from reconstituted mice was injected into secondary recipients to obtain individual spleen colonies (CFU-S). Southern blot analysis based integration study revealed genomic integration of lentivirus in 8 % of 203 total donor CFU-S-derived colonies; while every donor-derived colony from 12 studied mice contained apparently non-integrated circular lentivirus-derived DNA at 7 months after reconstitution. The presence of suggested episomal circular DNA was verified by two independent approaches: identification of viral-derived sequences in the episomal fraction of DNA and the inverse PCR technique. The proportion of colonies with episomal DNA decreased to 63±16 % and 7 % at 12–16 month after transplantation for mice reconstituted with 3000 and 300 cells respectively, suggesting that episomes slowly disappear proportionally to mitotic history of HSC. Nevertheless we identified the lentivirus-derived episomes in the bone marrow of secondary recipient as well. Southern blot analysis revealed lentivirus-derived sequences in hematopoietic tissues (peripheral blood, bone marrow, thymus), but not in any of non-hematopoietic tissues (liver, heart, kidney, lung, etc.). Flow cytometry analysis suggested that episomal circles are able to express transgene (GFP). The results suggest that episomal circular DNA can be amplified in hematopoietic cells by yet to be identified mechanism. Long term persistence of circular form of vector with capacity to express genes of interest could be helpful for gene therapy. On the other hand, in spite of our inability to reveal horizontal transfer of the vector, uncontrolled amplification is potentially hazardous and such vectors must be studied carefully.

Poster 52:

Ectopically expressed HOXB4 perturbs hematopoietic differentiation of mouse bone marrow cells in a dose dependent manner in vivo

Hannes Klump, Bernd Schiedlmeier, Elke Will, Ute Modlich, Sebastian Carotta, Sabine Knöß, Gökhan Arman-Kalcek, Wolfram Ostertag, Christopher Baum.

HOXB4 is an attractive candidate gene for use in gene therapy of the hematopoietic system due to its capability to confer a selective growth advantage to hematopoietic stem cells in vitro and in vivo. Nevertheless, conflicting reports exist on the question whether ectopically expressed HOXB4 influences differentiation of expanding stem cells. Although it has been reported that HOXB4 does not negatively influence stem cell differentiation of transduced stem cells, we have recently described that HOXB4 can lead to the inhibition of differentiation of human CD34+ cord blood cells (SchiedImeier et al., 2003).
In this work we asked, whether the described activities of HOXB4 correlate with its amounts expressed in vitro and in vivo. For this purpose, we transduced primary bone marrow cells of mice using different retroviral vectors which mediate distinct expression levels. To follow HOXB4 expression easily in transduced cells during the experiments, especially after transplantation into mice, in vivo, we coexpressed eGFP and an N-terminal HA-tagged HOXB4 using the 2A esterase of foot-and-mouth disease virus, which directs cotranslational separation of both proteins. In vitro, HOXB4 led to a selective growth advantage of the transduced (eGFP positive) cells, irrespective of the expression levels tested. Nevertheless, after transplantation into irradiated recipient mice, the repopulation efficiency of transduced bone marrow cells was severely reduced when ectopic HOXB4 was expressed in larger amounts. However, within the engrafted transduced cell population, a similar distribution of lineages was observed, independent of the expression level of HOXB4: the numbers of early progenitor cells (c-kit/Sca1+) were significantly increased and differentiation of myeloid (CD11b/Gr1+), erythroid (Ter119+) and B-cell (B220+) progenitors was perturbed in all recipient mice expressing HOXB4 ectopically in the bone marrow. Our observations suggest that increasing the amounts of HOXB4 gradually shifts the hematopoietic steady-state towards self-renewal of stem- and progenitor cells at the cost of differentiation.

Poster 53:

A novel “sort-suicide” fusion gene vector for adoptive immunotherapy

Olga S. Kustikova[1,2], Zhixiong Li[2,3], Anke Wahlers[1,2], Wolfgang Bohn[4], Winfried R. Beyer[2], David Chalmers[5], Pierre Tiberghien[5], Klaus Kühlcke[6], Axel R. Zander[1], Christopher Baum[2,3], Boris Fehse[1]

[1]Bone Marrow Transplantation, University Hospital Hamburg-Eppendorf;
[2]Dept. of Cell & Virus Genetics and [4]Dept. of Tumor Virology, Heinrich-Pette-Institute Hamburg;
[3]Experimental Cell Therapy, Dept. of Hematology & Oncology, Medical School Hannover, Germany;
[5]INSERM E-0119/UPRES EA-2284, Etablissement Français du Sang-Bourgogne/ Franche-Comté, Besançon, France;
[6]EUFETS AG Idar-Oberstein, Germany

Background/Objective: Adoptive immunotherapy using suicide gene-modified allogeneic T lymphocytes is a promising strategy to control graft-versus-host disease (GvHD) while conserving graft-versus-leukemia (GvL) and graft-versus-infection (GvI) effects. However, clinical as well as preclinical studies have revealed several problems that need to be resolved before broad application of this approach will be achieved, in particular impaired T cell function as a consequence of long ex vivo culture, and insufficient elimination of *Herpes simplex virus* thymidine kinase (HSVtk)-transduced T cells after ganciclovir administration due to the presence of inactive splice variants.

Methods: To address these problems, we have developed new retroviral “sort-suicide” vectors co-expressing truncated CD34, a gene transfer marker which ensures rapid enrichment of transduced cells using commercially available GMP-approved devices, and a splice-corrected variant of the suicide gene (scHSVtk) which confers high sensitivity to the prodrug ganciclovir. Different co-expression strategies were explored based on the retroviral backbone of MP71 hybrid vectors containing elements of the myeloproliferative sarcoma virus (MPSV) and the murine embryonic stem cell virus (MESV).

Results/Conclusions: We show that one of our vectors, which encodes a tCD34/scHSVtk fusion protein, mediates strong expression of the “sort-suicide” selection marker thereby allowing for highly efficient purification and selective elimination of transduced cells, including primary T lymphocytes. We therefore suggest that this new vector may be of use within the concept of adoptive immunotherapy with suicide gene-modified allogeneic T lymphocytes.

Poster 54:

Humanized Ouabain resistance selectable marker gene (OuaR)

Alexandra Treschow, Alar Aints, Sirac Dilber

Today, there is not a flawlessly suitable selectable marker gene for ex vivo cell selection of gene-modified cells for clinical application. The ouabain resistant selection marker gene (OuaR) described previously (Aints et al. Hum Gene Ther 2002) allows for rapid and efficient selection of human cells. However, the rat origin of this construct provides a non-neglectable risk of the marker gene becoming immunogenic in clinical settings. Therefore, our aim is to develop a ouabain resistant selectable marker gene with low risk of immunogenicity. The human homologue of the rat Na+,K+ ATPase alpha 1 gene displays significantly lower resistance to ouabain, a cardiac glycoside which binds to and inhibits the enzyme. The strategy for our approach is to modify the human Na+,K+ ATPase alpha 1 gene as to gain increased ouabain resistance property while demonstrating minimal or no immunogenicity when expressed in human cells in vivo. To date, the specific amino acids generating the difference in ouabain resistance between the rat and human homologues have not been identified. Hence, we prepared a set of human-rat chimeric Na+,K+ ATPase alpha 1 genes by PCR cloning to locate these critical residues. Ouabain sensitive HeLa and COS-7 cells were transiently transfected with EGFP plasmids C1and C3 carrying these constructs fused in frame with EGFP. These cells were further screened for resistance to 10mM ouabain. Our results showed that a gene construct, more than 99 % human, with substitutions of the first 10 of the 40 residue dissimilarities in the 1024 amino acid sequence made HeLa and COS-7 cells resistant to ouabain induced cell death. In fact, the difference in sensitivity to ouabain between wild type and this mutated form allows for efficient selection of human cells carrying the mutated construct with ouabain. This construct could serve as a selectable marker gene for in vitro gene-modification and selection of human CD34+ cells in order to increase engraftment rate in the bone marrow in situations where myeloablative conditioning is inappropriate. This would be the case in particular for treatment of genetic diseases. Immunotherapy may come to play an important role in replacing, repairing or enhancing immune functions in a variety of diseases and conditions. If the addition of a transgene is desired in the adoptive cells, the OuaR selectable marker could provide a safe and feasible system to rapidly obtain pure populations of gene-modified cells.