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## Editorial: June 2004

*In this issue of our Journal we are very pleased to announce the winner of the 2004 Pezcoller Foundation AACR International Award for Cancer Research.*
*The 2004 International Scientific Selection Committee, who met in Philadelphia on December 12th 2003, had to examine more than 20 nominees from many states in America, Europe, Asia and Africa.*

*The members of the Committee were the following:*
*Chairperson: Susan Band Horwitz, Albert Einstein College of Medicine, Bronx, NY, USA.*
*Members: Fedrick R. Appelbaum, Fred Hutchinson Cancer Research Center, Seattle WA, USA Riccardo Dalla Favera, Institute for Cancer Genetics, New York, USA. Hans H.Grunicke, Institut fur Med. Chemie und Biochemie, Innsbruck, Austria. Carl-Henrik Heldin, Ludwig Institute for Cancer Research, Uppsala, Sweden. Alberto Mantovani, Istituto Mario Negri, Milano, Italy. Alex Matter, Novartis Pharma AG, Basel, Switzerland. Lynn M. Matrisian, Vanderbilt School of Medicine, Nashville, USA.*
*Ex-officio members: Margaret Foti, Gios Bernardi.*

*2004 Pezcoller Foundation - AACR International Award for Cancer Research*
*Dr. Bernardi and Dr. Korsmeyer*

*The choice was not an easy one due to the high level of all the candidates.*

*Stanley J. Korsmeyer, from the Dana-Faber Cancer Institute of Boston, USA, was unanimously voted as the winner and was immediately endorsed by the AACR and Pezcoller Foundation Boards.*

*Dr. Korsmeyer received a commemorative plaque at the AACR meeting in Orlando in March, where he gave an impressive lecture. A further ceremony with the awarding of the Prize was held in the prestigious hall of Buonconsiglio Castle in Trento on May 7th, after which Stanley Korsmeyer was honoured by a long standing ovation.*

*The award motivation is: "for his pioneering observations that opened the molecular era of programmed cell death. His landmark experiments on the cells of patients with lymphoma established that BCL-2 plays a primary role in oncogenesis by blocking cell death. As a result, BCL-2 became the archetype of a new category of oncogenes: regulator of cell death. In the last two years, he has further advanced our understanding by identifying distinct roles for BCL-2 members. His rigorous analysis established a mammalian apoptotic pathway and placed multiple landmarks along its course. His outstanding contributions in defining the role of genetic mechanisms that govern programmed cell death and survival have encouraged clinical studies that have already had a significant impact on the development of tailor-made treatment of lymphomas and other cancer. It is truly rare for the research of a single investigator to have such wide impact and in particular such far-reaching applications that have affected so many disciplines in biology and medicine. His insights have altered our fundamental concepts of organ development, cellular homeostasis, and genesis and the treatment of cancer".*

*The Pezcoller Foundation is extremely proud to have added Stanley Korsmeyer to the list of the prestigious winners of the Pezcoller Foundation – AACR International Award for Cancer Research.*

*May we take this opportunity to draw your attention to the next Pezcoller Symposium entitled 'Stem Cells and Epigenesis in Cancer', to be held in Trento from 10th to 12th June 2004.*
*In this edition of our Journal you can also read the abstracts of the oral presentations and posters to be presented during this year's Symposium.*

*The call for the 2005 Pezcoller Foundation AACR International Award for Cancer Research have also been inserted towards the end of this copy.*

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# ABSTRACTS OF ORAL PRESENTATIONS

## Achieving blood engraftment from differentiated ES cells

***George Q. Daley***, *Division of Pediatric Hematology/Oncology, Children's Hospital, Boston, and Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School.*

*Bone marrow Hematopoietic Stem Cells (HSCs) are widely employed to treat genetic and malignant disease and are important targets for gene therapy, and yet success has been illusory because of the challenge of maintaining HSCs in culture, the intrinsic difficulty of expressing genes in HSCs, and the risk of insertional mutagenesis with viral vectors. Harnessing Embryonic Stem (ES) cells as a source of HSCs would facilitate genetic modification of stem cell populations precisely by homologous recombination, enable basic investigation into genetic and epigenetic influences on hematopoietic cell function, and empower pre-clinical models for gene and cellular therapy. Furthermore, if pluripotent ES lines can be generated by nuclear transfer, then truly isogenic autologous tissues could be created, obviating the need for post-transplant immune suppression and solving the donor shortage.*

*Nearly twenty years have passed since the first report of in vitro differentiation of ES cells into blood cells, yet achieving hematopoietic reconstitution of irradiated mice from ES cells has proven challenging. This difficulty has been ascribed to the fact that embryoid bodies (EBs) recapitulate the yolk sac stages of embryogenesis. Yolk sac blood progenitors are a distinct embryonic population that have limited lymphoid potential and may not persist into adulthood. It is unclear whether EBs support a microenvironment equivalent to the intra-embryonic para-aortic regions from which definitive HSCs emerge. Our studies have been aimed at defining the nature of blood progenitors that derive from ES cells when acted upon by transgenes that have been implicated in blood development or leukemogenesis. We have accumulated extensive data for the derivation of HSCs that support definitive, long-term lymphoid-myeloid engraftment of irradiated primary and secondary mice, and have defined this cell at the clonal level by retroviral marking. Using our methods, we have begun to model therapeutic transplantation strategies using ES cells to treat murine models of genetic disease, work aimed at establishing the feasibility and efficacy of ES-derived blood cell transplantation.*

*We began our investigations by asking whether a HSC could be identified clonally in differentiating cultures of ES cells. We dissociated EBs and infected with a retrovirus carrying the CML-associated BCR/ABL oncogene, noting that BCR/ABL transforms bone marrow HSCs without hindering their lymphoid-myeloid differentiation. Indeed, BCR/ABL enabled us to culture and clone lines of hematopoietic blast-like cells that formed primitive erythroid cells in vitro, and reconstituted definitive lymphoid-myeloid-erythroid hematopoiesis in irradiated mice (Perlingeiro et al., Development, 2001). This confirmed the previous demonstration by Keller and colleagues of a common progenitor for primitive and definitive hematopoiesis in ES culture, while extending the differentiation potential of these cells to include the lymphoid lineage. BCR/ABL-engrafted mice ultimately succumbed to leukemia. We therefore tested whether ectopic expression of STAT5, a target of BCR/ABL signaling, might enable normal blood engraftment. STAT5 stimulated normal hematopoietic proliferation from ES cells and enabled lymphoid-myeloid engraftment in*

*irradiated mice, though only transiently (Kyba et al., PNAS, 2003). Prompted by data that hoxb4 was expressed in bone marrow HSCs but not in yolk sac, and by studies from Humphries, Sauvageau and colleagues that showed enhanced reconstitution potential of hoxb4-transduced HSCs, we also tested hoxb4. We expressed hoxb4 in ES cells, cultured hematopoietic blasts on OP9 stroma, transplanted irradiated syngeneic mice, and observed long-term lymphoid-myeloid hematopoiesis in primary and secondary recipients, thereby demonstrating definitive HSC production from ES cells (Kyba et al., Cell, 2002). Together with Rudolf Jaenisch's laboratory, we used ES cells to model therapeutic HSC transplantation to treat genetic immune deficiency by therapeutic cloning. We performed nuclear transfer from tail-tip fibroblasts of the Rag2-/- mouse to produce cloned embryos, and then harvested ES lines that were isogenic to the donor mouse (ntESRag2-/-). We then repaired one of the two defective Rag2 alleles by homologous recombination to generate the ntESRag2+/- line. Using tetraploid complementation, we then generated cloned mice from the ntESRag2+/- lines, and showed that these mice had apparently intact immune systems, with normal levels of B and T cells and polyclonal IgH and TcR gene rearrangement. We then asked whether we could repopulate Rag2-/- mice with HSCs derived in vitro from the ntESRag2+/- line. Using genetic modification with a hoxb4 retrovirus, we engrafted Rag2-deficient mice and demonstrated regeneration of B and T cells, IgH and TcR gene rearrangement, and production of IgM, IgG, and IgA immunoglobulin in serum (Rideout et al., Cell, 2002).*

*In these early experiments, lymphoid reconstitution was modest, perhaps owing to the primitive nature of the ES derivatives or to skewing of HSC differentiation by the actions of hoxb4. This limitation has now been largely overcome owing to recent work performed in collaboration with Leonard Zon's group on the cdx4 homeobox gene, which acts upstream of hox genes to promote blood formation in the zebrafish and the mouse (Davidson et al., Nature 2003). Activation of the cdx4/hoxb4 pathway in murine ES cells enhances blood development, promotes high degrees of hematopoietic chimerism in irradiated animals, and shows stable patterns of lymphoid-myeloid reconstitution (Wang et al., unpublished). Southern analysis of purified myeloid and lymphoid populations of engrafted primary and secondary mice shows multiple common integration sites and oligoclonal reconstitution. We are currently testing two models to account for the capacity of cdx4/hoxb4 to promote blood engraftment from ES cells: 1) genetic modification induces yolk sac-like progenitors to adopt definitive HSC fate; or 2) the EB microenvironment inefficiently supports definitive HSC formation, and cdx4/hoxb4 serves to expand the frequency of these rare cells. Identification of the morphogens that act upstream of the cdx-hox pathway may enable HSC production from ES cells without the need for transgene activation.*

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## Induction of mesoderm and endoderm from embryonic stem (ES) cells

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*The ability of embryonic stem (ES) cells to generate diverse cell types in cultures offers outstanding opportunities to study the molecular events governing lineage specification as well as a source of cell populations and tissues for transplantation for cell based therapy. For this potential of the ES cell system to be realized, however, it is essential to first understand the processes that lead to the induction of the three primary germ layers, ectoderm, mesoderm and endoderm, that ultimately form the various tissues of the body. To begin to address these questions, we targeted the green fluorescent protein (GFP) cDNA to the mesodermal gene, brachyury (bry) (1). Using these ES cells (GFP-Bry), it is possible to track the formation of mesoderm and isolate mesodermal cells by sorting GFP+ cells from the differentiation cultures. With this approach, we have shown that mesoderm that generates hematopoietic and vascular cells is distinct from mesoderm that gives rise to the cardiac lineage. This is an important observation as it demonstrates that cell fates are established early in development. In addition, it clearly shows that isolation of appropriate staged cells will provide access to large numbers of relatively pure progenitors of the hematopoietic/vascular and cardiac lineages. Together with the expected mesoderm derivatives, we have also shown that endoderm derivatives, including hepatocyte-like cells, lung cells and gut tissue also develop from a brachyury+ progenitor (2). These findings suggest that both mesoderm and endoderm may develop from a common mesendoderm progenitor and that the initial events involved in the induction of these lineages is regulated by the same molecules. Further specification to mesoderm and endoderm is likely mediated by distinct factors or sets of factors. Current experiments are aimed at defining these regulators as well as evaluating the function of the mesoderm- and endoderm-derived cell populations.*

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## Molecular Circuitry of Stem Cell Self-Renewal

***Austin Smith***, *Ian Chambers, Luciano Conti, Jenny Nichols, Steven Pollard and Qi-Long Ying*
*Centre Development in Stem Cell Biology*
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*Stem cells are characterised by dual capacities for expansion or differentiation. They are the foundations for generation, maintenance and repair of many mammalian tissues and organs. Consequently, ability to direct multiplication of stem cells and to programme their differentiation is likely to create novel opportunities for regenerative medicine and for biopharmaceutical discovery. On the other hand, dysregulation of stem cells or inappropriate acquisition of stem cell properties by more differentiated cells may be causative for many cancers. Identifying and targeting tumourigenic stem cells may therefore be crucial for effective cancer therapies.*

*Our laboratory seeks to elucidate the molecular machinery of stem cell self-renewal, the process whereby a stem cell suppresses differentiation during a given round of cell division but retains that potential for future divisions[1]. We are studying this process in pluripotent embryonic stem (ES) cells capable of producing all three germ layers, and in lineage-restricted neural stem (NS) cells that can form neurons or glia but not other germ layer types. These two stem cell types have extensive multiplicative capacity in vitro by virtue of symmetrical self-renewal divisions. Our long-term aim is to describe*

*the molecular networks that determine self-renewal versus differentiation choice in these two different stem cell types and in related cancerous stem cells.*

*ES cell identity is governed by intrinsic determinants, notably the POU transcription factor Oct-4[2] and the divergent homeodomain protein Nanog[3,4]. Genetic deletion of either of these components results in a loss of pluripotent character and differentiation into extraembryonic fates. Overexpression of Nanog, but not Oct-4, is sufficient to sustain ES cell self-renewal[3,5]. However, in the absence of genetic manipulation, extrinsic stimuli are essential for ES cell propagation.*

*Mouse ES cells are maintained in the self-renewal cycle by stimulation with a combination of the cytokine leukaemia inhibitory factor (LIF) and either serum or bone morphogenetic protein (BMP). LIF acts via the transcription factor STAT3[6,7] but the critical downstream targets have yet to be defined. The key contribution of serum or BMP is to induce expression of the ID class of negative regulators that block activity of basic helix-loop-helix (bHLH) and other transcription factors[5]. Neither LIF nor BMP appear to directly regulate expression of Oct-4 or Nanog.*

*Using LIF plus BMP it is possible to propagate ES cells without supporting stromal cells in media containing transferrin, albumin and insulin. Under such conditions differentiation is suppressed more efficiently than in serum-containing medium and the ES cells retain full developmental competence including germline contributions. Furthermore, ES cells can readily be derived de novo from pre-implantation embryos using the LIF plus BMP combination.*

*Established ES cells adapted to culture on a feeder layer often undergo deleterious genetic and/or epigenetic changes on transfer to feeder-free culture. This phenomenon is consistent with epigenetic variability between ES cell lines, influenced by the culture protocols employed in their derivation and subsequent propagation[8,9]. In practical terms this has previously limited the use of feeder-free culture systems to those ES cell lines that were derived without feeders or with only transient exposure to feeders. Significantly, we now find that feeder-dependent ES cells can readily be adapted to feeder-free conditions without genetic damage or developmental compromise by using serum-free medium supplemented with LIF plus BMP.*

*Finally, we suggest that ES cells could provide a useful system for studying the evolution of malignancy[10]. ES cells proliferate without G1 checkpoint control[11]. This may underlie their propensity to form teratocarcinomas when injected into adult mice as opposed to early embryos. EC cells derived from spontaneous or embryo-induced teratocarcinomas are invariably aneuploid and developmentally compromised. However, to what extent ES cells cycled through a teratocarcinoma may retain their genetic integrity and ability to be "normalised" by the blastocyst environment is currently unknown.*

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## Epigenetic regulation of Self-renewal in normal and cancer stem cells.

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*Understanding the cellular biology of the tissues in which cancers arise, and specifically of the stem cells residing in those tissues, could provide new insights into cancer biology. Several aspects of stem cell biology are relevant to cancer. Common Cancers arise in tissues that contain a large sub-population of proliferating cells that are responsible for replenishing the short-lived mature cells. In such organs, cell maturation is arranged in a hierarchy in which a rare population of stem cells give rise to the mature cells and perpetuate themselves through a process called self renewal[1]. Both normal stem cells and some cancer cells undergo self-renewal, and emerging evidence suggests that similar molecular mechanisms regulate self-renewal in normal stem cells and their malignant counterparts.*

***Stem cells, cancer and self-renewal.*** *HSCs are the most studied and best understood somatic stem cell population[2]. Hematopoiesis is a tightly regulated process in which a pool of hematopoietic stem cells eventually gives rise to the lymphohematopoietic system consisting of the formed blood elements. In the adult, HSCs have two fundamental properties. First, HSCs need to self-renew in order to maintain the stem cell pool and the total number of HSCs is under strict genetic regulation[3]. Second, they must undergo differentiation to maintain a constant pool of mature cells in normal conditions, and to produce increased numbers of a particular lineage in response to stresses such as bleeding or infection.*

*Maintenance of a tissue or a tumor is determined by a balance of proliferation and cell death. In a normal tissue, stem cell numbers are under tight genetic regulation resulting in maintenance a constant number of stem cells in the organ. By contrast, cancer cells have escaped this homeostatic regulation and the number of cells within a tumor that have the ability to self renew is constantly expanding, resulting in the inevitable growth of the tumor.*

*Differences in self-renewal pathways between cancer stem cells and normal stem cells that allow for the continuous expansion of self-renewing can be exploited to more effectively treat cancer. To develop assays to identify these pathways, one must first realize that self-renewal is not synonymous with proliferation. Self-renewal is a cell division in which one or both of the daughter cells remain undifferentiated and retain the ability to give rise to another stem cell that has the same capacity to proliferate as the parental cell. Proliferation does not require either daughter cell to be a stem cell nor retain the ability to give rise to a differentiated progeny. Note that in normal tissues such as the blood, both stem cells (HSCs) and committed progenitor cells have the ability to proliferate extensively and to completely restore the hematopoietic system for up to two months. However, the progenitor cell is destined to eventually stop proliferating. With each cell division its potential to proliferate decreases until it is finally lost altogether. On the other hand, a single normal HSC can restore the blood system for the life of the animal. Thus, normal stem cells share with at least some of the cancer cells within a tumor the ability to replicate without losing the capacity to proliferate. Therefore, the identification of pathways that regulate self-renewal within the cancer cells is critical to our understanding of these diseases.*

*Not surprisingly, some oncogenes function to promote stem cell self-renewal. Adult stem cells of the hematopoietic and neuronal tissues express the proto-*

*oncogene* bmi-1*, a member of the polycomb family involved in the epigenetic repression of target genes. Although the number of HSCs in the fetal liver of* bmi-1$^{-/-}$ *mice was normal, the number of HSCs was markedly reduced in postnatal* bmi-1$^{-/-}$ *mice. Transplanted fetal liver and bone marrow cells obtained from* bmi-1$^{-/-}$ *mice contibuted only transiently to hematopoiesis. There was no detectable self-renewal of adult hematopoietic stem cells, indicating a cell autonomous defect in* bmi-1$^{-/-}$ *mice. A gene expression analysis revealed that the expression of stem cell associated genes[4], cell survival genes, transcription factors, and genes modulating proliferation including* p16$^{Ink4a}$ *and* p19$^{Arf}$ *was altered in bone marrow cells of the* bmi-1$^{-/-}$ *mice. Expression of* p16$^{Ink4a}$ *and* p19$^{Arf}$ *in normal HSCs resulted in proliferative arrest and p53-dependent cell death, respectively. Similarly,* bmi-1 *regulates self-renewal of adult neuronal stem cells partly via* p16$^{Ind4a}$*. Thus, expression of* bmi-1 *is essential for the generation of self-renewing adult hematopoietic and neuronal stem cells[5,6].*

***Breast Cancer Stem Cells.*** *Recently, tumorigenic and non-tumorigenic subsets of cancer cells have been isolated from human breast cancer tumors, providing the first direct evidence for cancer stem cells in solid tumors. To assay the tumorigenic cancer cells, a xenograft model for human breast cancer was developed that allowed breast cancer tumors isolated directly from patients to be passaged reliably* in vivo*. In this model, only a subset of the breast cancer cells had the ability to form new tumors[7]. In cancer cells isolated from most patients' tumors, tumorigenic cells could be distinguished from non-tumorigenic cancer cells based upon surface marker expression. In eight out of nine patients, tumorigenic cells could be prospectively identified and isolated by flow cytometry as CD44$^{+}$CD24$^{-/low}$Lineage$^{-}$ cells[7]. Limiting-dilution assays demonstrated that as few as one hundred tumorigenic cancer cells were able to form tumors, while tens of thousands of the other populations of cancer cells failed to form tumors in NOD/SCID mice. These tumorigenic cells have been serially passaged, and each time cells within this population generated new tumors containing additional CD44$^{+}$CD24$^{-/low}$Lineage$^{-}$ tumorigenic cells as well as the phenotypically mixed populations of non-tumorigenic cancer cells. Importantly, the phenotypic distribution of cells closely resembled that of the original tumor. These data demonstrate the presence of a hierarchy of cells within a breast cancer tumor in which only a fraction of the cells have the ability to generate a new tumor which contains similar populations of tumorigenic and non-tumorigenic cancer cells, suggesting that the tumorigenic cells can both generate both populations of cells. Thus, tumorigenic breast cancer cells from most tumors appear to exhibit the properties of cancer stem cells.*

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## Mouse Models of Glioblastoma: Cellular Origins

***Luis F. Parada****, Center for Developmental Biology, UT Southwestern Medical Center, Dallas, TX, 75390*

*Astrocytomas are a sporadic form of cancer for which no effective therapies have been developed. The prognosis remains unchanged over the past three decades. According to the WHO classification, four*

*grades of astrocytoma exist. Grade 1 is benign and also referred to as pilocytic astrocytoma. Grade II or low grade astrocytoma is characterized by infiltrative cells that home on neuronal bodies (perineural satellosis). Grade III or anaplastic astrocytoma is cell dense and highly proliferative. Grade IV or glioblastoma multiforme is characterized by pseudopalisading, necrotic foci, and intense microvascularization. All forms of astrocytoma express primitive cell markers such as nestin. In addition all forms of astrocytoma appear throughout the brain but do not leave the CNS. These observations have led to the suggestion that the CNS provides a niche that is required for tumor growth and that spontaneous tumorigenesis occurs throughout.*

*Historically, the prevalent model for astrocytoma formation invoked mechanisms of dedifferentiation of glial cells, followed by genetic and epigenetic signals that drive neoplastic transformation. The more recent appreciation of the existence of stem cells in the lateral ventricles and dentate gyrus have raised the question of a potential role for stem cells in tumor formation.*

*We have developed tumor suppressor based mouse models of glioblastoma that histologiaclly and molecularly resemble human glioblastoma, including progression from Grades II through IV. Through analysis of a variety of criteria, our results provide concrete evidence that stem cells could account for the origin of glioblastoma.*

## Human Leukemia Stem Cells

***John E. Dick*** *Ph.D.Division of Cell and Molecular Biology, University Health Network, and Dept of Molecular Genetics and Microbiology, University of Toronto 620 University Ave, Toronto, Ontario, M5G 2C1Leukemogenesis*

*Our understanding of the leukemogenic disease process has, to a large extent, been formed from many decades of research on human subjects involving characterization of the cellular phenotype of acute leukemia and other aspects of the clinical picture. One of the major difficulties with this approach is the limited ability for experimental intervention in human subjects. Moreover, it is almost impossible to gain insight into the early events of the leukemogenic process before they become clinically apparent. Until the last decade, most experimental approaches have involved the study of naturally occurring animal (mostly murine) leukemia and experimentally induced disease following transgenic or gene knock-out methods. However, while many aspects of these murine leukemias recapitulate the human disease, there can be significant differences with the human disease. Moreover, marked differences in genomic stability between humans and inbred mice strains suggest that the leukemogenic process might be subtly different. Ultimately, one would like to complement murine experiments with model systems that utilize human leukemia to ensure that they are relevant to the human situation and that therapies based on this knowledge will have a higher likelihood of efficacy in humans. The transplantation of normal and leukemic human cells into immune-deficient mice provides such a system.*

*Two fundamental problems in cancer research are identification of the normal cell within which cancer initiates and identification of the cell type capable of sustaining the growth of the neoplastic clone. There is overwhelming evidence that virtually all cancers are clonal and represent the progeny of a single cell. What is less clear for most cancers is which cells within the tumor clone possess tumor initiating or "cancer stem cell" (CSC) properties and are capable of maintaining tumor growth. The concept that only a minor subpopulation of so-called cancer stem cells (CSC) is responsible for maintenance of the neoplasm emerged about 50 years ago[1] with the best evidence coming from the hematological malignancies. Key to these studies is the depth of understanding of normal hematopoietic development that has been gained in the past 4 decades. Functional in vitro and in vivo assays are available for all stem and progenitor cell types ranging from the primitive pluripotential stem cells to multipotential and unipotential progenitors. In addition, a rich collection*

*of cell surface differentiation markers enable detailed characterization of normal hematopoietic development, as well as providing insight into how normal differentiation becomes disrupted in leukemia. It is clear that leukemic tissues, while abnormal, still retain remnants of normal differentiation and developmental programs.*

*With the advent of clonogenic assays for normal hematopoietic progenitors, it became possible to determine that the vast majority of acute myeloid leukemia (AML) blasts do not proliferate and only a minor proportion (~1%) of human leukemic cells are clonogenic progenitors (AML-colony-forming units-AML-CFU). However, it was not known if AML-CFU represented true leukemic stem cells (LSC). Conclusive evidence for the existence of LSC came from our identification of human SCID Leukemia-Initiating Cells (SL-IC) that were capable of propagating acute myeloid leukemia in a xenograft transplant system we developed for leukemic and normal stem cells[2]. These studies provided functional proof that the AML clone in humans is organized as a hierarchy that originates from SL-IC, which produce AML-CFU and leukemic blasts. SL-IC could be purified based on the CD34-CD38- cell surface phenotype and transplanted into NOD/SCID mice where they "differentiate" albeit abnormally into AML-CFU and leukemic blasts characteristic of the donor indicating that the leukemic clone is not characterized by blocked differentiation. AML could be serially transplanted into secondary mice demonstrating that SL-IC possessed extensive self-renewal capacity, a key determinant of stem cell function. Interestingly the cell surface phenotype of SL-IC showed significant similarities to normal human hematopoietic stem cells (HSC), suggesting that there maybe a relationship between these two stem cell populations and that the cell of origin of AML derives from the pool of normal HSC [3, 4].*

## Normal hematopoietic stcm cells

*The mammalian hematopoietic system is a hierarchy derived from stem cells that possess extensive self-renewal, proliferative, and differentiative capacity. Hematopoietic stem cells (HSC) maintain the hematopoietic system throughout life, and stem cell regulation is a critical element in the control of normal hematopoiesis. HSC can only be conclusively examined by in vivo repopulation. We have used repopulation of immune-deficient mice to develop a quantitative assay for human stem cells that have been termed SCID-repopulating cells (SRC). A detailed characterization of SRC is emerging in terms of frequency, cell surface phenotype, and cytokine responsiveness [2]. To understand the composition of the human hematopoietic stem cell compartment, we tracked the in vivo fate of individual SRC during repopulation of NOD/SCID mice by analysis of the unique clonal markers that were introduced with retroviral vectors [5]. The vector integration site provides a marker that is stably inherited by all progeny of an active stem cell. Analysis of serial BM aspirations from NOD/SCID mice transplanted with transduced cord blood (CB) demonstrated that the repopulation was oligoclonal with extensive variability in self-renewal capacity as well as in the lifespan and proliferative capacity of individual SRC. Some clones only contributed for several weeks after the transplant and disappeared, while others appeared later and persisted. Secondary repopulation experiments demonstrated that there was heterogeneity in the self-renewal capacity of the transduced SRC. These data point to the existence of different classes of human stem cells with short- and long-term-repopulating capacity (ST- and LT-SRC, respectively).*

## Leukemia stem cells

*In order to determine whether SL-IC represent a homogenous population of LSC where each member possesses equivalent repopulating function or whether there is functional heterogeneity, we undertook an clonal tracking approach as outlined above[9]. We found that some clones contributed transiently while others were long term and stable indicating that SL-IC are in fact*

*heterogeneous and the entire pool is comprised of different classes of short term (ST) and long-term (LT) SL-IC. We found that the mechanism that underlies this heterogeneity is variation of the self-renewal capacity of each SL-IC type. The self-renewal capacity was determined by carrying out serial transplant and assessment of whether the clone persisted or disappeared. Some SL-IC persisted in secondary and tertiary mice providing conclusive proof for the self-renewal of a CSC. In addition, we found that some LT-SL-IC generated a transient graft in secondary mice indicating that ST-SL-IC derive from LT-SL-IC. The fact that both LSC and normal HSC compartments are structured as a hierarchy as a consequence of progressive loss in self-renewal capacity provides strong support for the hypothesis that in AML the initial target cell for transformation lies within the HSC compartment [3, 4, 6]. Of course since leukemogenesis is a mulitistep process, the additional "hits" that are required could arise in these abnormal stem cells or in more downstream progenitors to result in a fully transformed LSC. Our data shows that the leukemogenic program does not abolish all the pathways that regulate normal hematopoiesis at the stem cell level. Thus, the intrinsic self-renewal capacity, as well as the decline in self-renewal capacity (i.e. regulation of self renewal) due to commitment processes, of HSC targeted by the initial leukemogenic event(s) continue to function in the resultant LSC. Indeed the recent finding that the stem cell-specific gene, Bmi-1, plays a key role in the self-renewal of both normal and leukemic murine stem cells supports this idea [7, 8].*

## Conclusions

*Leukemic stem cells hold the key to understanding the origin and maintenance of AML and possess biological properties that are different from the bulk of the leukemic clone making them difficult to eradicate. Thus, elucidation of these LSC-specific properties will aid in the development of more effective therapy that can be targeted to the most primitive LSC. The paradigm we have developed to examine the complexity of the LSC compartment should provide a roadmap to examine similar questions about the properties of CSC from other cancers.*

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## Functional analysis of Polycomb-silencing: Implications for stem cell biology and Cancer.

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*Repressive Polycomb-group (Pc-G) protein complexes and the counteracting Trithorax-group (Trx-G) of nucleosome remodeling factors are involved in the dynamic maintenance of proper gene expression patterns during development, acting at the level of chromatin structure (reviewed in: Jacobs and van Lohuizen, 2002; Lund and van Lohuizen, 2004). As such,*

*they are important controllers of cell fate. Important developmental targets include the* Hox *gene clusters but also critical cell cycle regulatory genes, such as the Ink4a/Arf tumor-suppressor locus (Jacobs et al., 1999a; 1999b; Voncken et al., 2003). When deregulated, both Trx-G and Pc-G genes are strongly implicated in formation of a diverse set of cancers. For instance, we have previously shown that overexpression of the Polycomb gene Bmi1 promotes proliferation and induces leukemia through repression of the Ink4a/Arf tumor-prevention fail-safe mechanism (Jacobs et al., 1999a). Conversely, Bmi1 deficiency leads to hematological defects, loss of hemapoietic stem cell renewal and severe progressive neurological abnormalities, in which de-repression of the Ink4a/Arf tumor-suppressor locus is critically implicated. Together with the labs of S. Marino, Zurich and Y. Arsenijevic, Lausanne, we have recently investigated the brain phenotypes in detail and have shown that Bmi1 is essential for proliferation of granule precursor cells (CGNPs) and for maintenance of self-renewal of cortical neural stem cells (Leung et al., 2004; Zencak et al, submitted). Deregulated proliferation of CGNPs, due to mutations in the Shh signalling pathway in ±25% of cases, is one event that leads to medulloblastoma development, a major malignant infant brain tumour of which other contributing lesions currently are poorly understood. Importantly, we were able to show that Bmi1 is critically required for CGNP proliferation and that Bmi1 is a target of the Shh pathway. Furthermore we demonstrated that Bmi1 is highly overexpressed in a majority of primary human medulloblastomas, which display aberrant constitutive-active Shh signalling. Lastly, using a conditional knockout approach, we have shown an essential role for Polycomb silencing in maintenance of Embryonal stem cell fate. Together with the recently established roles for Pc-G silencing in controlling proliferation of both normal and leukemic stem cells, these results link the Shh morphogen and development signaling pathway to Pc-G regulation and indicate a common conserved role for epigenetic Pc-G repressive complexes acting in a cell-intrinsic manner to allow maintenance of stem cell fate. The implications of these findings for stem cell biology and cancer will be discussed.*

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## Imprinting and epigenetic reprogramming in mammalian development

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*We are interested in expression, organisation, and function of imprinted genes. We find that differentially methylated regions in the imprinted Igf2 and H19 genes interact physically. These interactions are epigenetically regulated so that maternal and paternal chromatin is partitioned into distinct loops. In the neighbouring IC2 (Imprinting Centre 2) domain we find that imprinting in the placenta is maintained despite hypomethylation in the Dnmt1- mutant; these genes are instead marked by repressive histone modifications which depend on the imprinting centre IC2. Different epigenetic marking systems may thus be involved in imprinting; these may have different stability and hence show different susceptibility to being disrupted in human Assisted Reproduction Technologies (ART) such as IVF.*

*Epigenetic reprogramming of DNA methylation and chromatin marks occurs in early embryos and in primordial germ cells, and is likely related to pluripotency and erasure of imprints. We are investigating the interplay of DNA methylation with chromatin dynamics, and are interested in identifying factors that play a role in reprogramming.*

## From Nucleosome to Heterochromatin, Their Formation and Maintenance

*Speaker:* ***Geneviève Almouzni***
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*The ordered assembly of chromatin produces a nucleoprotein template capable of epigenetically regulating the expression and maintenance of the genome.*
*Histone chaperones have been isolated from cell extracts that stimulate early steps in chromatin assembly* in vitro. *The function of one such factor, chromatin assembly factor-1 (CAF-1), might extend beyond simply facilitating the progression through an individual assembly reaction to its active participation in a marking system. This marking system could be exploited at the crossroads of DNA replication and repair to monitor genome integrity and to define particular epigenetic states. We will discuss our recent findings on this topic. The interrelationships with other assembly factors and their respective role in specific assembly pathways will be covered. We will also present our data concerning the quality and stability of pericentric heterochromatin organization in interphase nuclei of mouse cells. This model system will be used to discuss the maintenance of heterochromatin during replication. We will discuss how these information can be relevant for events associated with nuclear reprogramming.*

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## Micro-Domains of HP1-Mediated gene silencing in euchromatin: mechanisms for establishing, maintaining and ensuring heritability

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*Establishing, maintaining and controlled differentiation of the of the stem cell niche in many diverse tissues is undoubtedly critical for mammalian development and homeostasis. These cells must retain and exploit the ability to reverse "memorized" states of gene activation and repression and be able to essentially "re-set the switches" of complete transcriptomes in order spawn new cell phenotypes. It is also clear that a new breed of therapeutic agents may have the ability to target these mechanisms. In order to both develop new transcriptional therapies and exploit the stem cells therapeutically, we must continue to define the molecular mechanisms that allow for coordinated activation or repression of specific sets of target genes. Targeting the enzymatic machinery which modifies chromatin structure is a promising tactic as this has emerged as a primary mechanism of gene regulation. Post-transcriptional modification of core histone tails via phosphorylation, acetylation/de-acetylation, methylation and ubiquination and sumoylation are key. Proteins such as HP1 directly recognize modified histone tails and lead to gene silencing. Reversing HP1 mediated gene silencing must be overcome in order to re-program gene expression. We have been interested in how this can be accomplished in mammalian stem cells. Heterochromatin protein 1 (HP1) in* Drosophila *is required for stable epigenetic gene silencing classically observed as position effect variegation of a transgene integrated adjacent to constitutive heterochromatin. However, mammalian HP1 proteins may be euchromatic, can be deposited on active genes by specific corepressors and anchored there by histone H3 containing the lysine 9 methylation mark. Little is known about the physical properties of chromatin that contains euchromatic genes which are silenced via HP1 recruitment. We have been characterizing the KRAB-zinc finger superfamily of silencers which, via association with there obligate co-repressor KAP1 can coordinate histone deacetylation, histone methylation and HP1 deposition to silence euchromatic genes. A mammalian cell culture based system for hormone regulated recruitment of this machinery to an expressed transgene has resulted in the following discoveries. In the presence of hormone, the transgene is rapidly repressed, the gene is spatially recruited to HP1-rich nuclear regions, assumes a compact chromatin structure, and is physically associated with HP1/KAP1 over a highly localized region centered around the promoter. Remarkably, once repression is established (via a 48 hour pulse of hormone) the silenced state is stably maintained for >50 population doublings in the absence of hormone at high frequency in clonal cell populations. This stable silencing is maintained in the absence of the DNA binding component and is highly reminiscent of HP1 dependent gene variegation in flys. The frequency of silent clone generation is increased by HP1alpha dose but not by HP1gamma. However, unlike variegation in flys, the silent state does not spread*

*to adjacent euchromatic transcriptional units. Detailed analysis of clonal silent cell lines has shown a region comprising only 3-4 nucleosomes at the promoter is highly enriched in trimethylated H3-MeK9, the SETDB1 methyltransferase and KAP1, thus showing that once the machinery is nucleated via a DNA binding protein, it can be maintained at the locus in its absence. Thus, in mammalian cells, highly localized recruitment of HP1 to a euchromatic promoter establishes a mitotically heritable silenced chromatin state. Further characterization of this system will be presented.*

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## Nuclear Cloning and the Reversibility of Cancer

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*The cloning of amphibians raised questions of nuclear potency and differentiation that are as controversial today as they were half a century ago. Unresolved issues include whether the capacity of a nucleus to direct the formation of an animal is lost with increasing age of the donor cell and whether its state of differentiation influences the type of abnormalities seen in the clone. Clearly, the central issue of nuclear cloning is an epigenetic one, i.e. how the donor genome is reprogrammed after transfer into the egg to ensure correct activation of those genes that are needed for embryonic development. In this talk I will review the nuclear transfer approach and how it can be used as a general tool to distinguish between genetic and epigenetic alterations that restrict the developmental potential of the genome (Hochedlinger and Jaenisch, 2003).*

### Nuclear cloning of mammals and the reprogramming of the genome:

*The full term development of sheep, cows, goats, pigs 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. One of the most interesting issues of nuclear cloning is the question of genomic reprogramming, i.e. the question whether successful cloning requires the resetting of epigenetic modifications which are characteristic of the adult donor nucleus. Our data indicate that inappropriate expression of key developmental genes contributes to lethality of cloned embryos*
*(Bortvin et al., 2003). However, even cloned mice that develop to birth and beyond display major dysregulation of gene expression (Humpherys et al., 2002). This argues that adult cloned animals may have widespread epigenetic abnormalities due to misexpression of many*

*genes even if they appear normal by superficial inspection.*
*The talk will review our recent studies on the reprogramming of nuclei from terminally differentiated B and T cells. We have derived monoclonal mice that carried in all tissues the genetic rearrangements of the IgG and TCR present in the mature lymphoid cell that served as a donor nucleus (Hochedlinger and Jaenisch, 2002). This result demonstrates that the nuclear cloning represents an general approach to identify genetic alterations that are present in genome of a single cell.*

## Reprogramming of cancer cell genomes by nuclear transplantation in frogs:

*The malignant state of tumor cells is known to be caused by genetic as well as epigenetic changes of the genome. Nuclear cloning technology represents an unbiased and general approach for distinguishing and for defining such alterations and for elucidating the basic epigenetic mechanisms that are involved in embryonic development and disease. The seminal experiments with amphibians conducted half a century ago suggested that the nucleus from a cancer cell could be partially reprogrammed upon transfer into an oocyte and accomplish development though the cloned embryos were abnormal and formed tumors. In this approach nuclei from a transplantable kidney carcinoma were transferred into eggs. Only about 1% of the transplanted eggs developed to abnormal tadpoles. In another study triploid cells from a tumor that had been induced in metamorphosing tadpoles were used as nuclear donors resulting in about 6% of abnormal cloned tadpoles. These experiments indicated that the genome of cancer cells is amenable to epigenetic reprogramming and is able to direct embryonic development although normal development was compromised. Thus, these classical experiments with amphibians demonstrated that the nuclear transplantation can be used as a tool to investigate whether nuclei from well-defined tumor cells and stem cells can support development after transfer into the oocyte.*

## Nuclear cloning of embryonic and somatic cancer cells:

*I will discuss our strategy of using nuclear transplantation procedures to compare the potency of stem cells, differentiated cells and of transformed cells to direct embryonic development after nuclear transfer. The potential of nuclei to serve as donors for cloning depends on (i) the state of differentiation and transcriptional competence and (ii) the chromosomal integrity of the donor genome. As nuclear donors we are using embryonic carcinoma cells and somatic cancer cells including defined leukemia cells and solid tumors such as sarcoma and melanoma cells. Our results suggest that the genome of embryonic carcinoma cells as well as of some somatic cancer cells can be reprogrammed to direct at least some embryonic development after transfer of the nucleus into the oocyte. The similarities and differences between stem cells and tumor cells represent an old issue in cancer biology. Though it is clear that genetic alterations accumulate during neoplasia, the importance of epigenetic alterations has become recognized only recently. Nuclear cloning represents an unbiased functional approach to define the epigenetic state of a nucleus and is a tool to distinguish between genetic changes and epigenetic alterations. Tumor specific epigenetic alterations are, in contrast to genetic changes, reversible opening potential therapeutic opportunities. It is one of the goals of our work to define the epigenetic state of somatic stem cell and cancer cell nuclei by their capacity to be reprogrammed after transfer into the egg. It is our hope that these studies will contribute to a better understanding of molecular parameters that distinguish malignant from normal cells and thus may help to devise rational strategies for reversing the malignant state.*

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## Cancer as an Epigenetic Disease: The Disruption of the DNA Methylation and Histone Codes

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*We are in an era where the potential exists for deriving comprehensive profiles of DNA alterations characterizing each form of human disease, specially cancer. DNA methylation is the main epigenetic modification in humans. Tumor cells show aberrant methylation of several CpG islands, but global demethylation versus the counterpart normal cells. Our results show that CpG island promoter hypermethylation has a tumor-type specific pattern. Epigenetic silencing affects all cellular pathways: from DNA repair (hMLH1, MGMT, BRCA1) to cell cycle ($p16^{INK4a}$, $p14^{ARF}$, $p15^{INK4b}$, p73). Promoter hypermethylation of particular genes have important consequences for the biology of that particular tumor. This is for example the case of the DNA repair gene MGMT which methylation-mediated silencing leads to transition mutations, but, at the same time, "marks" chemosensitivity. Those genes inactivated by epigenetic mechanisms are not only silenced and hypermethylated in sporadic cases, but also hypermethylation can be observed in hereditary tumors as a "second hit". We have also developed massive genomic screenings to find new hypermethylated genes in cancer cell. From these assays we have identified new candidate tumor suppressor genes with important potential roles in the pathogenesis of human cancer.*
*It is also widely accepted that methyl-CpG binding proteins (MBDs) couple DNA methylation to gene silencing through the recruitment of histone deacetylase and chromatin remodelling activities that modify chromatin structure. Chromatin Immunoprecipitation (ChIP) assays and restriction nuclease accessibility analysis demonstrate how the vast majority of tumor suppressor genes with CpG island promoter hypermethylation-associated inactivation also present histone hypoacetylation and histone methylation. Furthermore, an exquisite specific profile of different methyl-binding proteins (MBD1, MBD2, MBD3, MeCP2) exists in function of the gene studied.*
*Overall, our data demonstrates that human tumors suffer a profound, but specific, disturbance in their DNA methylation and chromatin patterns. Further research is required to understand the epigenetics of cancer.*

*Our group approaches this goal using different approaches:*

1) *Systematic analysis of human primary tumors and cancer cell lines for their 5-methylcytosine DNA content by High Performance Capillary Electrophoresis and study of its consequences in human tumorigenesis.*
2) *Systematic analysis of human primary tumors and cancer cell lines for their CpG island promoter hypermethylation profile using Methylation-Specific PCR in all known bona-fide tumor suppressor and candidate genes, and massive profiling using a CpG island microarray approach.*
3) *Search for new genes undergoing promoter hypermethylation in human cancer using candidate gene approaches; global genomic-methylation methodologies, such as Amplification of Intermethylated Sites (AIMS), derived from the*

*original arbitrarily primer PCR technique, and Restriction Landmark Genomic Scanning (RLGS) with methyl-sensitive enzymes; and cDNA microarrays analysis after the use of demethylating agents.*

4) *Functional and Morphological Identification of all the DNA methylation and chromatin elements involved in gene silencing in human tumors: DNA methyltransferases, Methyl-binding proteins, Histone Deacetylases, Histones Acetyltransferases, Histone Methyltransferases and Chromatin Remodelling Factors.*
5) *Identification of new genetic syndromes associated with germline mutations in the DNA methylation and chromatin genes and molecular characterization of the current ones (Rett syndrom, ICF, ATRXÉ).*
6) *Discovery of new agents and rescue of old drugs with a putative use in the treatment of cancer bassed on cancer epigenetics: DNA demethylating agents and Inhibitors of Histone Deacetylases.*
7) *Translational use of the DNA methylation aberrations found in the above described points for the early diagnosis, prognosis and follow-up of cancer patients studying all types of biological fluids and primary tumors.*

*These poweful lines of study will provide us with a unique view of the DNA methylation setting present in human cancer and their dramatic differences from the balanced scenario of the normal cells.*

## The Dual Role of DNA Methylation and Histone Modifications in Aberrant Gene Silencing in Cancer.

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*It is becoming clear that the progression of cancer, in addition to be driven by key genetic changes, is mediated by numerous epigenetic abnormalities, the best characterized of which are heritable gene transcription silencing events associated with aberrant promoter region hypermethylation. The maintenance, and establishment, of these epigenetic abnormalities involve a complex interaction between key histone amino acid modification changes and their interaction with the DNA methylation. Particularly, methylation of lysine 9 of histone H3 (me-K9-H3) may be a critical signal which helps to recruit DNA methylation to the promoters of silenced genes. In turn, this mark, in association with deactylation of K9-H3, is integral to maintenance of the silencing while the DNA methylation, once established, appears to act as a dominant "lock" which must be removed for reactivation of gene expression and reversal of the above repressive histone modifications to those associated with active transcription. The DNA methylation appears to be established, and maintained, by interactions between three known, biologically active, DNA methyltransferases (DNMT's), and these interactions may differ from one cancer cell type to another. These DNMT's may also bring their own inherent transcription repression activities, recruitment of other transcription repressors, and targeting of histone deactylases to the silencing process ( all above reviewed in references[1,2]).*

*One of the most important questions in the field of epigenetic gene silencing in cancer concerns the identification of the genes involved, and particularly their position and function in the process of tumor progression. This has become known for several classic tumor suppressor genes, which also are frequently mutated in cancer, and defining the promoter hypermethylation of these genes, indeed, established the importance of epigenetic gene silencing in tumorigenesis[1,2]. However, as genomic screening is occurring for genes hypermethylated in cancer, many epigenetically silenced genes are being found that do not have mutations. Defining the role and position of these silenced genes in tumor progression is an extremely important goal for full understanding of the impact of epigenetic alterations in cancer. Data will be presented to firmly suggest that silencing of such genes, and indeed perhaps the majority of promoter hypermethylated genes in cancer, may be critically involved in the earliest steps in tumor progression and lead to events which provide the pre-cancerous cell populations which become the substrate*

*for subsequent genetic events that lead to the full progression to cancer. In this regard, a role for the hypermethylated genes in the inappropriate activation of developmental pathways which are normally only active in undifferentiated, or stem cell like cells, but which should be silent in mature cells, may be a particularly key aspect of cancer progression.*

*Two examples will be discussed to illustrate the immediately above points. The first involves the epigenetic silencing, in colon cancer progression, of the secreted frizzled related protein (*SFRP's*) gene family[3], which encode for proteins that act as antagonists to the frizzled proteins, a key family of receptors that mediate Wnt pathway signaling at the cell membrane level. It will be shown how epigenetic silencing of SFRP's provides an early, pre-cancerous stage, event which renders the Wnt pathway abnormally active at the ligand level, prior to downstream mutations in the pathway that also lead to Wnt pathway overactivity. In cancer progression, the* SFRP *silencing (a postulated epigenetic gateway step) appears to lead to increased cellular -catenin levels which, in the presence of the downstream mutations ( an accepted genetic gatekeeper step) which disrupt complexes which shuttle this protein for proteosomal degradation, result in abnormal accumulation of β-catenin in the nucleus. This nuclear localization facilitates transcriptional activation by the protein of growth promoting genes, and inhibition of apoptosis signals, to fully drive colon cancer progression[4,5].*

*In a second example, the gene hypermethylated in cancer-1 (HIC-1), which is frequently hypermethylated and silenced in early tumor progression stages, but is not mutated, in multiple human cancer types, is shown to be a transcriptional repressor for which loss of functions leads to abnormal regulation of developmental pathways. Homozygous disruption of this gene leads to embryonic lethality in mice[6] while heterozygotes develop tumors late in life[7]. Disruption of Hic-1 leads to a complete alteration of the tumor spectrum produced by disruption of one allele of the powerful tumor suppressor gene,* p53 *( Chen et al, submitted, 2004).*

*Thus, heritable epigenetic gene silencing may actually play a key role in the very earliest stages of tumor development. The interaction of the epigenetic changes with genetic disruption of classic tumor suppressor genes may underly the full progression of human cancers.*

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## Hypomethylation Therapy of Cancer

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*Cytosine analogues that specifically and efficiently inhibit DNA methyltransferases were developed 40 years ago and shown to result in DNA hypomethylation 25 years ago, yet their full potential as anti-cancer agents had to await the demonstration of the central role methylation changes play in cancer development and progression. These two drugs, 5-azacytidine and 5-aza-2'-deoxycytidine (decitabine) have*

*shown efficacy in myeloid malignancies and applications for their approval as anti-cancer drugs are currently being considered by the US Food and Drug Administration. In-vitro, both drugs show a narrow window of activity, with substantial cytotoxicity and loss of hypomethylating activity at high doses. In the setting of a low-dose, phase I study in hematologic malignancies, we have shown that decitabine induces hypomethylation in a dose-dependent way, with a rapid plateau in hypomethylation at a dose of 15 mg/m2 IV daily for ten days, which coincided with the optimal clinical dose (based on responses). Using methylation of repetitive elements or total methylation measured by LC-MS, we estimated the degree of hypomethylation to range between 0% and 20% 5 days after initiation of therapy, depending on the dose. Decitabine-induced hypomethylation correlated with response at low doses but not at high doses. Remethylation begins very shortly after the drug is stopped, and is invariably complete by 30 days. After therapy, hypomethylation of single copy genes has been observed, including potential tumor-suppressor genes such as P15, but there appears to be gene-specific factors that control the degree of sensitivity to decitabine-induced hypomethylation, and the relationship between gene-specific demethylation and response remains unclear. Finally, although hypomethylation appears required for decitabine to work as an anti-cancer agent, the distal events that result in clinical responses remain undetermined. Clinically, studies to optimize and exploit gene reactivation induced by hypomethylating agents are at an early stage but are essential to determine the true potential of this approach to cancer therapy.*

## Histone deacetylase inhibitors: From discovery to clinical trials of these new targeted anti-cancer agents

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*The regulation of gene expression is largely determined by the proteins associated with DNA.[1] DNA is packaged into nucleosomes, the repeating unit of chromatin composed of about 146 base pairs of two superhelical turns of DNA wrapped around an octamer core of pairs of histones, H2A, H2B, H3 and H4. Posttranslational modifications of the amino-terminal tails of the histones, (acetylation of lysines, methylation of lysines and arginines, phosphorylation of serines and ubiquitination of lysines) play a major role in regulating gene expression. [2, 3] Acetylation of histones, determined in part by the activities of two sets of enzymes, histone acetyl-transferases (HATs) and histone deacetylases (HDACs), neutralizes the lysine positive charge, altering chromatin structure and allowing for greater access to promotor regions of genes for transcription factor protein complexes. The pattern of posttranslational modification of histones appears to represent a code that is recognized by non-histone complexes involved in the regulation of gene expression. [2-5]*

*This review will focus on HDACs and, in particular, the hydroxamic acid based HDAC inhibitor we discovered and developed, suberoylanilide hydroxamic acid (SAHA). [6]*

*There are three classes of human HDAC enzymes. Class I, HDACs 1, 2, 3, 8 and 11, which have homology in the catalytic sites with yeast RPD3 deacetylase, Class II, HDACs 4, 5, 6, 7, 9 and 10, which have homology in the catalytic sites to yeast HdaI and Class III, SIR-2 family of deacetylases which have an absolute requirement for NAD for activity. SAHA and related hydroxamic based compounds, eg., TSA, inhibit Class I and II, but not Class III HDACs. HDACs do not bind directly to DNA, but are recruited by protein complexes that may include HATs, corepressors and other transcription factors. There is abundant evidence that the different HDACs are not redundant in their biologic activities.[1, 7, 8] For example, HDAC1 knock-out is an embryotic lethal despite compensatory increase in HDAC2 and 3 expression. HDAC5 and possibly other Class II HDACs play a role in myogeneses (reviewed 7, 8).*

*We have shown by crystallographic analysis that SAHA*

*and TSA interact directly with the catalytic site of HDAC like protein, with the hydroxamic acid moiety binding to a Zn at the base of the catalytic pocket, inhibiting its enzyme activity.*[9]

*Contrary to what might be expected by the wide distribution of HDACs in chromatin of both normal and transformed cells, HDAC inhibitors do not cause a global alteration in gene transcription. Indeed, SAHA or TSA increase or decrease the expression of as few as 2 to 5% of expressed genes in transformed cells. Among the genes whose expression is frequently increased are $p21^{WAF1}$, TBP2 and cyclin E, and among genes whose expression may be decreased are cyclin D1, ErbB2 and thymidylate synthetase. Further, transformed cells are much more sensitive to SAHA induced death than normal cells.*[1,7,8]

*To gain an understanding of this differential sensitivity, we are studying the normal human fibroblast cell line (W138) and its SV40 transformed counterpart (VA13). SAHA induces growth arrest in both cell lines at the same concentration, but VA13 cells undergo rapid apoptosis, while the W138 remain viable and, on removal of SAHA, proliferate normally. SAHA induces changes in the pro-apoptotic pathway in VA13, but not W138 cells. It is clear that proteins that regulate gene transcription are not the only targets of HDAC inhibitors. Acetylation alters the structure and, presumably, the activity of proteins that play a role in signal transductions, cell cycle regulation, apoptosis, mitosis and cytokinesis. (Alterations in HATs and HDACs occur in many cancers.)*[8,14]

*A broad spectrum of transformed cells are sensitive to SAHA induced growth inhibition, apoptosis and/or terminal differentiation as shown by studies with cells in culture and with tumor bearing animals.* [1,8,15] *The phenotype induced by SAHA appears to be determined by intrinsic characteristics of the particular transformed cells. For example, SAHA treated human neuroblastoma and multiple myeloma cell lines undergo rapid apoptosis, human bladder cell lines arrest in G1 and certain human colon cancer cell lines become polyploidy with failure of cytokinesis and cell death.*

*In preclinical studies, both in transformed cell cultures and animal bearing tumors, SAHA has been shown to be synergistic with radiation, various cytotoxic agents, Gleevac and retinoids in suppression of cancer cell growth.*

*SAHA is in Phase I and II clinical trials.*[16,17] *SAHA administered either intravenously or orally, causes accumulation of acetylated histone in peripheral mononuclear cells and in tumor cells, which is a marker of its biologic activity. SAHA has induced responses (including stable decrease, partial responses and complete responses) in hematological and solid tumors, including cutaneous T-cell lymphoma, non-Hodgkin's lymphoma, mesothelioma, laryngeal carcinoma, thyroid cancer and others. SAHA is well tolerated, with side-effects that include fatigue, diarrhea, and dehydration which are reversible upon stopping drug.*

*In summary, HDAC inhibitors, such as SAHA, are promising new targeted anti-cancer agents. SAHA targets cells at several molecular levels which may be aberrant in transformed cells. The greater sensitivity to SAHA of transformed cells compared to normal cells and the selectivity in activation or repression of gene expression likely explains, in part, the favorable therapeutic index of this HDAC inhibitor. Discovering inhibitors that are selective for the different HDACs should contribute to a fuller understanding of the mechanism of the anti-cancer activity of these agents and facilitate development of even more effective and less toxic anti tumor drugs.*

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## Switching Genes On and Keeping Them On

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*The abnormal methylation of CpG islands located near the transcriptional start sites of human genes plays a major role in carcinogenesis. The methylation of cytosine residues in these regions is associated with alterations in chromatin structure including the binding of methylated DNA binding proteins and changes in the state of modification of histone residues in nucleosomes. These alterations serve to reinforce each other and may lead to the heritable silencing of genes which can have profound implications for human cancer development. Unlike mutational changes, epigenetic alterations are acquired in a gradual process which is associated with cellular division. Thus, these progressive alterations are potentially susceptible to interventions to reverse silencing. Epigenetic changes can be observed in premalignant tissues so that understanding what causes the alterations and development of potential strategies to reverse them could have an impact on carcinogenesis. Aberrantly methylated CpG islands can be detected with a high degree of sensitivity making some of these changes also suitable as potential biomarkers. The mechanisms underlying progressive methylation of CpG islands leading to altered chromatin configuration are now beginning to be understood.*

*Zebularine [1-(beta-D-ribofuranosyl)-1,2-dihydropyrimidin-2-one] acts as an inhibitor of DNA methylation and exhibits chemical stability and minimal cytotoxicity both in vitro and in vivo. Continuous application of zebularine to T24 cells induces and maintains p16 gene expression and sustains demethylation of the 5' region for over 40 days, preventing remethylation. In addition, continuous zebularine treatment effectively and globally demethylated various hypermethylated regions, especially CpG-poor regions. The drug causes a complete depletion of extractable DNA methyltransferase 1 (DNMT1) and partial depletion of DNMT3a and DNMT3b3. Last, sequential treatment with 5-aza-2'-deoxycytidine followed by zebularine hinders the remethylation of the p16 5' region and gene resilencing, suggesting the possible combination use of both drugs as a potential anticancer regimen.*

# ABSTRACTS OF POSTERS

## The role of HMGB1 protein in cell migration and metastasis

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*HMGB1 protein (High Mobility Group Box 1) is a chromatin protein that, when released passively, signals the traumatic and/or unprogrammed death of the cell which has released it. Significantly, cells that die in a programmed way never release HMGB1, because they bind it irreversibly to chromatin during the process of apoptosis. Moreover, HMGB1 can also be actively secreted by monocytes and macrophages. In all these cases, extracellular HMGB1 binds to a receptor (RAGE, receptor for advanced glycation endproducts) and signals tissue damage to the cells in the rest of the organism.*
*Two groups (Taguchi et al 2000 Nature 405:354-60; Huttunen et al 2002 Cancer Res 62:4805-11) have shown in mouse models that anti-HMGB1 antibodies or a decoy receptor (soluble RAGE) can retard the growth of primary tumors, and significantly reduce the spread of metastases. A large number of reports link HMGB1 or RAGE overexpression (or both) to the occurrence of human tumors, and to their invasiveness. We have now shown that extracellular HMGB1 and its receptor RAGE induce both migration and proliferation of vessel-associated stem cells (mesoangioblasts) and thus may play a role in muscle tissue regeneration. In vitro, HMGB1 induces migration and proliferation of both adult and embryonic mesoangioblasts, and disrupts the barrier function of endothelial monolayers. In living mice, mesoangioblasts injected into the femoral artery migrate close to HMGB1-loaded heparin-sepharose beads implanted in healthy muscle, but are unresponsive to control beads.*
*Based on these results, we propose the following working hypotheses: extracellular HMGB1 can work as a soluble growth factor for (some) tumor cells, and as a motogen and/or chemoattractant in the dispersal of tumor cells to give rise to metastases.*

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## Combination treatment of hypomethylating agents and brostallicin in Human prostatic cell lines

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*Brostallicin is a new DNA minor groove binder anticancer drug, whose activity depends on intracellular levels of glutathione/glutathione S-transferase pi.*
*Prostate cancer is characterized by the absence of the enzyme glutathione S-transferase pi, due to the strong methylation of the promoter of its gene.*
*More than 90% of human prostate tumors present this molecular characteristic, which is retained by the tumor cell line LNCaP.*

*In vitro brostallicin shows a low antitumor activity on LNCaP cells ($IC_{50}$ >200 ng/ml), compared to the Du145 prostatic cell line, in which GSTP1 promoter region is unmethylated($IC_{50}$ £30 ng/ml).*
*LNCaP cells were transfected with human GST-pi cDNA and two clones overexpressing GST-pi were selected.*
*Brostallicin was tested and its antitumor activity on these clones is about 5 - folds higher ($IC_{50}$ @ 50ng/ml) than on LNCaP wild type.*

*Combination treatments of demethylatig agents and brostallicin were also performed.*
*The best hypomethylating drug, which has shown to sensibilize LNCaP cells to brostallicin, has been the cytidine analog, zebularine.*
*Zebularine has a lower toxicity on LNCaP cells compared to 5-aza-deoxycytidine, which was the first and the most potent hypomethylating agent sinthetyzed.*
*LNCaP cells were pretreated with zebularine at the concentrations of 50,75,100, 125µM for 96h and 120h; then these cells were treated with brostallicin for 72h and the growth inhibitory activity tested using MTT assay.*
*In these experiments, brostallicin showed a higher antitumor activity respect to controls, not treated with zebularine.*
*GST total activity measurement was performed with cytosolic proteins from LNCaP cells treated with zebularine and using 1-chloro-2,4-dinitrobenzene as substrate.*
*GST total activity was detectable in cells treated with zebularine, but not in the controls.*
*Other hypomethylating agents, which aren't cytdine analogs, were used, such as procaine and procanaimide, but these drugs didn't improve brostallicin antitumor activity.*
*These results indicate that re-expression of the hypermethylated GSTP1 gene increases the activity of brostallicin in LNCaP prostate cancer cells and this could have important clinical relevance for prostate cancer.*

## Vinorelbine,sodium phenylbutyrate and 5-azacytidine eradicates breast cancer cells characterised by epigenetic alterations such as DNA methylation and histone deacetylation.

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*In this study,we prove that DNA methylation is very important in the management of breast cancer patients and determination of patient prognosis. Generally, promoter hypermethylation is an epigenetic alteration*

*which is linked to histone deacetylation and causes repression of gene transcription in breast cancer cells.We examine tumor suppressor genes,candidate tumor suppressor genes and other genes involved in transcription factors,cell differentiation, apoptosis, growth inhibitory signals,metabolism, antiproliferative signals,replicative senescence,angiogenesis,cell adhesion,tissue invasion,metastasis and other important regulatory proteins which have been transcriptionally inactivated due to aberrant methylation of promoter region CpG islands and histone deacetylation. We obtain DNA from serum, ductal lavage fluid and tumor cells excised by surgery from breast cancer patients and we analysed the specimens with RLGS,MCA-RDA,MS-AP-PCR,DMH, Microarray and Gene re-expression analysis, Bisulfite sequencing,Southern blot, Immunoprecipitation,Western blot and IHC.We analyze the specimens before and after monotherapy with vinorelbine and combined regimen composed of demethylating agent 5-Azacytidine,HDAC inhibitor Phenyl-butyrate and MT depolymerizing agent Vinorelbine-tartrate. Before treatment,we observed DNA promoter hypermethylation, gene associated histone deacetylation and transcriptional repression of the following genes: p16(INK4a/CDKN2A), p73, p15 (INK4b/CDKN2B), p14ARF, CCND2, SFN,RARb2, HIN-1,BRCA1, GSTP1,FABP3,HOXA5,p21WAF1/CIP1/SDI1,E-cadherin(CDH1), TIMP3, MGMT,APC, RASSF1A, NOEY2(ARHI), RARb2, MDGI, P27KIP1, Gelsolin, 14-3-3 Sigma(Stratifin),Mad,HMLH1,Nm23-H1,3-OST-2,Maspin, HIC1,MDG1 and Rb.Older patients exhibited enhanced CpG island methylator phenotype (CIMP).Results remained stable after monotherapy with vinorelbine.In contrast,after combined treatment with Vinorelbine,Phenylbutyrate and 5-Azacytidine,we observed inhibition of DNA methylation resulting in activation of gene expression which was accompanied by increased acetylation of DNA associated histones such as H3 and H4 and activation of caspases 3,7 and 9. There was decondensation of chromatin allowing access to endogenous proapoptotic endonucleases. Furthermore, there was activation of genes relevant to irreversible apoptotic cell death,type D2.In contrast,there was downregulation of cyclin D1 and bcl-2. Ki-67 exhibited inhibition of tumour proliferation and MTT assay exhibited inhibition of metabolic activity.Also,we observed inhibition of protein prenylation and downregulation of angiogenic VEGF and HIF-1a. TEM and SEM exhibited zeiosis, cytoplasmic and nuclear condensation leading to karyorexis with endolytic cleavage of the DNA into small oligonucleosomal fragments which were phagocytosed by macrophages and adjacent tumor cells.Concluding,the combined administration of 5-Azacytidine,Phenylbutyrate and Vinorelbine is responsible for the re-expression of genes characterised by DNA methylation and histone deacetylation leading to eradication of breast cancer cells offering a promising new therapeutic option against breast cancer.*

## Close association between DAP-Kinase-positive tumor-associated macrophages and apoptotic cancer cells in the colorectal carcinogenesis

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***Background:*** *The death-associated protein kinase (DAPK) gene is a positive regulator of programmed cell death and is inactivated by promotor hypermethylation in many tumor types. To date little is known about the significance of DAPK inactivation and its role for apoptosis in colorectal carcinogenesis.* ***Methods:*** *We investigated fresh-frozen specimens obtained from 103 patients with primary colorectal carcinomas of advanced stage and 22 formalin-fixed and paraffin-embedded T1 carcinomas showing normal colon mucosa, adenoma and carcinoma tissue on the same slice. We determined aberrant promotor hypermethylation using methylation-*

*specific PCR. DAPK mRNA expression was detected by* real-time *RT-PCR, the protein expression by immunohistochemistry. Apoptotic status was investigated using TUNEL-assay.* ***Results:*** *DAPK methylation was found in 58% of fresh-frozen tissue samples whereas DAPK promotor hypermethylation was even higher in the dissected carcinoma regions from paraffin tissues (80%). Adenomas showed aberrant methylation in 40% of cases with a significant increase in hypermethylation frequency from low-grade to high-grade dysplasia. DAPK promotor hypermethylation correlated significantly with lower mRNA expression. In the unmethylated cases DAPK protein expression dominated in the CD68$^{+}$ macrophages and the tumor epithelium as shown by double immunofluorescence stainings. DAPK-methylated cases showed a complete DAPK protein loss in tumor-associated macrophages and the staining was much weaker in the epithelium. Normal colon mucosa showed a complete lack of DAPK protein expression. Tumors with DAPK promotor hypermethylation showed a reduced apoptotic rate and overexpression of Bcl2 protein. There was no significant association between DAPK inactivation and tumor localization, Dukes classification, age or sex.* ***Conclusions:*** *Our data suggest that hypermethylation of the DAPK promotor occurs early in the colorectal carcinogenesis. DAPK-associated CD68$^{+}$-macrophages induce apoptosis in colorectal cancer cells.*

## Extracellular HMGB1, a signal of tissue damage, induces mesoangioblast migration and proliferation

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*HMGB1 is an abundant chromatin protein that acts as a cytokine when released in the extracellular milieu by necrotic and inflammatory cells. We show here that extracellular HMGB1 and its receptor RAGE may play a role in muscle tissue regeneration, because their interaction induces both migration and proliferation of vessel-associated stem cells (mesoangioblasts). In vitro, HMGB1 induces migration and proliferation of both adult and embryonic mesoangioblasts, and disrupts the barrier function of endothelial monolayers. In living mice, mesoangioblasts injected into the femoral artery migrate close to HMGB1-loaded heparin-sepharose beads implanted in healthy muscle, but are unresponsive to control beads. Surprisingly, mesoangioblasts migrate into dystrophic muscle of alpha-sarcoglycan null mice even if their RAGE receptor for extracellular HMGB1 is disabled. This implies that the HMGB1/RAGE interaction is sufficient, but not necessary, for mesoangioblast homing; a different pathway might co-exist. Although the role of endogenous HMGB1 in the reconstruction of dystrophic muscle remains to be clarified, injected HMGB1 may be used to promote tissue regeneration.*

## Shear Stress Modulates Chromatin Structure and Acts as a Vascular Phenotype Determinant in Mouse Embryonic Stem Cells.

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*Laminar shear stress (SS) modulates histones modifications and activates trancriptional complexes bearing acetyl-transferase activity in adult endothelial cells. Recent evidences, however, indicate that SS may exert morphogenetic functions during cardiac development of zebrafish embryos. In this study we evaluated the effect of SS on histones acetylation, phospshorylation, methylation and activation of specific transcription complexes in mouse embryonic stem cells (ES) as an* in vitro *model of early vascular and cardiovascular differentiation. ES were exposed to a laminar SS of 10 dyne/cm$^2$/sec$^{-1}$, or kept in static conditions (ST) in the presence or absence of the histone deacetylase inhibitor Trichostatin A (TSA). SS enhances lysine acetylation of histone H3 at position 14 (K14) as well as serine phosphorylation at position 10 (S10) and lysine methylation at postion 79 (K79) and cooperates with TSA inducing acetylation of histone H4 and phospho-acetylation of S10 and K14 of histone H3. ES exposed to SS activated the trancription factors MEF-2C and Smad4, which were found complexed to CREB Binding Protein acetyltrasferase (CBP/p300). This effect was paralleled by an early induction of VEGFR-2 expression. These results provide a role for SS in the modification of histones and in the activation of specific transcription factors in mouse ES and indicate a potential molecular mechanism by which vascular differentiation may be regulated by laminar flow.*

## Interruption of tumor dormancy by a short-term angiogenic burst

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*It has long been known that several T cell leukaemia-derived cell lines are non-tumorigenic in immunodeficient mice. We have established an experimental system with one of these cell lines (MOLT-3 in NOD/SCID mice, and have found that these cells persist* in vivo *close to the injection site and are viable up to 4 months after injection; yet progressive tumor growth did not occur. To investigate the possibility of reverting this phenotype, we co-injected irradiated cells from an highly angiogenic Kaposi's sarcoma cell line (KS) or its conditioned medium and observed that this could dramatically improve engraftment and led to progressive growth of a lymphoid tumor formed by MOLT-3 cells.*
*Dormant tumors, found in about 20% of the sites injected with MOLT-3 cells alone, were small and whitish and presented with reduced proliferation compared to the progressively growing and reddish tumors formed in the presence of irradiated KS cells, which arose in 100% of the injection sites. The possible involvement of angiogenesis in this phenomenon was suggested by (I) the broad expression of angiogenic factors by KS but not MOLT-3 cells; (II) the marked induction of angiogenesis by KS cells in vivo, and (III) the observation that co-injection of angiostatin- or interferon-a$_1$-producing KS cells prevented escape from tumor dormancy. On the other hand, KS cells had no measurable effect of MOLT-3 cell proliferation in vitro nor did they affect the behaviour of these cells exposed to apoptosis-inducing agents. More importantly, MOLT-3 cells pre-treated with conditioned medium of KS cells did not become tumorigenic, thus implying that the phenomenon observed mainly depends on an influence of KS-produced soluble factors on the host microenvironment which, in turn, shapes the behaviour of the leukemia cells. The differences between dormant and progressively growing tumors in terms of gene expression profiles and the ERK/p38 phosphorylation status are currently being investigated. These findings*

*indicate that cancer cells may remain dormant due to the lack of an angiogenic potential and that short-term angiogenic bursts may represent an epigenetic change sufficient to break tumor dormancy.*

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*Keywords: angiogenesis/dormancy/leukemia/Kaposi's sarcoma/gene transfer*

## *CDC27* is involved in radiation response in squamous cell carcinoma of the cervix

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*Introduction: Cancer of the cervix is the most common cancer among Indian women. The annual new cases have been projected to be 100,000/year in the country. Most of the cases presenting in the Institute are in Stage IIB and IIIB (80%) and since nearly 90% are poorly differentiated cancers, stage and grade have limited use as prognostic indicators. In spite of adequate conventional radiotherapy treatment, nearly 30-50% fail treatment. Hence, additional prognostic and predictive markers are needed.*
*Purpose: To identify genes involved in radiation response in carcinoma of the cervix.*
*Materials and Methods: Differential display was used to study the expression profile of tumour biopsy samples from patients, responding and not responding to treatment, obtained prior to radiotherapy and subsequent to treatment with Tele-radiation at 10Gy. One of the differentially expressed cDNA's when sequenced and was identified to be CDC27 and Immuno-histochemistry was used to study the expression of this protein. Cervical cancer cell lines were irradiated and their nuclei stained for expression of CDC27 and analyzed using FACS.*
*Results: One of the differentially expressed cDNA was sequenced and was found to be* CDC27 *gene sequence. Immuno-histochemical analysis of pre- and post treatment tumour samples from 15 patients showed that there was down-regulation of expression of CDC27 protein in seven patients. Down-regulation was associated with poorer response. The cell line data (SiHa and C33A) corroborate the above findings - SiHa which is less responsive to radiation than the C33A, had a marked down-regulation of CDC27.*
*Conclusion: This is the first study to suggest a role for* CDC27 *in radiation response. A larger cohort will be needed to confirm the value of CDC27 protein as a predictive marker for radiation response in carcinoma of the cervix. Methylation induced gene silencing, as a mechanism of this down-regulation of CDC27 will also be explored.*

## DNA Deamination in Epigenetic Control

***Svend Petersen-Mahrt****, Heather Coker, Hugh Morgan, & Wolf Reik*

*Epigenetic control can broadly be defined as alteration in the somatic phenotype without direct change in the genotype. Cancer on the other hand was generally believed to be the consequence of phenotypic change due to genetic alteration in the somatic lineage. The recent discovery of programmed DNA deamination as a means to alter genetic information is at the crossroads of epigenetics and cancer, and may provide further evidence for an epigenetic alteration causing cancer. Controlled targeted DNA deamination can lead to a better somatic immune response or elimination of intracellular viral DNA. Consequently mis-regualtion of DNA deamination will lead to cancer. To gain a better understanding of the intriguing process of DNA deamination we have undertaken in situ, genetic and biochemical experiments, with the result that we are able to more directly link DNA deamination with epigenetic control. This has led us to re-evaluate our notion of genetic and epigenetic causes of cancer.*

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## Multiple Markers for Melanoma Progression Regulated by DNA Methylation: Insights from Transcriptomic Studies

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*The incidence of melanoma is increasing rapidly, with advanced lesions generally failing to respond to conventional chemotherapy. Here, we utilized DNA microarray-based gene expression profiling techniques to identify molecular determinants of melanoma progression within a unique panel of isogenic human melanoma cell lines. When a poorly tumorigenic cell line, derived from an early melanoma, was compared with two increasingly aggressive derivative cell lines, the expression of 66 genes was significantly changed. A similar pattern of differential gene expression was found with an independently derived metastatic cell line. A considerable proportion of the differentially expressed genes found have been previously associated with melanoma development and progression, including* CDKN2A, IL-24, AIM1 *and* MAGEA4. *In addition, a range of novel markers were identified that correlated here with melanoma progression. Most notable was* TSPY, *a Y chromosome-specific gene that displayed extensive down-regulation in expression (between 137 and 317 fold) between the parental and derivative cell lines. Examination of a putative CpG island within the* TSPY *gene demonstrated that this region was hypermethylated in the derivative cell lines. Moreover, treatment of the derivative cell lines with the DNA methyltransferase inhibitor, 2'-deoxy-5-azacytidine (DAC), restored expression of the* TSPY *gene to levels comparable to that found in the parental cells. Additional DNA microarray studies uncovered a subset of 13 genes from the above-mentioned 66 gene cohort that displayed re-activation of expression following DAC treatment, including* TSPY, CYBA, *and* MT2A. *These data support the hypothesis that multiple genes are targeted, either directly or indirectly, by DNA hypermethylation in this melanoma model system. DAC suppressed tumor cell growth and migration* in vitro. *Moreover, systemic treatment of mice with DAC attenuated growth of melanoma xenografts. This study provides further support for the incorporation of demethylation agents into clinical trials for the treatment of melanoma.*

## Genome-wide measurement of genomic instability in somatic stem cells during aging.

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*It has been hypothesized that genomic instability within somatic stem cells leads to dysfunction in this cellular compartment and contributes to both cancer and other age related phenotypes. However, direct measurement of the extent of genomic instability in aging tissues has been problematic. Analyses that rely on information*

*derived from whole tissues can miss specific changes present in a subset of cells due to the fact that they are being averaged among the progeny of multiple stem cells. Sampling mutation rates at specific loci, or using artificial reporters, can result in biased estimates of mutation rates and does not allow detection of endogenous genes that are under positive or negative selective pressure* in vivo. *Here we have utilized clonally derived neural stem cells and comparative genomic hybridization (CGH) technology to survey the changes present in derivatives of individual neural stem cells (NSCs) and compared the frequency with which changes are observed in young (2 month) and old (2 year) mice. Neural stem cells derived from the brains of young and old C57BL/6 mice were isolated and expanded 6-7 divisions. DNA isolated from the neurospheres was analyzed using CGH. Initial CGH results performed on a small set of neurospheres identified a definitive deletion on chromosome 8 at position 22921K-23146K. PCR was used to confirm the CGH results and screen additional neurospheres from young and old animals. Events identified in neurospheres analyzed from an old animal ranged from no events, to a complete absence of specific product corresponding to a full deletion event, as well as events indicating loss of one allele. In contrast, neurospheres analyzed from a young animal showed no full deletion events and only one sample showed the loss of one allele. These data demonstrate the accumulation of genetic changes in the NSC compartment as a function of age and suggest that as many as 30 – 40% of the NSCs found in old animals carry at least some genomic defects.*

## Derivation and characterisation of neural stem cell lines from ES cells

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*Neural stem cells can be defined as cells capable of undergoing unlimited cell divisions whilst maintaining potency to generate both neurons and glia. Cells with such properties have been isolated from mammalian embryos and adults and are typically cultured as aggregates known as neurospheres. It is difficult, however, to culture mouse or human neural stem cells indefinitely as homogeneous populations in adherent culture without use of immortalisation strategies. Here we report the efficient and reproducible isolation of neural stem cells from mouse ES cells that undergo prolonged symmetrical self-renewal in monolayer culture. These EGF-dependent cells have elongated bipolar extensions, and express molecular markers of radial glia (RC2, GLAST, BLBP and Pax6). Our cell lines display neural stem cell properties both in vitro and in vivo upon transplantation in the embryo and adult brain. These neural stem cells can readily be derived from wild-type or genetically modified ES cells and offer a new experimental window into self-renewal and fate choice in the nervous system. They may also represent an unlimited homogeneous source of material for cell based neural therapies. We are currently investigating the derivation of similar adherent neural stem cells from primary mouse and human tissues.*

## Epigenetic Dynamics of X-chromosome Inactivation During Pre-iplnatation Develop-<ment of mouse

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*During the early development of female mammals, one of the two X chromosomes is transcriptionally repressed and transformed into heterochromatin. This*

*process, known as X inactivation. We are investigating the events underlying imprinted (extra-embryonic) and random (embryonic) X inactivation in mouse embryos. In particular, we have shown that although initially active, the paternal X chromosome (Xp) undergoes imprinted inactivation much earlier than previously thought, from the cleavage stages, well before the first signs of cellular differentiation. Reversal of this inactive state then occurs in the inner cell mass (ICM), which gives rise to the embryonic lineage. This reveals the remarkable plasticity of the X-inactivation process during early development and underlines the importance of the ICM in the reprogramming of epigenetic marks.*
*Our current work is focused on understanding the reprogramming events in the ICM and the epigenetic difference between imprinted and random X inactivation.*

## Apoptosis induction in poorly differentiated thyroid cancer cells: effect of the histone deacetylase inhibitor valproic acid

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*Thyroid tumors evolved towards dedifferentiation as well as those that are poorly differentiated from the start, have accelerated growth and, especially, fail to respond to traditional therapy. Histone deacetylase inhibitors are a new class of therapeutic agents able to induce tumor cell apoptosis and/or cell cycle arrest. Among others, valproic acid (VPA), a potent anticonvulsant that is able to inhibit histone deacetylase, is being evaluated for its application in the treatment of thyroid cancer.*
*Two different cells lines of poorly differentiated papillary carcinoma (N-PA and BHT-101 cells) were treated with VPA (0-3 mM) for different times up to 11 days. Cell proliferation was evaluated with the MTT technique during the whole period. For apoptosis studies, cells were treated for 72 hours with VPA and apoptosis was evaluated using Cell Death Detection ELISA* [PLUS]*. Finally, proteins extracted from N-PA and BHT-101 cells treated with VPA were used to evaluate PARP cleavage as apoptosis marker.*
*VPA significantly reduced N-PA and BHT-101 growth in a dose-dependent manner. The anti-proliferative effect of VPA might be due to apoptosis induction, since an increase in apoptotic index was evident both in N-PA and BHT-101 VPA treated cells. A specific western blot for PARP showed a significant cleavage consistent with apoptosis induction in cells treated with VPA.*
*In conclusion, the histone deacetylase inhibitor valproic acid is effective* in vitro *in reducing the proliferation of poorly differentiated thyroid cancer cells, acting through apoptosis induction.*

## Nuclear Reprogramming in *Xenopus laevis*.

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*Nuclear reprogramming are modifications in the genetic activity occurring artificially when a nucleus is transferred into a different cytoplasmic environment. If the nucleus is transferred to an egg, the differentiated nucleus recovers its totipotent capacity. Despite the fact that several decades ago it was shown that a frog egg was able to reprogram a somatic nucleus, the mechanisms of how this occurs are still poorly understood. Epigenetic marks are likely to be involved in differentiation and hence erased upon reprogramming. We are using* in vitro *assays, derived from* Xenopus laevis *to try to follow marks that could be removed or brought in during such events. We will present our data on this topic.*

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## DNA methylation as a basis for the aberrant expression of the human kallikrein 6 gene in breast tumor cells

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*The gene encoding human kallikrein 6 (protease M/zyme/neurosin) (KLK6) was originally identified by differential display based on its absent expression in a metastatic breast tumor as compared to the primary tumor derived from the same patient. In addition, the KLK6 gene was found highly overexpressed in a subset of breast and ovarian primary tumor tissues and cell lines, but downregulated or inactivated in corresponding metastatic tumors. Recent studies suggest that kallikrein 6 is a serum biomarker for ovarian cancer but may also be involved in pathologies of the CNS, such as Alzheimer's disease, Parkinson's and multiple sclerosis. The mechanism of the aberrant regulation of the KLK6 gene expression in tumor cells is currently unknown. Southern analyses showed no gross genetic alterations of the KLK6 gene in non-expressing tumor cell lines suggesting a transcriptional mechanism of KLK6 regulation during tumor progression. Expression profiling in squamous carcinoma cells as well as in colon cancer cells revealed that induction of differentiation by vitamin D3 analog EB1089 [1a±,25-(OH)2D3] is associated with remarkable induction of KLK6 gene expression.*
*Here, we show that methylation of CpG islands located in the KLK6 promoter underlies the aberrant expression of KLK6 in breast tumor cells. Demethylating agents, such as 5-aza-2'-deoxycytide, strongly induced expression of the KLK6 gene in non-expressing breast tumor cell lines both at the mRNA and protein levels. Trichostatin A, a potent inhibitor of histone deacetylases, could partially restore KLK6 expression in some tumor cell lines, indicating a dominant role of DNA methylation over histone deacetylation in KLK6 gene silencing. Both methylated and unmethylated alleles were detected in different cell lines. Using methylation-specific PCR and sequence analyses of sodium bisulfite-treated genomic DNA, we found a strong correlation between hypermethylation at CpG islands clustered in the promoter region, at positions -72, -64, -56, -53, -35, +98, +115, +128, and tumor-specific loss of KLK6 expression. On the other hand, complete hypomethylation of the identified positions underlies the overexpression of KLK6 in a subset of breast tumor cell lines. The identified CpG islands were found semi-methylated in normal mammary epithelial cells known to express low mRNA levels of KLK6. Our data shows that stable DNA methylation leads to silencing of KLK6 gene in metastatic breast tumor cells allowing its pharmacological modulation.*

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## Stem Cells: watch out for the neighborhood

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*Stem cells gather considerable attention for their ability to differentiate into various dedicated cell types. Apparently this holds true for embryonic stem cells as well as adult, bone marrow derived stem cells. There is increasing evidence that the latter physiologically take part in repair of peripheral tissue. The fact that bone marrow derived stem cells differentiate at a distant location into specific organ cells implies that the differentiation signal is generated in the local*

*environment of tentative tissue repair. This local instruction has to guide the changes from a stem cell, by definition endowed with the ability for self-renewal, to a differentiated, dedicated cell with limited potential for cell division. This implies that stem cells find the complete set of information in the local milieu where the transdifferentiation takes place. The information could be transmitted through soluble factors (e.g. cytokines), cell-cell contact (e.g. cell-adhesion-molecules), cell fusion or a combination thereof.*
*Conceivably an incomplete set of instructions could lead to a cell whose phenotype is "in between" that of an indefinitely dividing cell and an organ specific, terminally differentiated cell with limited potential for cell division. A cancerous tumor is an environment that itself has*
*lost many of the characteristics of the tissue of origin. It is therefore hypothesized that the tumor surroundings would give an ambiguous and incomplete set of signals to a stem cell on its way to full differentiation, raising the possibility of stem cells being "recruited" into the proliferative characteristics of the malignant process.*
*However, very little is known about the detailed mechanisms of the transdifferentiation process and its possible pitfalls. Nevertheless, in spite of these obvious uncertainties, various clinical trials presently try to leverage the tissue repair potential of stem cells. May be after all stem cells are not the cure all they are taken for.*

## Study of lentiviral vectors with two marker genes driven by two independent promoters for gene transfer applications.

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*Recombinant lentiviruses are widely used vectors for in vitro and in vivo long term gene transfer. Many of the initial gene transfer studies were conducted using single reporter genes. However, for many gene delivery applications, expression and detection of two genes are useful. The goal of this study is to explore the feasibility of directing the independent expression of two marker genes from a single lentiviral vector derived from HIV-1. We used the enhanced green fluorescent protein (EGFP) and the enhanced yellow fluorescent protein (EYFP). Because of spectral overlap of these fluorescent proteins, we modified a FACScalibur flow cytometer with insertion of Omega Optical filters. To coexpress independently two marker genes, we used two different promoter associations in lentiviral vectors. In a first strategy, EGFP transcription was driven by the EF1alpha promoter, and EYFP was translated from spliced RNA transcribed from the LTR promoter. In a second strategy, two internal promoters : EF1alpha and CMV were inserted in a SIN (Self Inactivating) vector. In order to coexpress efficiently both transgenes, different vectors were generated from the pTRIP deltaU3 EF1alpha EGFP and pTRIP EF1alpha EGFP lentiviral vectors (gifts from P. Charneau), with transcriptional units and viral elements in variable positions. Following transduction of hematopoietic and non hematopoietic cell lines, FACS and PCR analyses allowed to quantify transduction and integration efficiencies, respectively. Addition of EYFP in the original single gene lentiviral vector resulted in a decrease in the overall percentage of fluorescent cells and in the integration efficiency. Moreover, vectors using splice mechanism produced a weak coexpression of both genes (less than 4% of double positive cells). However, one of the SIN vector that contains two internal promoters transduced 32 to 100% of cells, depending on the MOI ; of these fluorescent cells, 12 to 78% were double positive.*
*Thus, a dual-promoter lentiviral vector can coexpress two transgenes efficiently in a single transduced cell. Insulator sequences could be inserted on both sides of one transcriptional unit to reduce transcriptional interference between promoters. This vector could enable many gene transfer applications, especially targeting studies with a lineage-specific promoter.*

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## Nuclear b-catenin expressing tumor cells at the invasive front of colorectal carcinomas: migrating tumor stem cells?

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*Most colorectal adenocarcinomas (CRC) have mutations in the APC tumor suppressor gene leading to overexpression of the Wnt-pathway effector b-catenin. Membranous b-catenin is associated with E-cadherin. In contrast nuclear b-catenin has an oncogenic function by acting as a transcriptional activator of target genes associated with tumor progression.*
*We have shown, that nuclear b-catenin predominantly accumulates in de-differentiated tumor cells at the invasive front, which stop prolifation and have the ability to migrate. The amount of these cells strongly correlates with clinical outcome and metastasis formation. In contrast tumor cells in central tumor areas are differentiated, forming tubular structures and often show membranous, E-cadherin-bound b-catenin. Strikingly after dissemination, tumor cells in metastases also show again a differentiated phenotype lacking nuclear b-catenin.*
*Nuclear b-catenin is decisively involved in two fundamental processes in embryonic development: epithelial to mesenchymal transition (EMT) and generation of stem cells. Based on these facts and our data we propose, that the de-differentiated tumor cells at the invasive front of CRCs act as "migrating tumor stem cells" which can re-differentiate and, depending on the range of dissemination, give raise to the primary carcinoma or metastases.*
*In support of this hypothesis, we could define various b-catenin target genes, selectively overexpressed in this cell population of invading tumor cells, by using reporter assays, EMSA, ChIP, siRNA techniques, in situ hybridisation, immunohistochemistry and animal models: A stem cell phenotype is supported by the b-catenin target genes hTERT and $p16^{ink4A}$, which also explains the observed growth arrest in the invasive tumor cells. Furthermore three other identified target genes, MMP-7, MT1-MMP and the g2 chain of laminin-5, synergize to exert a strong promigratory activity.*
*Taken together we suggest that both primary tumor and corresponding metastases of CRC are derived from a pool of de-differentiated "migrating tumor stem cells", which are defined by strong nuclear b-catenin accumulation and subsequent expression of its target genes. This gives the cells a feature, which drives malignant tumor progression including metastasis: the unusual combination of abilities allowing simultanously to migrate and behave as an epithelial tumor stem cell.*

## Ex vivo propagation of breast tumor-initiating cells with stem/progenitor cell properties.

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*Several lines of evidence have been reported demonstrating that tumors are heterogeneous in their cellular composition and organized according to a hierarchical model. Tumor-initiating capability has been recently demonstrated to be restricted to a limited subpopulation of stem-like cells in acute and chronic myeloid leukemia, as well as in brain and breast cancer; in particular, breast cancer tumor-initiating cells have been identified as $CD44^{+}/CD24^{-/low}$ cells.*
*According to these findings, we have tried to isolate and propagate stem/progenitor-like cells from solid breast cancer lesions. Single cell suspensions deriving from mechanical digestion of 15 surgical specimens were plated at clonal density (less that 1000/ml) in serum-free cell culture medium, supplemented with EGF, bFGF and insulin. In 5 cases, 8-10 days after plating formation of non-adherent spherical clusters of cells could be observed in culture. Single cells*

*deriving from primary spheres dissociation gave rise to secondary spheres; this procedure could be repeated for several passages resulting in an extensive amplification in cell number. Non-adherent spherical clusters formed even when 1 single cell/well was plated, thus demonstrating that cultured cells comprise a self-renewing population. Isolated cells were undifferentiated since they lack lineage-specific markers of the mammary gland epithelium; in presence of serum they could adhere and differentiate giving rise to heterogeneous cell cultures. Taken together these data suggest that isolated cells are endowed with stem/progenitor cells properties. Interestingly, the majority of cultured cells are CD44+/CD24-, exhibit telomerase activity, over express the inhibitor of apoptosis proteins* survivin *and can give rise to a new tumor even when only $10^3$ cells are injected in the mammary fat pad of SCID mice. When an histone-deacetylase inhibitor was added to culture medium, we observed a marked inhibition in cell proliferation and decrease in the percentage of S-phase cells; interestingly, in these experimental conditions, spheres appeared to be more regular in their morphology, most likely because of an enhancement in cell-to-cell interaction.*

*In conclusion, our isolated cells represent purified cultures of tumor-initiating cells with stem/progenitor cell properties and could therefore provide a unique in vitro model to study breast cancer stem cells.*

## Cancer prevention in growing countries NCI, Cairo experiment

***Prof. Ibrahim Abdel Salam***
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*Environmental carcinogenesis is one of the most important causes of cancer in growing countries due to environmental pollutants.*

*The cleaning out of the environment from environmental carcinogens may take long time, instead the use of some natural products in diet may prevent or minimize cancer incidence and cellular transformation .*

*The NCI Cairo experiment is the use of Nigella sativa (N.S.) in cancer prevention.*

*N.S. is a natural product cultivated in Egypt and many other African and Asian countries and known also as black seeds. N.S. ethanolic extract could inhibit the growing up of E.A.C. cells inoculated in mice, inhibited ascitic fluid formation, elongated the life span of the animals and had no side effects on liver, kidney functions and many other blood chemistry. N.S. could inhibit bilharzial infestation induced in experimental animals and reduced DNA increase due to bilharzia ova and kept albumin m-RNA normal.*

*The oil of N.S. was shown to have radioprotection property when used 3 days before total body irradiation of rats.*

*Also N.S. is considered as a new inhibitor of both arylamine activating cytochrome P-450 and O-acetyltransferase and an antimutagenic natural product with a promising role in limiting and controlling the carcinogenic effect of contaminated food with carcinogens. Hepatocarcinogenesis, collangiocarcinoma and breast carcinogenesis were also inhibited using 5% N.S mixed with animals diet.*

*The combined effects of lead, nitrate, noise stress and tobacco smoke as common pollutants in the Egyptian society on the incidence of malignant transformation among offspring rats through transplacental transfer from mother to fetus were shown, while the protective effect of N.S. was also confirmed. Chromatographic techniques on N.S. revealed the presence of essential oils ( thymoquinone 30%, P- cymene 38% and α - pinene 14% while expressed oils were stearic 47% arachidic 32% beside lesser amounts of plamitic 7% and myristic 4% α - amyrin, B-sitosterol and oleanolic acid were isolated from the unsaponifiable matter of lipid portion. Chemical analysis of N.S. revealed the presence of protein 21.1%, fat34.3% fiber5.1% Ash 3.4%, moisture 0.51% and CHO, 35.59% .*

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## Stem cell biology of the human prostate epithelium: Implications for prostate carcinogenesis

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### Introduction

*The human prostate epithelium is composed of a basal and luminal cell compartment. While luminal cells secrete proteins like Prostate Specific Antigen (PSA) into the glandular lumina, basal cells are proposed as stem cells of the entire prostate epithelium. Although prostate adenocarcinoma is the most frequent malignancy among western men, its progenitor cell of malignant transformation is still unknown.*

### Subjective

*Study of prostate epithelial differentiation and its implications for prostate cancer development*

### Methods

*Immunofluorescent triple-staining for keratin (K) 5, K14 and K18 were performed on epithelial cell cultures as well as normal and malignant prostate tissues. Epithelial subpopulations were further characterized using antibodies to* c-met *receptor, p27kip1, Ki-67.*

### Results

*Within the human prostate epithelium four cell populations are discriminated by their keratin expression-profile. While basal cells co-localize K5 and K14 combined with low levels of K18 ($K5^{++}/14^{++}/18^{+}$), luminal cells highly express K18 ($K18^{++}$). In addition, two intermediate subpopulations are characterized either by basal $K5^{++}/18^{+}$- or luminal $K5^{+}/18^{++}$- expression. In vitro, immunophenotypically basal cells ($K5^{++}/14^{++}/18^{+}$) give rise to intermediate cells and luminal cells ($K18^{++}$) indicating that the entire prostate epithelium is derived from a basal stem cell. It is postulated that the stem cell population ($K5^{++}/14^{++}/18^{+}$) gives rise to intermediate cells (basal $K5^{++}/18^{+}$ and luminal $K5^{+}/18^{++}$) that indeed express the HGF-receptor* c-met, *the proliferation marker Ki-67 and are negative for the cell-cycle-dependent kinase inhibitor p27. After migration and expansion the intermediate subpopulation ultimately shows terminal differentiation ($K18^{++}$,* c-met *negative).*
*Within human prostate carcinoma luminal and intermediate cells ($K5^{+}/18^{++}$) can be discriminated while basal cells are absent. Interestingly, the number of intermediate cells (K5,* c-met*) is enhanced in androgen-deprived and hormone-escaped prostate cancer. Stimulation of the intermediate prostate cancer cell line DU-145 with HGF leads to cell scattering and increased invasive capacity, suggesting that intermediate cells play a role in prostate cancer invasion.*

### Conclusion

*Intermediate cells are postulated as progenitors for prostate carcinogenesis and targets for androgen-independent tumour progression. Androgen-independency is associated with an enrichment of intermediate cells and over-expression of peptide growth factor receptors like*
c-met. *Targeting intermediate cells by inhibition of their peptide growth factor receptors, therefore, offers novel treatment modalities for prostate cancer.*

## Global loss of mono-acetylated histone H4 in cancer

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*It has been well established that a wide variety of CpG island-associated tumor suppressor genes become methylated and silenced in cancer. Concurrently, several heterochromatic regions rich in repetitive DNA lose 5-methylcytosine. In this work, we attempt to answer whether the observed opposite status of genomic DNA methylation in promoters and repetitive sequences is associated with specific modifications of the histone H4 tail. We compared a panel of 24 human cancer cell lines with 80 samples of normal lymphocytes from healthy volunteers and found a global decrease of monoacetylated histone H4 (35%) and trimethylated histone H4 isoforms (15%) in tumor samples using three independent methodologies (high performance capillary electrophoresis, mass spectrometry and western blotting). Colon and lymphoma human primary tumors also showed a reduction of monoacetylated histone H4 (10-20%) and trimethylated histone H4 isoforms (5-10%) when comparing with their normal counterparts. Western blotting with antibodies against specific acetylated and methylated residues within the histone H4 tail and also tandem mass spectrometry of tryptic histone H4 peptides derived from the same samples showed that the decrease in monoacetylated histone H4 in tumors mainly occurs at lysine 16, whilst loss of trimethylation takes place at lysine 20. The relationship between DNA methylation and histone H4 acetylation at specific positions was studied by chromatin immunuprecipitation revealing that only lysines 5 and 12 were significantly hypocetylated in the promoters of genes that became hypermethylated in cancer. Moreover, the satellite 2 repetitive DNA sequences, which are hypomethylated in colon cancer cells lacking DNMT1 also lack acetylation at lysine 16, thus suggesting a relationship between DNA hypomethylation and histone H4 acetylation in these regions. These data have important implications for understanding the relationship between DNA methylation and histone H4 modification, suggesting discrete epigenetic states for CpG island-containing promoters and repetitive DNA associated chromatin regions in cancer.*

## Possible role of genes related to DNA methylation process as modifiers of BRCA1/2 gene penetrance

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*BRCA1 and BRCA2 gene mutations are responsible for about 30% of all families with hereditary breast and/or ovarian cancer. However, significant variations in the level of risk associated with a mutation of BRCA1/2 genes have been highlighted, suggesting the existence of modifiers of BRCA1/2 gene penetrance. DNA methylation is an important mechanism of transcriptional regulation. Cancer cells might have aberrant patterns of DNA methylation. Mutations in enzymes involved in SAM (S-adenosylmethionine) metabolism, such as MTHFR (methylene tetrahydrofolate-reductase), MS (methionine synthase) and CBS (cystathionine beta-synthase) are good candidates for affecting the pattern of DNA*

*methylation. The aim of the present study is to investigate the role of genes related to DNA methylation process as possible modifiers of the BRCA1/2 gene penetrance. To address this matter we genotyped 71 affected and 27 unaffected BRCA1/2 mutation carriers (of which 47 affected and 18 unaffected BRCA1-carriers and 24 affected and 9 unaffected BRCA2-carriers) and two control groups: one group of healthy individuals and one group of cancer patients matched by age and by tumour type. Four polymorphisms have been considered: C677T and A1298C for the MTHFR gene, A2756G for MS gene and Ins68bp for the CBS gene. The polymorphism analysis has been performed by means of PCR and RFLP's. The Hardy-Weinberg equilibrium was respected in each group. A Kaplan-Meier estimate on age of disease onset showed that the BRCA1 carriers with the genotype CT at the position 677 of the MTHFR gene showed a lower age of onset of the disease compared to the CC genotype, whereas the BRCA2 carriers heterozygous for the A2756G of the gene MS exhibited an higher age of onset of the disease. A multiple logistic regression analysis revealed an increased risk of breast cancer (Odd Ratio 3.8; P=0.04) in BRCA1 individuals older than 45 years of age, carrying the ins68bp allele of the CBS gene. The identification of specific genetic profile that act as BRCA gene penetrance modifiers opens up the possibility of a better cancer risk evaluation and a better rational approach to preventive strategies.*

## Promoter hypermethylation of the DNA repair gene MGMT is more frequent in secondary glioblastomas and is indipendent from other prognostic factors

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*$O^6$-methylguanine-DNA methyltransferase (MGMT) is a DNA repair protein that specifically removes promutagenic alkyl groups from the $O^6$ position of guanine in DNA. Repair of cytotoxic DNA damage by MGMT is a potentially important factor of resistance to alkylating chemotherapeutic agents, commonly used in the treatment of glioblastoma multiforme (GBM) since it reduces the cytotoxicity of these drugs.*
*We assessed the inactivation of the DNA-repair gene MGMT by promoter hypermethylation using Methylation-Specific PCR (MSP) in 45 GBM obtained from patients subsequently treated by conventional radiotherapy and CDDP+BCNU. We observed that the MGMT gene was methylated in 15 patients (33%).*
*This finding was associated with prolonged overall survival (25 versus 14 months; log-rank p=0.026) and with a longer Progression Free Survival (PFS) (11 versus 7 months; log-rank p=0.037). Secondary GBMs had prolonged overall survival (30 versus 11 months; log-rank p=0.0030) than*
de novo *tumors, whereas other prognostic factors were not statistically associated with ST or PFS. Moreover, methylation status was more frequent in secondary than in primary GBMs (70% versus 23%, p=0.0091), but was not associated with other clinical parameters. Other genetic markers as EGFR amplification, p53 mutations and microsatellite analysis for loss of heterozigosity are under study to assess their influence on the treatment response and overall survival of patients with GBM.*

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# 2005 Pezcoller Foundation-AACR International Award for Cancer Research

*The Pezcoller Foundation-AACR International Award for Cancer Research is given annually to a scientist anywhere in the world who has made a major scientific discovery in the field of cancer, who continues to be active in the field, and whose ongoing work holds promise for future substantive contributions to cancer research. The Award recognizes extraordinary basic or translational cancer research. The Award will be presented to a single investigator for his or her highly original work. In extraordinary circumstances, two individuals may be selected to share the award when their investigations are clearly related and have resulted in prizeworthy work. The Awardee will be selected by an International Committee of AACR members appointed by the AACR President with the agreement of the Council of the Pezcoller Foundation. The selection will be made solely on the basis of the Awardee's scientific accomplishments without regard to race, gender, nationality, geographic location, or religious or political views.*

*The Pezcoller Foundation was established in 1980 by Professor Alessio Pezcoller, a dedicated Italian surgeon who made important contributions to medicine during his career and who, through his foresight, vision and generous gift in support of the formation of the Foundation, stimulated others to make significant advances in cancer research. Over the past decade the Pezcoller Foundation, in collaboration with the ESO-European School of Oncology, gave a major biennial award for outstanding contributions to cancer and cancer-related biomedical science.*

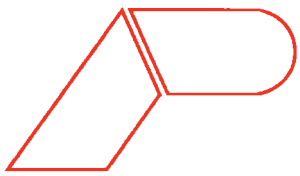
*The American Association for Cancer Research (AACR) was founded in 1907 by eleven physicians and scientists dedicated to the conquest of cancer and now has over 19,000 members in more than 60 countries who are experts in basic, clinical, and translational cancer research. The AACR is dedicated to its mission of preventing and curing cancer through the communication of important scientific results in a variety of forums including publications, meetings and training and educational programs. Because of the commitment of the Foundation and the AACR to scientific excellence in cancer research, these organizations are now collaborating annually on the presentation of this Award.*

*This will strengthen international collaborations and will be a catalyst for advancements in cancer research internationally.*

*The winner of the Pezcoller Foundation-AACR International Award for Cancer Research will give an award lecture during the AACR Annual Meeting and will receive the award in a ceremony at the Foundation's headquarters in Trento, Italy, after the AACR annual meeting. The award consists of a prize of 75.000 Euro and a commemorative plaque.*

**Nomination Deadline: Friday, September 17, 2004**

*Questions about the nomination process should be directed to the AACR Office (Philadelphia) via Fax at (215) 440 9372- Telephone (215) 440 9300 - or E-mail awards@aacr.org - www.aacr.org*



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