

# Identification and validation of new molecular and cellular targets for novel anti-cancer strategies

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The elucidation of the cascade of molecular alterations that lead to cancer is a prerequisite for the development of effective therapies through the identification of rational targets. Recent years have witnessed remarkable success with therapies that are based on a thorough understanding of the molecular pathogenesis of cancer. This progress has proceeded alongside a conceptual and experimental reframing of cancer as a complex developmental problem that requires the characterization of the stem cell compartments, the epigenetic levels of regulation and the interactions between the cancer cell and its host.

The unraveling of this complexity offers the opportunity to uncover several options for molecular therapy by drawing on the molecular pathways at different levels of regulation and hence on different aspects of the tumor phenotype. Consistent with this objective, we have focused on: i) the alterations in genetic and epigenetic regulatory networks; ii) the actual aberrations in cell division; iii) the emerging links between cancerous growth and the molecular environment of the host and iv) the diagnostic and predictive value of genetic and epigenetic markers, and their role in the choice of the most appropriate therapies. These four aspects frame cancer development as a cascade of molecular events amenable to experimental validation and therapeutic intervention, in which the derangement of growth and differentiation programs (through genetic and epigenetic mechanisms) leads to abnormal cell division in the context of the host tissue and its microenvironment. A fruitful interaction with the clinical departments enables us to test the relevance of new molecular markers for diagnostic, prognostic and therapeutic improvements, aiming at a rapid translation of basic findings to the clinical setting.

## 1. Genetic and epigenetic deregulation

### a. Probing the cancer networks with genome wide approaches (Myriam Alcalay)

The main focus of the lab is to understand transcriptional networks involved in oncogenesis by exploiting the integrated use of high-throughput technologies, which represent powerful tools for the discovery and analysis of genetic networks underlying cancer. We are currently focusing on two main lines of research:

### 1. *Molecular signature of Acute Myeloid Leukemia*

The AML1/ETO oncogenic transcription factor functions to initiate Acute Myeloid Leukemia by recruiting co-repressor complexes to DNA. We performed an integrated study exploiting genome-wide chromatin immunoprecipitation and expression profiling coupled to bioinformatics analysis with the aim of identifying determinants of AML1/ETO-dependent transcriptional regulation. We found that:

- i) surprisingly, AML1/ETO direct target genes can be up-regulated, and there is differential enrichment of transcription factor binding sites in the promoters of downregulated and upregulated genes.
- ii) AML1/ETO binding regions are characterized by the presence of the consensus binding sites for specific transcription factors, including AML1, AP.1, Ets-1 and HEB.
- iii) AML1 and AML1/ETO are often present simultaneously on the same DNA regions, demonstrating that the fusion protein follows the binding pattern of its native counterpart AML1, but does not function primarily by competing with it.
- iv) Upon expression of AML1/ETO, the DNA binding pattern of the E-protein HEB is grossly rearranged, and HEB is recruited to AML1/ETO target regions. We believe the functional interaction of AML1/ETO with AML1 and HEB a major role in transcriptional regulation determined by the fusion protein.

### 2. *The PRDM gene family in cancer*

PRDM5 functions as a transcriptional repressor by recruiting histone methyltransferase G9a to DNA, and behaves as a putative tumor suppressor in different types of cancer. We identified transcriptional targets of PRDM5 in U2OS cells and found critical genes involved in modulating Wnt signaling, which is a critical pathway during development. We therefore performed overexpression or depletion experiments for PRDM5 in the zebrafish model system, and found impairment of morphogenetic movements during gastrulation and during development of anterior neural structures and optic vesicles, leading to abnormal eye formation and cyclopia. Our results suggest that PRDM5 regulates components of canonical and non-canonical Wnt pathways, and overall negatively modulates Wnt signaling in vivo. Inactivation of PRDM5 may, therefore, represent a

yet unidentified mechanism of constitutive activation of Wnt signaling in human tumors.

#### b. Histone methylation in lineage commitment and maintenance (Giuseppe Testa)

The methylation of histones on lysine tails (HLM) is a central mechanism of lineage determination. HLM is developmentally regulated, undergoing changes at specific loci during the differentiation of both embryonic and tissues stem cells. Until recently the prevailing view held that histone methyl marks were stable and that HLM could be reversed only by histone replacement. Over the last three years the identification of several histone demethylases (HDMs) specific for particular lysines has challenged this model, suggesting that HLM is more dynamic than anticipated and that the establishment and maintenance of cell lineages results from a regulated process of addition and removal of methyl marks. In particular, the realization that Ezh2, a member of the Polycomb group (PcG) of proteins first discovered in the fly as stable repressors of homeotic genes, catalyzes the methylation of histone H3 on lysine 27 (H3K27) suggested a central role for this modification in the process of gene silencing that accompanies differentiation. In ES and neural stem cells several genes bound by PcG and marked by H3K27 trimethylation are repressed and become activated during differentiation, suggesting that PcG-mediated repression keeps cells multipotent by repressing lineage commitment. Recent studies have refined this notion revealing that key developmental genes in ES cells are kept in a state poised for activation by a bivalent chromatin signature that features both H3K4 and H3K27 trimethylation (associated with, respectively, gene activation and gene repression). Upon differentiation these bivalent domains are resolved in a lineage specific fashion, hinting at HDMs as possible effectors of this crucial epigenetic transition. Consistent with the role of PcG in lineage choices, alterations in H3K27 methylation are hypothesized to be early key events in the cascade of epigenetic aberrations of cancer, particularly the hypermethylation of CpG promoters that is a hallmark of carcinogenesis and an important mechanism of tumor suppressor inactivation. The recent observation that the majority of genes that are hypermethylated in cancers are pre-marked by H3K27 methylation underscores the relevance of deranged H3K27 methylation in oncogenesis, and grounds in molecular detail the notion of cancer as a developmental disorder of aberrant differentiation. In order to dissect the contribution of histone lysine methylation and demethylation to lineage commitment and tumorigenesis, we study mouse ES cells because they can be genetically altered with precision and they can be differentiated *in vitro* into all cell types, and *in vivo* through the derivation of mutant mice.

#### 1. Conditional approaches to the role of histone methyltransferases (HMTs) *in vivo*.

Our work focuses on the functional characterization of MLL, MLL2 and SETD7, all of which are implicated in oncogenesis. MLL is one of the genes most frequently translocated in human leukemias and its close homolog MLL2 is amplified in human cancer cell. SETD7 is a unique HMT because, besides the methylation on lysine 4 of histone H3 (H3-K4) through which it promotes gene specific expression, it also methylates p53 and the basal transcription factor TAF10. Methylation by SETD7 stabilizes p53 and promotes its activity on the target genes, suggesting that SETD7 might operate at the crossroad of pathways that control lineage specific gene expression and proliferation.

In order to inactivate these genes in both a constitutive and a conditional manner, a versatile gene targeting strategy was employed that we recently pioneered and is now referred to as 'knock-out first' (Testa et al., *Genesis* 2004). It relies on phage mediated homologous recombination in *E. coli* ('recombineering'), a technology that allows the fluent engineering of DNA without size and site limitations and that combines the advantages of knock-out and Cre-loxP conditional alleles in a single targeting effort (Testa et al., *Nature Biotechnology* 2003).

We have generated SETD7 knock-out mice, and we started to dissect the role of MLL and MLL2 through conditional inactivation. In order to characterize their function in the establishment and maintenance of differentiation, we selected as a model the development of B cells, since it is one of the best characterized systems of cellular differentiation *in vivo*, with defined stages of maturation identified by well-established molecular markers. As such, it represents an ideal system to address an issue that is still largely unexplored, namely the relevance of an intact H3K4 methylation axis to the resilience of differentiated states. Within a fruitful collaboration with the laboratory of Stefano Casola (IFOM), a detailed analysis of MLL and MLL2 revealed specific patterns of expression at different stages of B-cell maturation. Hence, through the use of validated mouse lines that express Cre recombinase at the pro-B-, pre-B- and mature B-cell stage, we have now started to identify the requirement of MLL2 for the homeostasis of specific B-cell compartments.

#### 2. Conditional approaches to the role of histone demethylases (HDMs) *in vivo* and *in vitro*.

In order to explore the relevance of histone lysine demethylation to lineage commitment, we took a comprehensive approach and analyzed the expression patterns of all known and putative HDMs during the course of the differentiation of ES cells into neural stem (NS) cells. The choice of the neural stem cell differentiation system reflects both its biological relevance and its unique technical advantages.

This led to the identification of *Jmjd3* which was characterized with Dr. Gioacchino Natoli (IEO), as a H3K27 specific demethylase (De Santa et al., *Cell* 2007). Together with Utx, these are the first two activities shown to antagonize Polycomb-mediated gene repression, suggesting that they play an important role in the controlled H3K27 demethylation that accompanies cell differentiation. Consistent with this prediction we have uncovered an essential function for *Jmjd3* in neural commitment, and we have proceeded to engineer both constitutive and conditional knock-out mice to explore its function in vivo. These mice will allow us to assess the effect of perturbing the H3K27 axis of epigenetic regulation on tumor initiation and maintenance.

#### c. Genome accessibility as a tool for the identification of new targets (Saverio Minucci)

Genome accessibility in the context of cellular chromatin is a well-established epigenetic feature of active regulatory DNA. The accessibility of specific regulatory sequences is tightly controlled and defines the combinatorial codes that direct and specify gene expression patterns for any given stage of development.

In this study, we have used primary human hematopoietic stem and progenitor CD34+ cells as a model system to understand which fraction of the genome undergoes changes in accessibility during myeloid differentiation. In locus-wide analyses, detection of DNase Hypersensitive Sites (DHS) is widely used to map accessible DNA, but large-scale mapping of DHS presents several limitations. Thus, we have developed and applied a novel large-scale approach based on the use of restriction enzymes (RE) to probe and sequence DNA elements, which are accessible in chromatin. Among these sequences, we identified known and novel cis-regulatory elements, including enhancers, silencers and insulators.

Transcription start sites (TSS) were also well represented. Genome-wide analysis of accessibility indicated that low nucleosome density is a common feature of gene promoters, even when poorly transcribed (as inferred by gene expression profiling studies, performed in parallel). Of note, we located a significant fraction (60%) of the DNA accessible in chromatin at intergenic sites and repeated DNA, suggesting that these regions may be far more complex than previously thought. These data were then used to map large genomic domains (at a resolution of 1,2Mb) with differential accessibility during early step of hematopoiesis. These domains include clusters of genes involved in blood cell formation and function. Mapping changes in genome accessibility, therefore, can be also used to identify large-scale modification in chromatin structure. This approach will be extended to the analysis of the tumorigenic process, with the aim of identifying genomic regions which may host novel oncogenic targets.

#### d. The role of histone-acetyltransferase in tumor progression and therapeutic implications (Bruno Amati)

The main topic of our research is to understand the role of histone-acetyl transferases (HATs) in tumor development. We initially focused on Tip60, a HAT known for its functions not only in gene regulation, but also in the DNA damage response (Squatrito et al., *Trends Cell Biol.* 2006). This first part is the subject of a recent publication (Gorriani et al., *Nature* 2007). Briefly, we have shown that the state of heterozygosity for a “knockout” (KO) allele of Tip60 gives results in a marked acceleration of Myc induced tumorigenesis. This was determined by crossing Mice Tip60 + / - with a transgenic model of Burkitt lymphoma (E $\mu$ -Myc) where deregulated Myc expression in B lymphocytes causes the development of lymphomas. The analysis of various human tumor types has also revealed that the loss of one allele of Tip60 is a frequent early event during breast cancer development. The mechanism underlining Tip60 tumor suppression functions has been clarified by the study of the E $\mu$ -Myc mouse model: the Myc oncogene causes the activation of the DNA Damage Response (DDR) pathway, and recent data show how this response is important in limiting cancer progression. In fact, we demonstrated that Myc-induced DDR is dependent on Tip60. Overall, these clinical and experimental data have shown that Tip60 is a novel and relevant tumor suppressor. We are now breeding E $\mu$ -Myc mice with mice carrying KO alleles for two other HATs, GCN5 and PCAF. For this part of the project, we are waiting for the development of lymphomas in order to determine whether the loss of GCN5 or PCAF will affect tumor development. We have also begun the study of the function of another epigenetic regulator, PRMT6 in response to Myc activation. PRMT6 is a histone-methyl-transferase that we have recently shown to methylate arginine 2 of histone H3 (H3R2) (Guccione et al., *Nature* 2007). The observation that H3R2 methylation correlates with the methylation of lysine 27 (Guccione et al., *Nature Cell Biology* 2006), a chromatin modification known for its role in the repression of tumor suppressor loci, warrants further investigation of the role of PRMT6 in tumor development.

Finally, we took advantage the E $\mu$ -Myc model to understand the role of the CDK2 kinase, a regulator of cell cycle, in Myc induced lymphomagenesis. In E $\mu$ -Myc transgenic mice homozygous for a *Cdk2* knockout allele (*Cdk2*<sup>-/-</sup>), an enhanced senescence response retarded the progression of B-cell lymphoma. Thus CDK2 has a non-redundant role in tumor progression, which may have important implications for its use as a therapeutic target.

## 2. Aberrations of cell division

### The spindle assembly checkpoint as a therapeutic target in cancer (Andrea Musacchio)

Our research group is engaged in the characterization of the molecular mechanism of the spindle assembly checkpoint (SAC) and of its interaction with the kinetochore, the structure through which the mitotic chromosomes attach to the mitotic spindle, a microtubule-based structure. The SAC is a device that monitors the correct attachment and alignment of chromosome to the mitotic spindle. Errors in the progression of the attachment process in the mother cell may result in the distribution of incorrect numbers of chromosomes to the daughter cells, causing them to become aneuploid or polyploid. This form of genomic instability is particularly frequent in cancer cells, but its underlying causes have remained largely unclear. A decade ago, it was proposed that cancer cells showing chromosome instability might have a compromised SAC. Since then, however, several studies have contributed to delineate the concept that the SAC may be weakened rather than abrogated in cancer cells, thus favouring infrequent but non negligible chromosome segregation errors that would then create the basis for genetic selection of more aggressive cell variants. Other studies have pinpointed that cancer cells that have already acquired gross imbalances in chromosome numbers, possibly as a consequence of a single aberrant mitosis, might be more strictly dependent on the SAC. Indeed, a growing body of evidence indicates that the impairment of the SAC is generally lethal for cancer cells. On these bases, it is important to explore the potential of SAC abrogation as an anti-cancer therapy.

In 2007, we continued to explore the molecular bases of the SAC. A main line of research in the laboratory is concerned with the validation of a model of checkpoint function known as the “Mad2 template model”. The model states that two conformers of the same checkpoint protein Mad2 (open-Mad2 and closed-Mad2) interact to stimulate the accumulation of an active conformer that promotes the arrest of a cell in mitosis. After several years of discouraging attempts, we were finally able to obtain well-diffracting crystals of the open-Mad2-closed-Mad2 dimer and to determine its 2.9-Å crystal structure by X-ray crystallography (Mapelli et al., Cell 2007). The work provides a snapshot of the mechanism of docking of the O-Mad2 conformer onto the C-Mad2 conformer and that allows O-Mad2 to bind its target in the checkpoint Cdc20.

We have also reiterated our efforts towards the functional and structural elucidation of Aurora B, a mitotic checkpoint protein that is currently indicated as one of the most promising targets for anti-tumor therapy. In collaboration with the research group of Prof. Stephen S. Taylor at the University of Manchester, we have characterized a series of Aurora B kinase mutants that render the kinase resistant

to the inhibition by different classes of small-molecule ATP-competitive inhibitors. These studies *in vitro* pave the way to the development of strategies to predict the type of resistant mutants that might arise during clinical treatment with a kinase inhibitor, and the type of alternative therapeutic opportunities available once resistance has developed.

## 3. Host-tumor interactions

### Chronic inflammation and cancer: Transcriptional mechanisms as potential therapeutic targets (Giacchino Natoli)

A large body of epidemiological and experimental data demonstrated a direct link between chronic inflammation and the development of several types of cancers. Moreover, most cancers contain an inflammatory infiltrate that is hijacked by tumor cells to promote angiogenesis, tissue invasion and cell proliferation.

*In vivo* experiments in mouse models have demonstrated that modulation of tumor properties by inflammatory cells requires the transcription factors of the NF-κB family, thus suggesting the possible use of anti-NF-κB drugs in tumor therapy. Drugs disabling the whole NF-κB signaling pathway are already being tested in clinical trials, but well-justified concerns about their safety have been raised because of the many physiological responses in which NF-κB is required. Conversely, therapies blocking the induction of subsets of NF-κB-regulated genes should provide more restricted and predictable effects, but the molecular bases for their design are not available.

Our unit is interested in the mutual relationship between the inflammatory response and the epigenome, namely the ensemble of chromatin modifications that maintain and propagate across mitosis a given program of gene expression. On the one hand the epigenome controls recruitment of transcription factors to inflammatory genes and generates tissue-specific and temporal profiles of inflammatory gene expression. On the other, inflammation affects epigenetic control leading to striking alteration of tissue differentiation in chronically inflamed tissues (e.g. intestinal metaplasia in chronic gastritis). Such alterations often precede and are associated with the development of malignant tumors. We have recently started to unravel the mechanistic basis of this phenomenon: we have identified an enzyme, Jmjd3, which is inducible by inflammatory stimuli and which erases a histone covalent modification that controls cellular differentiation by keeping repressed the genes that specify alternative cell fates (trimethylation of histone H<sub>3</sub> at lysine 27) (De Santa et al., Cell 2007).. In collaboration with the groups of Giuseppe Testa (IEO) and Stefano Casola (IFOM) we have generated conventional and conditional knock-out mice that will be used to directly test the possible involvement of Jmjd3 in differentiation abnormalities associated with chronic inflammation.

#### 4. Mechanisms of leukemogenesis (Pier Giuseppe Pelicci)

Animal models of acute myeloid leukaemia (AML) are invaluable in the study of disease mechanisms and for the development of targeted therapeutic strategies. In our laboratory, we have created animal models of acute promyelocytic leukaemia (APL) caused by PML/RAR $\alpha$  and of AML induced by AML1/ETO or overexpressed PRDM16, using a retroviral transduction and bone marrow transplantation approach. We are now investigating the ability of novel proteins - cMYB/GATA1 and mutant NPM - to cause leukaemia in mice.

**Cooperation of sPRDM16 and p53-loss in the expansion of hematopoietic stem cells, immortalization of progenitors and induction of myeloid leukemias.** Transgenic expression of the abnormal products of AML-associated primary translocations in hematopoietic stem/progenitor cells initiates leukemogenesis in mice, yet additional mutations are needed for leukemia development. We report here aberrant expression of *PRDM16* in AMLs with translocations of 1p36 or with normal karyotype, carrying, respectively, relatively high prevalence of mutations in the p53 tumor suppressor or nucleophosmin (*NPM*), a gene that regulates p53. Two isoforms are expressed from the *PRDM16* gene, differing in the presence or absence of the PR domain. Overexpression of the short isoform, *sPRDM16*, in murine bone marrow induces AML with full penetrance, but only in the absence of p53. The murine leukemias are characterised by multilineage dysplasia and dysmegakaryocytopoiesis, a common feature of human AMLs with 1p36 translocations or *NPM* mutations. *sPRDM16* overexpression increases the pool of hematopoietic stem cells *in vivo* and, *in vitro*, blocks myeloid differentiation and prolongs progenitor life span. Loss of p53 augments the effects of *sPRDM16* on stem cell number and induces immortalization of progenitors. Thus, overexpression of *sPRDM16* induces abnormal growth of stem cells and progenitors and cooperates with disruption of the p53 pathway in the induction of myeloid leukemia.

**Characterization of NPM mutants.** Mutations leading to aberrant cytoplasmic localization of Nucleophosmin (*NPM*) are the most frequent genetic alteration in acute myelogenous leukemia (AML). *NPM* is a nucleolar chaperone, which inhibits cell proliferation and possesses tumor suppressor functions *in vitro* and *in vivo*. *NPM* binds the Arf tumor suppressor and protects it from degradation. The AML-associated *NPM* mutant (*NPMmut*) also binds p19Arf, but is unable to protect it from degradation, suggesting that inactivation of p19Arf contributes to leukemogenesis in AMLs. Moreover, we have recently shown that *NPM* regulates turnover of the c-Myc oncoprotein by acting on the F-box protein Fbw7 $\gamma$ , a component of the E3 ligase complex involved in the ubiquitination and proteasome-degradation

of c-Myc. Remarkably, the expression of *NPM* mutant in AMLs might have the double effect of activating proliferation through c-Myc stabilization and attenuating the resulting checkpoint response through ARF degradation. These studies have increased our basic knowledge on the molecular basis of tumor development and will help us to design new anti-tumor strategies. There is, indeed, urgency for novel treatment modalities of leukemias, since only approximately 20% to 30% of the AML patients are cured with the available therapies. Since leukaemias with *NPM* mutations comprise up to a third of all AMLs, targeting of *NPMmut* is a major challenge of current research in leukemias.

#### Identification of oncogenes cooperating with PML/RAR $\alpha$ in leukemogenesis.

In order to identify secondary events that cooperate with PML/RAR $\alpha$  in the induction of leukemia we performed retroviral insertional mutagenesis analysis on PML/RAR $\alpha$  knock-in (KI) mice. As we previously reported, infection of PML/RAR $\alpha$  KI animals with Moloney Murine Leukemia Virus (MLV) significantly accelerated the development of promyelocytic leukemias that developed in PML/RAR $\alpha$  KI animals not infected (5,6 months compared to 10 months, respectively). We extracted genomic DNA from spleens derived from leukemic mice that developed an accelerated APL and, using the inverse PCR (IPCR) technology and the TOPO TA cloning kit, we were able to clone several viral insertions per tumor. Up to date, through sequencing of the cloned genomic DNA fragments and searching of the GenBank database, we were able to identify three common integration sites (CIS), defined as at least two integrations in a sequence of 30 kilobases (Kb). Two CIS are located on mouse chromosome 3: the first is located 15 Kb upstream of the Ecotropic viral integration site 1 (*Evi1*) gene; the second is in the TDPOZ gene cluster. Each of these insertions have been identified in 3 different leukemic samples. The third CIS was cloned from 8 different tumors and occurs 600 bp 3' of the coding sequence of mouse Dachs-hund1. All genes we identified as possible target of viral integration have been reported to act as transcriptional regulators and to be involved in cellular differentiation. In particular, the *Evi1* gene has been previously identified as a common site of viral integration and its inappropriate activation by chromosomal rearrangements has been detected in human myeloid leukemias. While analyzing more tumors in order to clone new CIS and to define the frequencies of the CIS described above, we are trying to define the effect of the viral insertion on the expression/function of the genes of interest and the possible mechanisms of cooperation with PML/RAR $\alpha$ . Moreover, we are currently performing studies *in vivo* in order to confirm the cooperation of PML/RAR $\alpha$  and the CIS identified in the development of

promyelocytic leukemia. Our experimental approach consist in infecting stem cells derived from PML/RAR $\alpha$  KI mice with retroviruses expressing the CIS of interest and transplant them in lethally irradiated, wild type mice.

## 5 Normal and Cancer Stem Cells

Tumors have been traditionally regarded as biologically homogenous populations of cells endowed with high proliferating activity. This view is changing with the realization that many, if not all cancers are organized as abnormal tissues containing a subset of cells with stem cell (SC)-like properties (cancer SCs), which produce differentiated progeny with limited replication potential. Notably, cancer SCs are responsible for sustaining tumor growth in model systems and are thought to drive the growth and metastasis of spontaneously occurring tumors.

The notion of cancer SCs, and the related kinetic models of tumor growth, have important implications for cancer treatment. Current therapies have been developed to decrease the bulk of the tumor mass and, though they may produce dramatic responses, are unlikely to result in long-term remissions if the rare cancer-SCs are not targeted as well. A corollary of this view is that, at least in principle, the selective ablation of cancer SCs, even in the absence of targeting of the whole tumor mass, should lead to the “sterilization” of the tumor and to its cure. If true, this would have enormous consequences on the way we design anti-cancer therapies, especially in light of the projected lower toxicity of such “specific” treatments. Notwithstanding these hopes, there is presently little experimental evidence in support of the concept of “cancer SC targeted-therapy”. This is largely due to our poor understanding of the biological and molecular differences between normal and cancer SCs. We do not know, for instance, whether cancers SCs display, as compared to their normal counterparts, different growth potential, life span, drug sensitivity and, more importantly, which are the relevant molecular pathways.

**Maintenance of quiescence by p21 sustains self-renewal of leukaemia stem cells.** Quiescence of hematopoietic stem cells (HSCs) is critical for their ability to self-renew under stress conditions. In the absence of the cell cycle inhibitor p21, HSCs replicate more frequently under homeostatic conditions, and are rapidly consumed after serial transplantation or myelosuppressive chemotherapy, suggesting that p21 expression and restricted cell cycling are critical to prevent premature HSC depletion under conditions of increased self-renewal. We investigated if leukemia-initiating oncogenes (PML-RAR and AML1-ETO fusion proteins) increase HSC self-renewal and if maintenance of HSC quiescence is critical to support clonal expansion of leukemic SCs. We report here that in vitro or

in vivo expression of fusion proteins into HSCs increases self-renewal and up-regulates p21. In the presence of fusion proteins, p21 expression is indispensable for the increased self-renewal of HSCs, maintenance of functional quiescence and protection of from premature depletion. Analysis of the contribution of p21 to leukemia development revealed that p21 expression is absolutely required for the initiation of leukemogenesis by AML1-ETO and for the maintenance of PML-RAR – induced leukemias. In the absence of p21, PML-RAR – leukemias show marked reduction of the pool of quiescent leukemic SCs and were unable to expand after transplantation into syngenic hosts. These data demonstrate that active maintenance of quiescence through p21 up-regulation is indispensable to support oncogene-induced hyper-proliferation of HSCs and self-renewal of leukemic SCs, and provide the first molecular target for anti-leukemic strategies aimed at eradicating quiescent cancer SCs. Extended life-span and increased frequency of symmetric self-renewing divisions in cancer stem cells, due to attenuated p53 signaling. Recent findings support the idea that cells with the properties of stem cells (SCs) are integral to the development and perpetuation of several forms of human cancer, and that eradication of cancer SCs may be essential to achieve cancer cure. However, direct proof of these concepts is still lacking, mainly due to our poor understanding of the biological differences between normal and cancer SCs, and of the underlying molecular mechanisms. Here, we investigated the self-renewal properties of normal and cancer SCs, using the mammary gland as model system. We found that normal SCs rapidly lose self-renewal potential in culture, while cancer SCs are nearly immortal and their number expands geometrically, due to increased frequency of symmetric divisions. Mechanistically, we showed that p53-signaling is attenuated in cancer SCs, and that p53-null SCs possess the same self-renewal properties of cancer SCs. Pharmacological restoration of p53 in cancer SCs restored normal kinetics of self-renewal, without affecting significantly the growth of progenitor cells. Importantly, this correlated with reduced tumor growth in vivo. These data demonstrate that extended life-span and increased frequency of symmetric self-renewing divisions in cancer SCs is critical for tumor growth, and suggest that asymmetric division functions as a mechanism of tumor suppression in mammals. Finally, our findings demonstrate that the selective ablation of cancer SCs leads to reduced tumor growth, thus providing experimental support to the concept of “cancer SC targeted-therapy”. In parallel to these studies, based on the emerging concept that tumorigenesis might be regarded as to normal morphogenesis gone awry and that pathways governing normal stem cell functions are often subverted in cancer, we performed a comprehensive transcriptomic analysis of human normal mammary stem cells. This study

enabled us to characterize the molecular identity of human normal mammary stem cells, with the ensuing identification of several molecular determinants and pathways, whose regulation might be important in the maintenance of the mammary stem cell compartment, and for the molecular strategies enacted by differentiating progenitors to exit this compartment. When used to interrogate independent expression profile datasets of breast cancers, the newly identified normal mammary stem cell signature was able to stratify breast cancers according to their biological, clinical, and molecular features. Strikingly, we found that poorly differentiated, more aggressive, breast cancers are enriched in cells with molecular features of human normal mammary stem cells. Collectively, our data support the notion that the complex features of human breast cancers are a function of their resemblance to the stem cell state, which, in turn, might reflect the number of stem cells (most likely cancer stem cells) contained in the different cancers. In conclusion, results of these studies strongly argue that the aberrant persistence of normal stem cell features in tumors might be the consequence of the alteration of regulatory mechanisms controlling key normal stem cell functions. More importantly, they show that the number of stem cells in a breast tumor can vary greatly, with discernable impact on several tumor features. Since cancer stem cells are held responsible for the majority of the failures of chemotherapeutic regimens, our results might have important practical applications.

## 6. Predictive molecular markers

### Predictive value of p53 gene status in breast carcinoma patients treated with taxanes-based adjuvant chemotherapy (Giuseppe Viale)

We have retrieved tumor tissue samples from 1264 patients enrolled in a randomized clinical trial evaluating the possible benefit of the addition of taxanes to anthracycline-based adjuvant chemotherapy. All tumor samples have been centrally re-evaluated for the expression of estrogen (ER) and progesterone receptors (PgR), and of HER2 status. We have also immunostained for the p53 protein all the cases, looking for the nuclear accumulation of this protein that is associated with the occurrence of gene mutations. The immunohistochemical reaction has been performed using the Do7 monoclonal antibody, that recognizes an epitope shared by the wild-type protein and by its mutated forms. The detection step of the reaction has been carried on using a highly sensitive reagent.

At the same time, we have also started to sequence the p53 gene on DNA extracted from the formalin-fixed paraffin-embedded tissue sections. From 269 (72%) of the 375 samples examined so far we managed to obtain good quality DNA, that has been sequenced. In 48 (18%) of the 269 cases a mutation in exons 5-8 of the p53 gene has

been identified. Most (36/48 or 75%) of the mutations were of the missense type, 6 of the truncating type, and 6 were silent or intronic. The majority of the missense mutations involved the DNA-binding motif. In 3 samples, 2 mutations have been detected.