Walking pathways at Heidelberg

Conference on oncogenes and growth control

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The leitmotiv of the *EMBL/Salk/EMBO Conference on Oncogenes and Growth Control* was the analysis of signal transduction pathways involved in cancer. Related themes were also touched on, including the new fields of genomics/proteomics, bioinformatics, and stem cell differentiation. The meeting took place in Heidelberg (Germany) on April 20–23, 2002, and was organized by A. Nebreda, G. Superti-Furga, M. Bienz, D. Bohmann, H. Land and C. Marshall.

Tumorigenesis is more than cancer cells

R.A. Weinberg (Boston, MA) set the tempo for the meeting, giving a holistic view of the genetic alterations required for the development of a human tumor. Unlike mouse cells, which need only two genetic events for transformation, their human counterparts require four genetic alterations, which can be mimicked in cell culture by expressing the molecular analogs of the Chinese “Gang of Four”: the large T antigen (affects the Rb and p53 pathways), the small T antigen (inactivates the PP2A phosphatase), oncogenic Ras (provides proliferative and survival signals) and hTERT (confers telomere length homeostasis and cell immortalization). Despite these advances in the understanding of the genetic alterations involved in cancer, it would be a mistake to become too complacent about this new ‘orthodoxy’ for oncogenic progression. Weinberg put forward his thesis emphasizing that the evolution of cancer *in vivo* cannot be fully explained without taking into consideration the social context in which cancer cells grow. In support of this, he presented data consistent with the idea that cancer cells and the surrounding stroma co-evolve, modifying each other to facilitate tumor progression. Although the mechanism by which this occurs is not yet established, Weinberg presented data indicating that the effect of cancer cells on stromal cell biology is mediated by the secretion of TGF- β (transforming growth factor- β). This action appears to induce long-term changes in stromal cell behavior that, in turn, favor the growth of cancer cells, possibly by paracrine signaling. One of the consequences of this cellular interaction *in vivo* is a reduction in the threshold of oncogenic signals required for a tumor to develop. The take-home message was that experiments with mouse cells or even cultured human cells are not adequate to uncover all of the intrinsic and extrinsic biological steps that lead to the development of a tumor *in vivo*. Weinberg discussed examples of transplants of human cancer and stromal cells into mice, whose study could help to explain the social aspects of tumor progression.

Introduction

There could be nowhere better than the EMBL in Heidelberg, where academic tradition and cutting edge innovation march hand-in-hand, to host the 2002 *EMBL/Salk/EMBO Conference on Oncogenes and Growth Control*. The talks and posters presented during the 4 days of the conference focused on how signaling pathways are fine-tuned and coordinated to ensure the generation of proper biological responses, on how cell behavior is conditioned by intracellular, autocrine and paracrine effects, and on the development of new genomic and proteomic strategies with which to dig deeper into the transcriptional programs associated with cell proliferation, renewal and differentiation in both physiological and pathological states. Some of the new concepts arising from this conference are summarized below.

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Regulation of signaling pathways by extracellular and intracellular events

The activation of cellular responses by the autocrine stimulation of signaling pathways and how this impacts on the biology of the cancer cell were discussed in several presentations. As previously revealed by microarray analysis (Schulze *et al.*, 2001), oncogenic Raf induces the survival of epithelial cells by generating an autocrine loop mediated by the secretion of growth factors of the epidermal growth factor (EGF) family. J. Downward (London, UK) extended these observations, demonstrating that this autocrine loop is responsible for 50% of the transcriptomal changes induced by activated Raf. Interestingly, all of the genes that are downregulated by Raf belong to the autocrine-dependent set. B.H. Land (Rochester, NY) indicated that cancer cell survival can also be regulated via a second autocrine loop involving the integrin $\alpha 6$ and its natural ligand, laminin $\gamma 2$. This loop is induced by the combined effect of *ras* oncogene expression and the deletion of the *apc* or *p53* gene. Remarkably, the survival of normal cells is independent of this signal, implying that oncogene cooperation not only confers cancer cell survival but also a selective vulnerability to cell death that could be of use in therapy. Consistent with this idea, Land showed that the pharmacological blockage of this autocrine loop induces selective cancer cell death, particularly of *p53*-defective tumors. Autocrine loops are responsible for regulating other tumor-promoting cellular functions besides apoptosis. Building on observations from genome-wide expression profiling, H. Beug (Vienna, Austria) demonstrated that the epithelial–mesenchymal transition, which is driven by joint TGF- β R (TGF- β receptor) and Ras signaling and is relevant for metastasis, also utilizes an autocrine loop. This is mediated by the PDGF-R (platelet-derived growth factor receptor), which is associated with increased expression of downstream elements of this pathway such as the transcriptional factor Stat1 (signal transducer and activator of transcription 1).

One important question discussed at the meeting is how cells insulate signaling pathways to avoid the generation of incoherent biological responses. W.A. Lim (San Francisco, CA) used the yeast Ste5 and Pbs2 proteins to illustrate the role of scaffolding proteins in assuring signal transmission and specificity. Ste5 is an anchor molecule that participates in the mating response of yeast induced by the α factor pheromone. It does so by forming a multiprotein complex with the signal transduction elements that participate in this response, including Ste20 (β/γ subunits of G protein activated by the α factor receptor), the mitogen-activated protein (MAP) kinase kinase kinase Ste11, the MAP kinase kinase Ste7, and the MAP kinase Fus3 (Figure 1A). Pbs2 is involved in the yeast response to high extracellular salt concentrations, working both as a MAP kinase kinase in this pathway and as a docking platform for the other elements of this route, Ste11 and the MAP kinase Hog1 (Figure 1B). The key role of Ste5 and Pbs2 as signaling insulators was demonstrated in yeast cells lacking the endogenous Ste5 and expressing a Ste5/Pbs2 chimera deficient in Ste7 and Fus3 binding (Figure 1C). In these cells, the addition of α factor triggered the osmotic stress response instead of the expected mating response. Lim also utilized genetic methods to analyze the role of Ste5 in the assembly of the MAP kinase cascade of the mating response, demonstrating

Fig. 1. Schematic representation of MAPK modules and scaffolding proteins of the yeast mating (A) and the high-osmolarity signaling (B) pathways. (C) A Ste5/Pbs2 chimera is capable of responding to a signal for one pathway (α factor) with a response appropriate to the other (high osmolarity). Defective binding sites for interacting proteins are indicated by crossed bars.

that the function of Ste5 is to assemble Ste11, Ste7 and Fus3 in a spatial orientation compatible with optimal signal output. J. Wrana (Toronto, Canada) presented a second mechanism that ensures signaling insulation by segregating antagonistic elements of the same route into different subcellular compartments. He demonstrated that TGF- β Rs are internalized through both endocytic vesicles and caveosomes, and that the first route is important for TGF- β R signaling, whereas the second is essential for TGF- β R degradation. The endocytic vesicles contain Sara (Smad anchor for receptor activation), an adaptor molecule that facilitates the association of the activated TGF- β R with Smads (Similar to mothers against decapentaplegic), the main transcription factors activated by the TGF pathway. In contrast, the caveosomes contain the inhibitory Smad7 protein and the E3 ubiquitin ligase Smurf2 (Smad ubiquitination regulatory factor 2), two negative regulators of the TGF pathway that promote TGF- β R degradation.

The generation of balanced biological responses in normal cells requires that the activation of signaling pathways is transient, to avoid pathological situations such as cancer or developmental diseases. Receptor internalization and degradation is one mechanism for inhibition of a particular pathway, but it is obvious that this can also be achieved by other strategies. One of these is to establish negative feedback loops in which activated effector molecules inhibit their upstream regulators. In this respect, A. Nebreda (Heidelberg, Germany) showed that the p38 α MAP kinase induces the repression of its activator, MKK6 (MAP kinase kinase 6), by promoting the degradation of its

Fig. 2. Schematic representation of some of the negative feedback mechanisms discussed at the meeting. The pathways and regulatory molecules responsible for the downmodulation of the pathway are shown in red.

mRNA. This effect appears to be mediated by the binding of a hitherto unknown protein to the 3' untranslated region of the *mkk6* mRNA. J. Schlessinger (New Haven, CT) discussed the regulation of FRS2 α (fibroblast growth factor receptor substrate 2 α), an adaptor molecule that plays a key role in FGF-R (fibroblast growth factor receptor) signaling via its association with Grb2 (growth factor receptor-bound protein 2) and Gab1 (Grb2 associated binding protein 1) (Figure 2A). The adaptor function of FRS2 α is inhibited by Erk-mediated phosphorylation of eight threonine residues (Figure 2A). Expression of FRS2 α mutants lacking these negative regulatory sites leads to enhanced signals by the FGF-R, indicating that this feedback mechanism plays a role in modulating the amount of signaling by this pathway. FRS2 α threonine phosphorylation is also mediated by receptors that do not activate FRS2 α (EGF-R, PDGF-R, insulin-R), suggesting a mechanism whereby the activation of one receptor can ensure the silencing of parallel pathways. Finally, FRS2 α contributes to the downmodulation of FGF signaling via binding to c-Cbl, an E3 ubiquitin ligase involved in FGF-R degradation (Figure 2A). Z. Lu (La Jolla, CA) described another means of signal downmodulation that is utilized by MEKK1 (MAP or ERK kinase kinase 1). During apoptotic responses induced by the treatment of cells with sorbitol, this kinase experiences a signaling dilemma since it can simultaneously activate pro-apoptotic and anti-apoptotic pathways (Figure 2B). Lu demonstrated that this Gordian knot is untied by the ubiquitylation and degradation of Erk, which is targeted for this process by means of the E3 ubiquitin ligase activity contained within the N-terminal PHD (plant homology domain) region of MEKK1 (Figure 2B). This inhibitory mechanism is required for the apoptotic function of MEKK1, since the expression of Erk mutants that cannot interact with MEKK1 inhibits sorbitol-induced apoptosis. E. Nishida

(Kyoto, Japan) discussed a mechanism of signal downmodulation that was elucidated based on studies with inhibitors that sequester key signaling regulators. Sprouty (Spy), an adaptor molecule that was initially isolated as an inhibitor of the FGF pathway in *Drosophila*, regulates the extent of Erk activation by competing with the Shp2 tyrosine phosphatase and FRS2 proteins for binding to the son-of-sevenless 1 (Sos1)/Grb2 complex (Figure 2A). The Spy/Grb2 association is mediated by the binding of the Grb2 SH2 domain to a single phosphotyrosine residue (Y55) present in Spy. Nishida also showed that Spy can be dephosphorylated by Shp2 (Figure 2A), suggesting that the signaling output of the pathway can be controlled rapidly and reversibly at the posttranslational level.

Given the above-mentioned complexity of signaling networks, computer and mathematical models may become important tools in understanding how signaling interactions and cross-talk are integrated by cells to induce specific biological responses. U. Klingmüller (Freiburg, Germany) gave us a vision of the future of this area in her discussion of theoretical models that have been generated for the regulation of the Jak/Stat pathway by the erythropoietin receptor. Using empirical data on the kinetics of the phosphorylation of Jaks and Stats, she developed a mathematical model that predicts the behavior of the signaling network and, more importantly, infers the regulatory forces that modulate this route. This model indicated that the main step in Stat regulation upon treatment with erythropoietin is the maintenance of the Stat nuclear import/export cycle, rather than the retention of Stat molecules in the nucleus. This was subsequently corroborated *in vivo*. Based on these results, we are afraid to say that some of us will have to go back to high school times and replace our copies of Maniatis with a good old calculus book.

Stimulation and infidelities flowing in the Wnt

The conference also covered new developments regarding the regulatory mechanisms operating within the Wnt pathway, its interconnection with the Hedgehog route, and the influence of Wnt on the transcriptome. S. Cohen (Heidelberg, Germany) discussed the importance of the regulation of ligand gradients in the extracellular medium to ensuring balanced developmental responses. He presented data indicating that the developmental effects induced by the activation of the β -catenin pathway in *Drosophila* depend not only on the availability of the *Drosophila* Wnt homolog Wingless (Wg), but also on the distribution of this protein in a defined gradient within the tissue undergoing morphogenesis. This gradient is shaped by the action of two groups of proteins. Heparan sulfate surface proteoglycans such as Dally and Dally-like bind to Wg in the extracellular matrix, leading to the accumulation of this protein near the receptors on the target cell. This action is counteracted by Notum, a secreted pectin acetyltransferase that modifies both Dally and Dally-like and reduces their binding to Wg (Figure 3). In agreement with this functional role, the Wg gradient is reduced in *Dally-like*, and increased in *Notum*, mutant tissues. Interestingly, Wg promotes Notum expression, indicating that Wg contributes to the shape of its own gradient by regulating the expression of a protein that modifies extracellular proteoglycans to which it can bind. A. Kikuchi (Hiroshima, Japan) showed new aspects of β -catenin regulation (Figure 3). He demonstrated that Axam (Axin-associating molecule) works as an inhibitor of the Wnt pathway at three different levels. It blocks the binding of Dvl (Dishevelled) to Axin (Axis inhibitor), liberating Axin for binding to casein kinase 1 (CK1) and glycogen synthase kinase-3 (GSK3), two kinases that promote β -catenin phosphorylation and degradation via the proteasome (Figure 3, right). Axam also works as a desumoylation enzyme that contributes to β -catenin destabilization by an as yet unknown mechanism (Figure 3, right). Finally, Axam inhibits the nuclear localization of TCF4 (T-cell factor-4), the main transcription factor of the Wnt pathway, apparently by promoting its desumoylation (Figure 3, left). M. Bienz (Cambridge, UK) surprised those who thought that everything was known about the β -catenin pathway by describing the discovery of *Drosophila* Pygopus (Pygo), a nuclear partner for β -catenin essential for the activation of TCF-target genes (Figure 3, left). Pygo has two human homologs that are also required for TCF-mediated transcription in colorectal cancer cells, as demonstrated by knock-down experiments using RNA interference (RNAi) techniques. H. Clevers (Utrecht, The Netherlands) reported recent results from microarray analysis of colorectal cancer cell lines expressing an inducible dominant-negative (DN) form of TCF4. He showed that blockage of the β -catenin pathway resulted in a rapid cell cycle arrest and the restoration of the differentiation program of these cells, a process that is dependent on the de-repression of *p21^{WAF}*, one of the genes detected in microarray analysis. Immunocytochemical studies indicated that many of the messages that are downregulated by DN-TCF are present in the crypt progenitor compartments of normal intestinal epithelium, suggesting that β -catenin promotes tumorigenesis by keeping crypt cells in the undifferentiated state.

Fig. 3. Schematic representation of the Wnt pathway. Dally-like and Notum influence the binding of Wnt protein family members to their receptors (members of the Frizzled protein family) positively and negatively, respectively. In the absence of Wnt, Dvl is inactive, and β -catenin is serially phosphorylated by CK1 and GSK3 (green when active), leading to β -catenin ubiquitylation and degradation in the proteasome (right side). Phosphorylation of β -catenin by these kinases is facilitated by two adaptor proteins, APC and Axin. Binding of ligand to the Frizzled (Fz) receptor activates Dvl, causing it to bind Axin. This step blocks the interaction of Axin with the β -catenin kinases, resulting in increased levels of β -catenin. β -catenin then binds to TCF transcription factors, leading to the transcriptional activation of target genes (left side). This process is facilitated by the sumoylation of TCF proteins by PIASy as well as by the incorporation of Pygo into the transcription complex. The Axam protein antagonizes β -catenin by promoting its degradation and by keeping TCF4 outside the nucleus. Phosphorylated (P) residues are shown in yellow. Ubiquitin (U) and sumo (S) proteins are shown in pink. New proteins or proteins better characterized at this meeting are shown in blue. Genes discussed whose expression is repressed or induced by the Wnt pathway are shown in red and black, respectively. β -cat, β -catenin.

Interestingly, another gene found in the microarray experiments, the Ephrin B2 (EphB2) tyrosine kinase receptor, has shed light on how cancer cells migrate within the villi to generate the typical

polyp histology. Clevers showed that the migration pattern of cancer cells is not cell autonomous, but dependent on repulsive forces set in place by cell contacts between EphB2-expressing cancer cells and differentiated epithelial cells expressing Ephrin, the EphB2 ligand. As a consequence of these repulsive forces, cancer cells migrate away from the differentiated zone and towards the center of the villi, leading to the classical morphology of colorectal polyps.

While most data place APC into the Wnt pathway merely as a regulator of β -catenin degradation, most recent experiments suggest that it may have its own agenda in tumorigenesis. Using an inducible, tissue-specific *apc* knockout, O. Sansom (Cardiff, UK) has been able to demonstrate that the loss of this protein leads to the rapid appearance of tumor cells, but that many of these do not show β -catenin upregulation. Thus, at least some of the tumorigenic properties of *apc*^{-/-}-derived tumours are β -catenin independent. M.A. Price (New York, NY) showed recent data from *Drosophila* indicating that other β -catenin partners can also have affairs outside the β -catenin home. She showed that GSK3 and CK1 also regulate the lifespan of Cubitus Interruptus (Ci), the transcription factor activated by the Hedgehog pathway. Although it was already known that Ci degradation is dependent on its phosphorylation by PKA, Price demonstrated that this modification primes further phosphorylation at adjacent sites, by GSK3 and CK1.

Prometheus was not such a Titan after all

The cancer troops are now metastasizing into other fields. Thus, for the first time in the *Oncogenes and Growth Control* conference series, there was a session devoted exclusively to stem cell biology where many of the usual suspects in oncogenesis made an appearance. A. Trumpp (Epalinges, Switzerland) showed that the tissue-specific knockout of the *c-myc* gene in bone marrow cells results in the blockage of stem cell differentiation, leading to larger populations of stem cells and highly reduced populations of mature hematopoietic cells. This effect could be derived, at least in part, from the enhanced adhesion of the dividing stem cells to stromal cells due to the upregulation of the adhesion molecule LFA1 in *c-myc*^{-/-} cells. This enhanced adhesion probably perturbs the developmental decisions taking place during stem cell mitosis (Spradling *et al.*, 2001), resulting in the generation of two equivalent daughter stem cells instead of the usual stem cell and committed cell. N. Rosenthal (Monterotondo, Italy) showed that adult muscle tissue can be regenerated by a population of bone marrow stem cells sensitive to IGF1 (insulin-like growth factor 1). Consistent with this, the generation of transgenic mice expressing IGF1 in muscle induces an Schwarzenegger-like phenotype characterized by muscle hypertrophy. IGF1 may turn out to be the elusive water from the 'Fountain of Youth', since the IGF1 transgene prevents muscle aging and can even rescue the muscle phenotypes of *mdx* (muscular dystrophy, X-linked) and *sod1* (superoxide dismutase) mice, two animal models traditionally used for studying muscle degeneration. Finally, E. Lagasse (Palo Alto, CA) gave a biological explanation for the mythological regenerative abilities of Prometheus' liver; after listening to his talk, we know that liver regeneration actually relies on the transdifferentiation properties of hematopoietic stem cells (HSC). Lagasse described transplant-

ation experiments in which different populations of bone marrow cells were introduced into FAH (fumarylacetoacetate hydrolase)-deficient mice, which die of progressive liver failure unless their dying hepatocytes are regenerated by the transplanted, enzyme-producing cells. He showed that highly purified HSCs, but not other bone marrow cell populations, can rescue of the lethal phenotype of *fah*^{-/-} mice. As few as 50 injected HSCs could do the job, underscoring the great potential of HSCs for clinical application in the future.

It only gets more complex

The use of genomics has already helped the understanding of transcriptomal changes associated with specific oncogenic events. However, the power of genomics and proteomics can be applied in more ambitious plans, such as the implementation of high-throughput screenings aimed at finding and assembling all the missing pieces in the cell signaling mosaic. In this context, T. Pawson (Toronto, Canada) and G. Superti-Furga (Heidelberg, Germany) showed how proteomics techniques are making it possible to chart protein-protein interactions at work in specific pathways. Examples included interactions mediated by SH3 proteins (T. Pawson), Polo-like kinases (T. Pawson) and TNF α (G. Superti-Furga). P. Bork (Heidelberg, Germany) described new *in silico* methods, based on interspecies comparative genomics, for identifying elements of metabolic pathways. All of these studies highlighted the power of proteomics to identify sets of proteins involved in defined pathways, as well as interconnections between them via linker proteins. Although this first batch of experiments shows the utility of these approaches for characterizing functional relationships on a whole cell scale, the Damocles' sword still lingering over our heads is the question of how accurate they are. Bork tackled this problem by comparing the results of genome-wide protein-protein interaction screenings using bioinformatic tools, and presented the unsettling finding that ~50% of all the interactions described so far are probably artifacts. The take-home message is that we will need to approach the study of cellular functions using multiple experimental techniques and integrated database approaches simultaneously. New methods of normalization and quality-control will have to be put in practice to make sure that close-to-reality data are obtained.

Alternative methods for obtaining a genome-wide view of biological processes were also discussed at the meeting. M. Boutros (N. Perrimon laboratory, Boston, MA) described ongoing high-throughput *loss-of-function* experiments aimed at addressing the roles of the 14,000 *Drosophila* genes in defined biological responses. He demonstrated that RNAi is a feasible approach for this kind of study by using the technique to inhibit key components of the Toll and Imd (immune deficiency) signaling pathways of *Drosophila*. D. Bohmann (Rochester, NY) presented another strategy to identify genes involved in developmental processes in *Drosophila*. His method is based on the labeling of specific cell types with GFP (green fluorescent protein), purification of GFP⁺ and GFP⁻ cells by flow cytometry, and subsequent analysis of the transcripts from each population by SAGE (serial analysis of gene expression). Expression of GFP in cells was achieved by placing the *gfp* cDNA under the regulation of tissue-specific promoters. When applied to the

characterization of cells from the proliferating and differentiating zones of the developing *Drosophila* eye primordium, this method revealed groups of genes associated with each. Interestingly, the search for common regulatory elements in the promoters of these gene pools revealed the presence of binding sites for DREF (DNA replication-related element factor) in the proliferation-related genes. This transcription factor is key in this process, because its ectopic expression can drive proliferation even in cells that have already begun to differentiate. Finally, C. Marshall (London, UK) presented the results of a study on mutations of *B-raf* in human cancer, a project that arose from a collaboration with the Cancer Genome Project at the Wellcome Trust Sanger Institute. The ultimate aim is to screen the exons and exon/intron boundaries of all of the human genes present in a collection of established cancer cell lines and primary tumors for mutations. As a proof of principle, the study has focused initially on mutations within genes of the *raf* family. In a collection of 1000 samples, activating mutations in *B-raf* were detected in 7.5% of cases, mainly in tumor types where Ras mutations were also present. Strikingly, mutations occurred with high incidence in melanoma (~60% of cases). This interesting approach will soon provide us with a comprehensive map of the genetic changes implicated in specific tumor types.

Perspectives

The signal transduction field seems to have reached maturity, since most topics discussed at the conference dealt with the mechanisms by which pathways propagate signals and communicate with each other rather than on the isolation of new signaling molecules. In addition, the conference illustrated the great complexity of the cellular responses mediated by the combination of cell–cell interactions, autocrine and paracrine events that are necessary for the development of cancer. In this context, it is now clear that the response of one cell to a specific stimulus is not cell-autonomous, but depends on the presence of synergistic and antagonistic extracellular stimuli and the type of cells living in the neighborhood. Due to these new advances, we

can approach pathological states such as cancer in a much more systematic way than previously. However, we must also develop new techniques to deal with this complexity, an experimental challenge that will have to be addressed through multifaceted approaches combining genomics and proteomics, bioinformatics, and mathematical models. Thus, one can predict that the techniques of the scientists working in this field in the near future will be as complex as the cellular responses studied by them.

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