Molecular mechanisms in signal transduction and cancer

Meeting on Oncogenes & Growth Control

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The EMBL/Salk/EMBO Conference on 'Oncogenes & Growth Control' was held at the EMBL in Heidelberg, Germany, between 17 and 20 April 2004, and was organized by M. Bienz, G. Evan, C. Marshall, A. Nebreda and R. Treisman

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Starting at the end

In the last session of the meeting, J. Campisi (Berkeley, CA, USA) looked at cancer from an evolutionary point of view and presented a model in which cancer is not only caused by the accumulation of mutations, but also facilitated by the senescent cells that accumulate in older people. Her model is based on the observation that senescent cells support the growth of preneoplastic cells much better than that of normal fibroblasts. Senescent cells may do so by secreting many products, including growth factors, cytokines such as interleukin-6 (IL-6), and proteases. Older people do indeed accumulate senescent cells as shown by β -galactosidase staining. Furthermore, older people have higher levels of IL-6, which is one of the causes of frailty. Campisi argued that the occurrence of senescent cells is a pleiotropic effect of our fight against cancer cells, and as they occur after reproductive age, there is no selective pressure to eliminate them. This fight against cancer has also resulted in the evolution of tumour suppressor genes, which fall into two groups: the caretakers, which are repair genes that protect the integrity of the genome; and the gatekeepers, which are apoptosis- and senescence-inducing genes that eliminate or arrest potential cancer cells. Caretaker genes are important for sustaining cell renewal. However, gatekeeper genes deplete the organism of cells and essential stem cells, which stalls cell renewal and therefore may cause ageing.

The p53 gene is both a caretaker (by inducing DNA repair) and a gatekeeper (by inducing apoptosis in the case of irreparable damage). Therefore, after a DNA-damaging insult, p53 is activated and the necessary responses are induced. G. Evan (San Francisco, CA, USA) addressed the question of what would happen if p53 is activated only when "it really matters," that is, when a tumour arises. He generated p53-null mice that express an oestrogen receptor (ER)-p53 fusion protein so that p53 could be switched on and off at will. When these mice were irradiated and p53 was not being expressed, the mice did not show severe damage responses but they developed tumours. However, if p53 expression was restored for just a brief period of time, after the DNA damage had been resolved, no tumours were observed. Evan speculated that if we could repress p53 for most of the time and only activate it when a tumour arises, perhaps we can keep the beneficial effect

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Introduction

Good weather and a pint of apple juice (although we thought we had ordered beer) at a farmhouse opposite the European Molecular Biology Laboratory (EMBL) heralded the start of the 15th conference on 'Oncogenes & Growth Control'. The participants had the opportunity to enjoy the friendly atmosphere at the EMBL, the hospitality of the staff and the tasty food. And of course, a series of excellent talks, which provided an in-depth view of several topics in signal transduction and cancer biology. In this report, we will give you an overview of what was discussed.

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of p53 in fighting tumour formation, but lose its unwanted effects in ageing.

One potential cause of ageing may be the depletion of stem cells, whereas tumour formation may in part be due to the failure of stem cells to differentiate. It is therefore very important to know how stem cells are regulated. The accepted view is that stem cells divide asymmetrically into one cell that remains a stem cell and one that is bound for differentiation. A. Trumpp (Epalinges, Switzerland) gave an interesting presentation on how stem-cell renewal might occur in mammalian systems and particularly on the role of Myc in this process. First, he indicated that haematopoietic stem cells are restricted to a certain region of the bone marrow—the stem-cell niche—where the stem cells interact with the supporting cells through adhesion anchors, such as N-cadherin and integrins. He showed that a conditional mouse strain that lacks Myc in the bone marrow compartment accumulates stem cells. This accumulation was not due to decreased apoptosis or increased cell proliferation, but rather to a failure to differentiate. Surprisingly, these cells can differentiate normally *in vitro*. Thus, the failure to differentiate is apparently due to the unique surrounding of the stem-cell niche. Conversely, when Myc is over-expressed in stem cells, the stem-cell niche is depleted of stem cells. This led Trumpp to propose a model in which Myc is involved in the switch of a stem cell to a cell that is on its way to differentiation. It is unclear how Myc is regulated, but the consequence of Myc activation is the repression of the cell-adhesion molecules N-cadherin and the integrins. This repression is likely to be a prerequisite for the cells to leave the niche.

Haematopoietic stem cells were also the topic of T. Graf's presentation (New York, NY, USA), who showed that under normal conditions, blood cells do not contribute to other tissues during mouse development: when these cells were irreversibly labelled with yellow fluorescent protein they could only be detected in the haematopoietic lineage. However, even differentiated haematopoietic cells retain a surprising degree of plasticity. Graf showed that the artificial introduction of the transcription factor C/EBP- α into B cells reprogrammed these cells to become macrophages, and that their immunoglobulin genes were rearranged. The switch involves the induction of the macrophage gene expression programme by C/EBP- α together with the transcription factor PU.1 (already expressed in B cells) and the down-regulation of lymphoid genes through the C/EBP- α -mediated inactivation of the transcription factor Pax5 (Xie *et al*, 2004).

What tumours do

Angiogenesis is considered to be a key step in tumour growth, but K. Alitalo (Helsinki, Finland) showed that the formation of lymph vessels (lymphangiogenesis) also has a role in tumour metastasis (Saharinen *et al*, 2004). Both vascular endothelial growth factor (VEGF)-C and -D are responsible for the induction of lymphangiogenesis by binding to and activating the VEGF receptor VEGFR3, which is a receptor tyrosine kinase. Indeed, the overexpression of VEGF-C induces the formation of lymph vessels and lymph metastasis, and thus provides a route for metastasizing cells to escape from the tumour. By contrast, a soluble VEGFR3 extracellular domain that functions as a decoy to trap VEGF-C and -D inhibits embryonic lymphangiogenesis and lymph metastases. In addition to the role of senescent cells in tumour formation as indicated above, immune cells also have an

important role in tumour initiation and progression. Strikingly, chronic inflammation is one of the causes of cancer. Z. Werb (San Francisco, CA, USA) discussed the importance of this interaction and showed that tumours also have a profound effect on the behaviour of lymphocytes and macrophages, as these cells become highly motile in the presence of a tumour.

How to get rid of tumours

At present, the best way to treat metastatic cancer is with chemicals. Although the majority of these drugs are still rather non-specific, more targeted drugs are entering the market. Imatinib (US, Gleevec; non-US, Glivec), an inhibitor of the Abl tyrosine kinase, is an example of a drug that is now being used successfully to treat chronic myeloid leukaemia, which develops because of a chromosomal translocation that fuses the B-cell antigen receptor (Bcr) to the Abl tyrosine kinase. The resulting kinase is constitutively active and causes a massive proliferation of white blood cells. G. Superti-Furga (Heidelberg, Germany) described the crystal structure of Abl in its inactive state. In this conformation, the amino-terminal myristyl group is inserted in the kinase domain causing a conformational change that allows the Src homology SH2 and SH3 domains to interact with and inhibit Abl. Bcr-Abl lacks the N-terminal myristyl group. Imatinib only binds to the catalytic site in its inactive state and thus stabilizes Bcr-Abl in the inactive conformation. Imatinib also targets Kit and the platelet-derived growth factor (PDGF) receptor. This led H. Beug (Vienna, Austria) to speculate that imatinib might also prevent the formation of metastatic cells. In his mammary epithelial cell system, the autocrine production of PDGF is required for epithelial-mesenchymal transition (EMT), as well as the induction and maintenance of metastasis. This confirms that EMT is a faithful *in vitro* correlate of tumour progression and metastasis.

Superti-Furga described further chemical proteomics experiments using imatinib and another kinase inhibitor, PD17. The compounds were immobilized at a permissive group and then used as affinity reagents to 'pull down' interacting proteins that were then identified by mass spectrometry and bioinformatics. These experiments nicely displayed the difference in selectivity between the two compounds. Imatinib, the more bulky compound of the two, pulled down all its known targets, as well as a few additional ones that may explain previous reports of imatinib affecting the amyloid precursor protein processing pathway involved in Alzheimer's disease. PD17 is much more promiscuous and interacts with a whole battery of kinases. This approach can be used to monitor the specificity of kinase inhibitors and other drugs that interfere with signalling pathways.

Another approach to treat tumours in the future may be to interfere with the hedgehog pathway. P. Beachy (Baltimore, MD, USA) elegantly described the important role of this signalling pathway in metastatic prostate cancer (Lum & Beachy, 2004). Hedgehog binds to the cell surface receptor patched (Ptc) thereby releasing smoothened (Smo) from inhibition by Ptc (Fig 1). Overexpression of two hedgehog ligands, Sonic hedgehog and Indian hedgehog, and constitutive activation of Smo, either by mutation of Smo or loss of Ptc, is observed in many tumours. Indeed, Beachy calculated that the hedgehog pathway is involved in as many as 25% of all cancer deaths. In prostate cancer, Smo expression seems to correlate with the metastatic behaviour of cells, leading to the proposal that the hedgehog

Fig 1 | A schematic version of the hedgehog signalling pathway. Hedgehog (Hh), when bound to its receptor patched (Ptc), will relieve the inhibition of Ptc on smoothened (Smo). Activated Smo in turn will activate members of the Gli transcription factor family.

pathway is promoting the metastatic programme of prostate cancer cells. Importantly, the natural compound cyclopamine, which was isolated from a poisonous plant that causes a developmental defect resulting in a cyclopic eye, binds to Smo and inhibits the pathway. This drug is now in clinical trials, as are other drugs that inhibit Smo.

The hedgehog signalling pathway also has a role in skin tumour formation. F. Watt (London, UK) showed that stem cells in the skin differentiate in different lineages depending on whether the β -catenin–T-cell-factor (Tcf) signalling pathway is switched on or off: high levels of stabilized β -catenin promote hair follicle formation, intermediate levels promote sebocyte differentiation and low levels promote interfollicular epidermal differentiation. Hair follicle tumours arise in mice that have a high epidermal expression of β -catenin and sebaceous tumours develop when β -catenin signalling is blocked by N-terminally truncated lymphoid-enhancer binding factor 1 (Lef1). This correlates with the expression of either Sonic hedgehog (in hair follicle tumours) or Indian hedgehog (in sebaceous tumours).

The spectacular recent finding that the B-Raf kinase is mutated in many tumours, such as melanomas, adenocarcinoma of the colon and papillary thyroid tumours, is another indication of the importance of the Ras–B-Raf–MEK–ERK pathway in human tumour formation. D. Barford (London, UK) presented the recently described crystal structure of B-Raf and explained the nature of most of the observed mutations (Wan *et al*, 2004; Fig 2). Most mutations can be explained by the destabilization of the inactive conformation or the stabilization of the active conformation. Interestingly, one group of mutations results in a B-Raf with reduced activity *in vitro*, but this protein still activates ERK *in vivo*, perhaps through the formation of heterodimers with Raf1. A candidate drug exists that can inhibit B-Raf and this Bayer–Onyx compound is now in clinical trials. Interestingly, similar to imatinib, this compound binds to and stabilizes the inactive conformation of the kinase. For the design of specialized treatments, the precise genetic constitution of the tumour might be very important. S. Lowe (New York, NY, USA) reported that similar tumours react completely differently to specific treatments. Thus, B-cell lymphomas induced by retroviral infection of haematopoietic stem cells with PKB/Akt in E μ -Myc-expressing mice are sensitive to a combination of rapamycin and adriamycin,

Fig 2 | The structure of B-Raf in the vicinity of the catalytic site. Mutation of Val 599 to Glu activates B-Raf by 500-fold. In the wild-type protein, the inactive conformation of the activation segment (red) is stabilized by buried hydrophobic interactions between the aliphatic side chain of Val 599 of the activation segment and Phe 467 of the P-loop. Conversion of the activation segment to the active conformation (green) requires exposure of Val 599. Val 599 therefore stabilizes the inactive conformation relative to the active state. By contrast, a Glu at position 599 stabilizes the active conformation after it is solvent exposed by interacting with Lys 506 of the α C-helix and destabilizes the inactive conformation by forming unfavourable interactions with Phe 467. The position of the Bayer–Onyx compound (BAY43-9006) that stabilizes the inactive conformation is indicated. For more details, see Wan *et al*, 2004.

but similar tumours induced by Bcl2 are not. Both Bcl2 and PKB/Akt induce cell survival by protecting cells from going into apoptosis, and apparently the rapamycin target Tor mediates the anti-apoptotic effect of PKB/Akt. Interestingly, one of the downstream targets of Tor signalling, the elongation factor eEF-4E, also induces lymphomas. As eEF-4E functions downstream of Tor, these tumours are insensitive to rapamycin/adriamycin treatment (Wendel *et al*, 2004).

p53 still has its surprises

The p53 protein is probably the most extensively studied molecule in the cancer field. One of the proteins that is involved in the regulation of p53 is ADP-ribosylation factor (Arf). In a series of elegant experiments, J. Lees (Cambridge, MA, USA) presented evidence that Arf is regulated both positively and negatively by members of the E2F family. It was shown previously that E2F1 is a transcriptional activator of Arf, but by using knockout cell lines, Lees showed that E2F3b is a transcriptional repressor of Arf, which directly binds to the Arf promoter. Thus, in normal cells, E2F3b represses Arf, whereas in tumour cells, in which E2F1 is frequently activated due to the absence of retinoblastoma protein (Rb), E2F1 takes over and induces Arf expression (Aslanian *et al*, 2004).

The effects of p53 on cell-cycle inhibition and apoptosis are produced by several downstream transcriptional targets. K. Vousden

(Glasgow, UK) showed further evidence for the role of Puma in p53-induced apoptosis. This protein has a Bcl-homology domain and interacts with Bcl2 to induce apoptosis. However, a deletion mutant that fails to induce apoptosis still retains some ability to bind to Bcl2, indicating that this interaction is not the sole function of Puma in inducing apoptosis. The key role of Puma in p53-mediated apoptosis was also investigated by Lowe in his E μ -Myc-induced mouse lymphoma model system. The use of RNA interference (RNAi) through the introduction of small hairpin RNA (shRNA) molecules that are homologous to either p53 or Puma strongly accelerated the formation of these tumours, indicating that in this model system, Puma phenocopies the effect of p53.

More kinases

J. Downward (London, UK) described the use of an shRNAi library containing three different constructs for all protein kinases. This library was used to identify novel intermediates in Ras-induced senescence including p110 α -PI3 kinase, p70S6 kinase and the kinase target Mink. Mink is an STE20/GCK-like kinase that activates the p38 and Jun N-terminal kinase (Jnk) pathways. Mink shRNA inhibits the Ras-transformation of certain cell lines, indicating that this kinase also has a role in Ras-induced cell transformation.

LKB1 is a kinase that is mutated in patients with Peutz Jeghers syndrome, which is characterized by multiple benign and malignant tumours. The protein functions in a complex with the Ste20-related adaptor protein (Strad) and the scaffolding protein Mo25. D. Alessi (Dundee, UK) discussed their recent result that LKB1 is the upstream kinase for the AMP-activated protein kinase (AMPK): LKB1 phosphorylates AMPK in the T-loop and in LKB1-null cells, AMPK can no longer become activated (Hawley *et al*, 2003; Fig 3). T. Mäkelä (Helsinki, Finland) studied intestinal polyps that appear in LKB1-heterozygote-null mice. Tumours from these mice have a high level of cyclooxygenase 2 (Cox2) expression. Interestingly, in LKB1-heterozygous-null/Cox2-null mice the number of polyps is strongly reduced, which suggests that Cox2 contributes to the formation of the polyps. This result is the basis of a clinical trial of Cox2 inhibitors to treat these polyps. H. Clevers (Utrecht, the Netherlands) showed that when LKB1 is ectopically expressed in colon cell lines in the presence of Strad, these cells become polarized and form brush borders. Importantly, neighbouring cells are

not required for this process. This indicates that cells can polarize apical and basolateral membranes outside the context of an epithelial sheet (Baas *et al*, 2004; Fig 3).

T. Hunter (La Jolla, CA, USA) reported that F-actin inactivates a cytoplasmic pool of Abl in suspended cells and, conversely, that Abl has a role in actin-mediated microspike (filopodia) formation. Indeed, when spreading on fibronectin, cells null for both Abl and the Abl-related gene *Arg* have fewer microspikes than wild-type cells, whereas cells overexpressing Abl have more. Two proteins that can interact with Abl, p62Dok1 and Nck, mediate this Abl effect. The direct phosphorylation of Dok1 by Abl at Trp 361 creates a binding site for Nck, which can then activate local F-actin assembly (Woodring *et al*, 2004). M. Frame (Glasgow, UK) showed that Src, together with integrins, regulates focal adhesion kinase (FAK), which in turn deregulates the transport of E-cadherin to cell-cell junctions through the action of myosin light-chain kinase (MLCK) and other kinases. Interestingly, in the DMBA-TPA mouse skin-tumour model, keratinocytes that lack FAK still form papillomas, although these occur less frequently than in those that express FAK. However, these fail to convert into squamous cell carcinomas.

On small GTPases and transcription factors

In the field of small GTPases, C. Marshall (London, UK) reported on the Ral effector pathway that operates downstream of oncogenic Ras. He described a new effector for Ral, Zonab, which is a transcription factor that also binds to the tight junction protein ZO-1. Zonab is known to bind to CCAAT motifs and repress transcription. The model is that either ZO-1 or Ral keeps Zonab inactive in the cytosol and relieves transcriptional repression by Zonab. J. Bos (Utrecht, the Netherlands) reported on the crucial role of Rap1 in inside-out signalling to integrins: Rap1 is both necessary and sufficient to induce $\alpha_4\beta_1$ -integrin-mediated cell adhesion. This increased adhesion process is most probably due to an increased clustering (avidity) of integrins. In addition, Bos presented evidence that Rap1 is also involved in E-cadherin-mediated signalling. When Rap1 was inhibited, MDCK cells failed to bind to E-cadherin molecules that were coupled to plastic. Furthermore, activation of Rap1 inhibited hepatocyte growth factor (HGF)-induced scattering of MDCK cells, which supports a role for Rap1 in the maintenance of cell-cell junctions.

In the transcription factor field, R. Treisman (London, UK) reported further on his recently published work to decipher how serum response factor (SRF) is regulated by actin. One of the proteins involved in this process is Mal, a transcriptional coactivator (Miralles *et al*, 2003).

Interestingly, P. Rørth (Heidelberg, Germany) reported that *Drosophila* Mal has a crucial role in guiding border cells to their proper location in the oocyte. This process is induced by epidermal growth factor (EGF) or by PDGF/VEGF, depending on the destination. Border cells migrate as a group of six cells, and tension between the cells activates Mal to coordinate migration. This tension is induced by long extensions of the cells towards their destination. Indeed, when Mal is mutated, the cell body does not migrate, but the extensions break loose and continue to do so.

Lessons from the beginning

Our understanding of cancer-related processes often originates from studies in *Drosophila*. M. Bienz (Cambridge, UK) discussed the role of Pygopus (Pygo) in the regulation of the Wnt signalling pathway in *Drosophila*. Pygo is a protein that forms a complex

Fig 3 | LKB1 a master regulator. LKB1 has been shown to act as a T-loop kinase for several kinases (Lizcano *et al*, 2004) including the partitioning-defective kinases (PARs) and AMPK. The work discussed by H. Clevers suggests that LKB1 through PARs can act to regulate cell polarity (Baas *et al*, 2004). D. Alessi showed that LKB1 acts to regulate AMPK, a kinase that is involved in sensing the cellular ratio of AMP/ATP (Hawley *et al*, 2003).

with Legless (Lgs), and together they interact with Armadillo (Arm; the *Drosophila* homologue of β -catenin). This interaction enhances the localization of Arm to the nucleus, where Arm interacts with Tcf to induce transcription. In addition, Pygo itself can interact with transcriptional coactivators and induce transcription. Indeed, a fusion of the carboxyl-terminus of Pygo to a mutant of Tcf rescued the dominant-negative phenotype of this mutant. B. Conradt (Hanover, NH, USA) reported on the mechanisms by which *Caenorhabditis elegans* regulates the apoptosis of cells that are destined to die. During the development of serotonergic neurons, the BH3-domain-containing protein egg laying abnormal 1 (EGL-1) is induced in the daughter cell that undergoes apoptosis. This induction is transcriptionally regulated through two helix-loop-helix proteins, HLH-2 and HLH-3, which compete with the snail-like transcription repressor CES-1 for binding to the EGL-1 promoter. How HLH-2 and -3 are induced is still unclear.

Concluding remarks

The field of signal transduction and cancer is still progressing at a rapid pace. It is encouraging that the translation of this knowledge into the development of potential therapeutics is also continuing to take place. This meeting was certainly a stimulating experience in the continuing efforts to combat cancer. We are already looking forward to the next one.

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