Understanding cancer: from the gene to the organism

Conference on genes and cancer

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Received January 21, 2002; revised March 21, 2002; accepted March 22, 2002

The 18th meeting of the UK Molecular Biology and Cancer Network took place at the University of Warwick, UK, December 10–12, 2001, and was organized by Caroline Hill, Helen Hurst, Steve Jackson, Tony Kouzarides, Chris Marshall, Jon Pines, Patti Pinto, Stefan Roberts, Andy Sharrocks, Richard Treisman and David Wynford-Thomas.

The many faces of cancer

Cancer is a complex disease arising from multiple genetic lesions. Cellular transformation is accompanied by changes in numerous processes, including DNA repair, cellular proliferation, cell-cycle control and apoptosis, and acquired capabilities such as tissue invasion and metastasis (Hanahan and Weinberg, 2000). The complex interplay between genes altered in these different processes can lead ultimately to tumorigenesis. This conference covered some of the latest discoveries that address the molecular causes of cancer, and its broad subject base reflects the diversity of genes involved. As changes in gene expression are a major driving force behind tumorigenesis, this was a prominent topic of discussion, as were cell-cycle control and the maintenance of genome integrity. The influence of the local cellular environment in determining the normal versus oncogenic functions of genes was a key concept to emerge and,

finally, new technologies that permit us to study genes and cancer in a more global context were covered.

Chromatin modifications and remodelling

Changes in gene expression profiles are among the key determinants of tumorigenesis. One of the first steps in activating gene expression is altering the accessibility of chromatin to the transcription machinery. This can involve either modification of histone tails and/or changes in the chromatin structure (Figure 1). Proteins related to components of the SWI/SNF complex form parts of important chromatin remodelling machines. Studies by T. Owen-Hughes (Dundee, UK) on one such protein, the chromatin remodelling ATPase, ISWI, suggest how it may play a role in transcriptional regulation by mobilizing nucleosomes along the DNA. The key finding presented was that ISWI binds to free DNA next to the nucleosome and promotes translocation of the DNA, which then loops around the nucleosome in a stepwise manner. Obviously, these remodelling machines must be targeted to the correct promoters. Work on the yeast homologue, ISW1, by C. Goding (Oxford, UK) suggests a mechanism by which this could be accomplished. His studies on the *PHO8* promoter demonstrate a role for ISW1 in repressing transcription and implicate the basic helix–loop–helix leucine zipper (bHLHZip) transcription factor Cbf-1 in recruiting it to the DNA *in vivo*.

The importance of the histone H4 tail for the repressive activity mediated by ISW1 was also demonstrated by Goding. Histone tails are subject to covalent modification with a number of different functional groups, including phosphate, acetyl, methyl and ubiquitin groups. These modifications can act positively or negatively to regulate gene expression and often act in a combinatorial manner.

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Fig. 1. Chromatin modifications and remodelling in gene activation. Activation and repression of transcription involve both reconfiguration of nucleosome positioning (chromatin remodelling) and post-translational modification of the histone tails (chromatin modification). Transcription factors (TF) can recruit a variety of enzymatic activities that can either add or remove acetyl groups (Ac), add methyl groups (Me) or add phosphate groups (P) to histone tails. Different combinations of these enzymatic activities are recruited to particular promoters to elicit their regulatory effects.

A further emerging area of interest in this field is histone methylation. T. Kouzarides (Cambridge, UK) presented a series of interesting findings on the mechanisms and consequences of arginine and lysine methylation of histone tails. One key observation is that the tumour suppressor retinoblastoma (RB) protein (a critical regulator of progression through G₁ phase of the cell cycle) recruits the Lys-methylase Suv39 to DNA via association with the transcription factor E2F (Nielsen *et al.*, 2001). This, in turn, methylates Lys 9 in histone H3 tails, leading to binding of the repressive heterochromatin protein 1 (HP-1) to the methylated tail and subsequent shut-down of E2F-regulated cell-cycle genes. Further studies from his group have led to the identification of a lysine methylase, SET1, with a different specificity (H3 Lys 4), which is important for the activation of around 200 genes in yeast. The observation that methylation can be an activating signal changes our way of thinking about this type of modification, which was hitherto thought to represent only a repressive signal. Finally, Kouzarides reported that arginine methylation of histones also occurs *in vivo* and demonstrated the function of one arginine methylase, CARM1, in activating a specific target gene, the estrogen-receptor-inducible *PS2* gene.

Transcriptional repression due to histone deacetylation is mediated by histone deacetylases (HDACs). A. Dejean (Paris, France) demonstrated that HDACs themselves are regulated by post-translational modifications, adding a further tier of control. Members of both class I (HDAC1) and class II (HDAC4) deacetylases can be sumoylated, and SUMO conjugation is inhibited by activation of the calcium-calmodulin dependent kinase, CaMKIV. As SUMO modification promotes nuclear localization, this event is thought to regulate the nuclear-cytoplasmic shuttling of HDACs.

Chromatin modifications play a major role in transcriptional regulation. However, modification of the higher-order structure

of chromatin is also critical to generate specialized chromosomal regions such as centromeres and telomeres. R. Allshire (Edinburgh, UK) demonstrated the importance of maintaining highly methylated chromatin at centromeres to promote SWI-6 (the yeast homologue of HP-1) binding and cohesin recruitment. In the absence of cohesin, the centromeres of chromosomes do not pair up correctly and multiple aberrations occur during their segregation following DNA replication.

Finally, S. Jackson (Cambridge, UK) indicated the potential importance of chromatin modifications in DNA repair, using the example of phosphorylation of histone H2A, which leads to chromatin reconfiguration and access by the DNA repair machinery.

Gene regulation

Although chromatin modification and remodelling are key steps in gene regulation, it is the transcription factors that direct these activities to promoters and ultimately dictate the specificity of gene activation. S. Roberts (Manchester, UK) demonstrated that a specific splice form of the Wilm's tumour suppressor protein WT-1 recruits the coactivator protein Par4 and that this rescues cells from apoptosis after UV treatment. WT-1 is also regulated by a cosuppressor protein that downregulates its transcriptional activity. BASP-1 (brain acid soluble protein) was identified by Roberts as a candidate cosuppressor that is expressed in the same subset of cells (podocytes) as WT-1 in the developing kidney.

Members of the E2F transcription factor family share many similar properties and exhibit overlapping DNA binding specificities, making it difficult to determine functional distinctions. However, studies on E2F transcription factors by J. Lees (Boston, MA) demonstrated that these proteins do not all function in the same manner. Whereas E2F1–E2F3 act as activators, E2F4 and E2F5 are repressive transcription factors. Elegant knockout experiments demonstrated that *in vivo*, E2F4 and E2F5 exert their repressive activity by recruiting the RB-related proteins p107 and p130, which only bind to E2F1–E2F3 once the pool of E2F4 and E2F5 is depleted.

Context-dependence of tumorigenesis and cell-cycle control

The use of cultured cells has led to significant progress in understanding the mechanisms of tumorigenesis. However, although these studies have been valuable, they do not take into account the microenvironments in which tumours form *in vivo*. Furthermore, classic knockout and transgenic studies probing the role of oncogenes or tumour suppressors do not fully recapitulate the sporadic nature whereby the majority of tumours arise, and developmental complications often mask the tumorigenic effects that manifest themselves in later life. A. Berns (Amsterdam, The Netherlands) introduced several modifications that could be used in mouse studies to more closely relate the findings to the function of oncogenes and tumour suppressors *in vivo* and, hence, enable the elucidation of downstream pathways or therapeutic testing (Meuwissen *et al.*, 2001). For example, mutations can be introduced in the context of additional, already characterized mutations, thereby mimicking the

Fig. 2. Specific expression of luciferase reporter gene in pituitary tumours. Luciferase expression is driven by a pituitary-specific promoter and its expression monitored in a mouse in which pituitary tumours have been generated using a pituitary specific Cre-recombination system to knock out RB conditionally (A. Berns, Amsterdam, The Netherlands).

multiple defects found in cancer cells. Similarly, by expressing mutant alleles or deleting genes in a specific temporal and spatial manner using conditional tissue-specific transgenic and knockout models, the sporadic nature of tumours can be recapitulated. Using such approaches, it was demonstrated that, in the lung, only cells that express the oncogenic small GTPase K-Ras, a member of the Ras superfamily, develop adenomas and that these develop in a synchronous manner, indicating that K-Ras is sufficient to initiate tumorigenesis. Similarly, the loss of both RB and p53 has been shown to be sufficient to promote the formation of malignant and metastatic lung tumours that have characteristics of small-cell lung cancers. Finally, Berns reported that, by driving luciferase gene expression under tissue-specific promoters in transgenic mice (Figure 2), it is possible to monitor the growth of tumours, and hence their regression, following drug treatment. This non-invasive tumour monitoring technique is a promising approach for following the long-term effects of anti-cancer drug treatment.

A further example of context dependence was given by P. Sicinski (Boston, MA), who demonstrated that, whereas Ras promotion of tumours in mammary cells is absolutely dependent on the presence of cyclin D1, this is not the case in fibroblasts. In this cell type, cyclins D2 and/or D3 are sufficient for Ras-mediated tumour formation.

The importance of location for correct signalling at the intracellular level was raised by I. Hagan (Manchester, UK), who described how a mutation in the fission yeast spindle-pole protein Stf1/Cut12 was able to overcome the loss of the mitotic inducer, Cdc25. Cdc25 is a phosphatase, and yet Stf1/Cut12 shows no homology to any enzymes. Thus, one potential role for Stf1/Cut12 might be to anchor other regulators to the spindle pole.

Mouse models of signalling *in vivo*

A further use of mouse models is to unravel the function of signalling pathways *in vivo*. This is particularly important, as many pathways employ related proteins and it is not clear whether they have unique or overlapping functions. A good example of this is the Ras–Raf–MEK–ERK pathway, which was the subject of discussion by several speakers (summarized in Figure 3). It has generally been accepted that Raf-1 is the kinase that links Ras to the activation of the mitogen activated protein

kinase ERK, despite the existence of the related A-Raf and B-Raf proteins. However, C. Pritchard (Leicester, UK) demonstrated that ERK activation is normal in mouse embryonic fibroblasts (MEFs) derived from Raf-1 (and A-Raf)-deficient mice but is ablated in B-Raf-deficient mice. This firmly places B-Raf, rather than Raf-1, in the signalling cascade, although, previously, B-Raf had been thought only to play a minor role in Ras signaling in a few cell types such as neurones. The B-Raf-deficient mice also exhibit some unique properties, including vascular hemorrhaging due to apoptosis of vascular endothelial cells. In contrast, Raf-1-deficient mice exhibit a more general enhancement of apoptosis that leads to embryonic lethality.

Further studies on Ras signalling by D. Tuveson (T. Jacks laboratory, Boston, MA) illustrated the importance of studying transgenes with normal expression levels to circumvent pathway crosstalk that might occur in the presence of higher doses of protein. By using transgenic mice in which K-Ras was expressed from its own promoter, it was shown that MEFs had unexpected cellular and biochemical properties that were distinct from situations where oncogenic Ras was overexpressed.

The importance of cyclin D1 in tumorigenesis was also emphasized by knockout studies, carried out by Sicinski. Cyclin D1 was shown to be essential for Ras- and Neu-induced tumorigenesis, whereas neither cyclin D2 or D3 were important for Ras-mediated tumour formation. This requirement appears to be due to Ras and Neu acting via the ERK MAP kinase pathway to upregulate the cyclin D1 promoter rather than the nature of the cyclin itself, since the replacement of the D1 coding region with that of cyclin E had no effect on Ras-mediated tumour formation.

Finally, the importance of particular members of transcription factor families was revealed by the work of A. Roberts (Bethesda, MD). The Smad proteins are the nuclear effectors of TGF β signaling and, when stimulated, these transcription factors translocate to the nucleus. There are five known receptor-activated Smad proteins that act in multimeric complexes in concert with the common subunit Smad4 to stimulate target genes. Smad2 and Smad3 both act in response to TGF β signaling, but knockout studies revealed important differences. In Smad3 knockout mice, processes such as macrophage chemotaxis and inflammatory responses are impaired, indicating a lack of compensation by Smad2. Furthermore, by comparing MEFs derived from knockout animals, microarray analysis (in collaboration with E. Bottinger, New York, NY) demonstrated that nearly all TGF- β -dependent genes were also Smad3-dependent, whereas

Fig. 3. Novel insights into Ras signalling arising from mouse models. The expression of K-Ras at physiological levels is sufficient to induce adenomas in the lung. In contrast, high levels lead to senescence in MEFs. B-Raf, rather than Raf-1, links K-Ras with ERK activation. One target of the Ras–ERK pathway is the cyclin D1 gene that promotes proliferation.

only a small subset of genes showed dependence on Smad2. Thus, each Smad protein has distinct targets *in vivo*.

Although these studies marked significant advances in understanding the signalling mechanisms in tumour development, a cautionary note about the differences between mouse and man was raised by G. Peters' (London, UK) analysis of the human *INK4/ARF* locus, in which primary dermal fibroblasts from individuals with familial melanoma were used. These cell lines were effectively null for one of the two genes encoded by this locus, p16^{INK4a}, while retaining full p14^{ARF} function. However, in contrast to the situation in mouse cells, human-derived fibroblasts lacking p16^{INK4a} are insensitive to Ras-inducible growth arrest, although, like the mouse cells, they do undergo replicative senescence in culture.

Cell-cycle and genome integrity

Given the multitude of genetic changes that can lead to cancer, it is vital that the integrity of the genome is maintained as fully as possible. The genome is under constant attack from a range of DNA-modifying agents and has the challenge of maintaining linear chromosomes. Elegant checkpoint signal transduction cascades termed 'checkpoint pathways' (Weinert, 1998) address this issue, as they prevent the cell from dividing when the DNA has been damaged. These pathways consist of sensors of DNA damage and a transduction cascade that stops cell-cycle progression by altering the activity of a target molecule in the cell-cycle control machinery. In every system, ataxia telangiectasia (ATM)-related kinases are critical intermediates between the sensor and the transduction cascade. Jackson described results of recent work that have provided a molecular mechanism for recruitment of the ATM-related kinase, Mec1p, to sites of DNA damage. Once recruited, Mec1p has the potential to phosphorylate various downstream targets, including histone H2A, which leads to chromatin reconfiguration and access by the DNA repair machinery.

The free ends of linear chromosomes present the cell with a number of challenges: it must replicate its DNA without any loss during replication, the free ends must not activate checkpoint signals such as the ATM pathway, and the free chromosome ends must be prevented from fusing by non-homologous end joining. These challenges are met by the generation of unique structures called 'telomeres' at the ends of each chromosome. In an elegant keynote lecture, T. de Lange (New York, NY) described how the DNA at telomeres is arranged into specific 't loop' structures that resemble recombination intermediates and the relative contribution of two telomere binding factors to loop formation. A specific telomere associated factor, TRF2, is able to generate the loop-like structures, where the end of the chromosome loops back and binds further upstream on the telomere. This, in turn, protects telomere ends and prevents them from activating the DNA damage checkpoint and being reduced in length. A different factor, TRF1, contributes to the maintenance of telomeres by acting as a negative regulator of telomere length.

Global screening technologies

The recent and impending sequencing of the genomes of numerous model organisms, in particular the human genome,

provides us with a wealth of information for furthering our understanding of biological systems, especially cancer. However, technologies and approaches must still be devised to harness this vast amount of data and to use it to elucidate cellular pathways and regulatory networks.

The most obvious immediate benefit of having access to large amounts of sequence information is the ability to use microarrays that contain either complete complements of expressed cDNAs or large numbers of known genes. One way in which this has been used is in tumour profiling, as illustrated by L. Staudt (Bethesda, MD), whose studies of lymphomas showed that each type of tumour exhibits a unique genetic profile that correlates with its stage of progression (Alizadeh *et al.*, 2000). 'Lymphochips' have been developed that contain 18 000 genes that are expressed in lymphoid tissues and also include all the genes implicated in regulating cellular growth. These microarrays have been used to probe gene expression profiles in different B cell lymphomas and permit their subclassification, providing better prognostic and diagnostic indicators of survival and treatment regimes than do current classification methods. These subclassifications are based on similarities to different kinds of normal B cell populations, like, for example, germinal-centre-like tumours that have a good prognosis or activated B-cell-like tumours that have a poor prognosis. By constructing more sophisticated algorithms with which to analyse and depict the data, tumour heterogeneity can be further dissected to reveal novel subtypes with different survival outcomes. Furthermore, potential therapeutic intervention strategies can be devised based on the pathways that become deregulated in particular tumour subtypes. For example, in diffuse large B cell lymphoma cells that resemble activated large B cells, a characteristic feature is the upregulation of targets and components of the NF- κ B pathway, including the gene encoding the NF- κ B transcription factor subunit *c-Rel* and the amplification and upregulation of the target gene *bcl-2*. As Bcl-2 is an anti-apoptotic regulatory protein, this has important implications for cell survival and, hence, tumorigenesis.

One problem with microarray studies is that they do not distinguish between direct and indirect targets. However, by using chromatin immunoprecipitation analysis with specific antibodies (ChIP), it is possible to identify which genes are directly bound by particular transcription factors and, hence, are under their direct control. For example, promoter regions have been arrayed and then probed with labelled DNA derived from ChIP analysis. J. Zeitlinger (R. Young laboratory, Boston, MA) illustrated this powerful approach, in which regulatory networks of key transcription factors involved in control of the yeast cell cycle were analysed (Simon *et al.*, 2001). Each regulator was shown to be controlled, in turn, by different regulators, thereby creating a closed loop. An analogous approach has been used for mammalian genes, where arrays have been constructed with 1500 promoters from genes involved in the cell cycle. E2F4-specific ChIPs identified several known E2F targets, including Mcm3 and CDC6, but also revealed unexpected roles for E2F in regulating DNA repair and in checkpoint control.

In addition to the expression data and regulatory networks that can be built up from array analysis, another important requirement is to develop genetic techniques whereby the function of individual open reading frames can be determined in a global manner. Although this is relatively straightforward in yeast, it

represents a more difficult problem in multicellular eukaryotes. J. Ahringer (Cambridge, UK) demonstrated how RNA interference (RNAi) can be used to study gene function in *Caenorhabditis elegans*. By introducing double-stranded RNA molecules into this organism, expression of the corresponding gene can be ablated. This technique can be applied globally by creating libraries of double-stranded RNAi constructs; in the case of *C. elegans*, an elegant way of introducing these is to feed them on *Escherichia coli* that harbour expression constructs for these short RNA molecules. This approach was used to investigate the role of components of the NuRD complex in chromatin remodelling, where it was shown to be required for Ras induction in vulval patterning. RNAi can also be taken advantage of in screens for suppressors or enhancers of activated Ras in the multi-vulva phenotype. This powerful approach has obvious implications if it can be developed for higher eukaryotes.

One of the ultimate strategies in genetics is to let biology do the talking. The ability to ask a model organism directly what is most important for a particular process accounts for the disproportionate contribution of yeasts, *Drosophila* and *C. elegans* to our understanding of the molecular basis of cancer. Two elegant approaches described by A. Bradley (Cambridge, UK) now also let the mice tell us directly which genes to investigate. The identification and study of tumour suppressor loci are often hampered by the need for loss of function of both alleles before a change is observed. Creating mouse strains that harbour genetic deletions or that are more susceptible to DNA damage because of changes to the repair machinery permits the generation of genetic backgrounds that facilitate the discovery of recessive tumour suppressor genes. In the first approach, mice with large deletions of regions of the genome were monitored to identify the genes within these zones that are lost or mutated in tumours. In a second approach, excessive mitotic recombination is driven by conditional ablation of the Bloom's helicase, which usually maintains the fidelity of recombination. Combining this genetic background with mutations in genes such as the adenomatous polyposis coli (APC) gene facilitates the rapid identification of the recombinational breakpoints that are associated with tumour development.

Perspectives

Several important take-home messages emerged from this conference. One of the most important with regard to understanding and combating tumorigenesis was the necessity of generating and testing good *in vivo* models that are relevant to human disease. The context-dependent function of several genes and the lack of redundancy of others points to the importance of studying these genes within their natural expression domains. We are now beginning to exploit the information gleaned from genome sequencing projects, and technologies are still being developed to aid in this exploitation. In the context of

tumorigenesis, gene profiling appears to have great potential both as a prognostic indicator and for the design of therapeutic strategies. However, although these global technologies are clearly important, the detailed study of individual genes and proteins remains critical in terms of both basic science and in generating therapeutic agents. The recent advances in our understanding of transcription control illustrate how the study of basic elements of molecular and cell biology in a range of systems might ultimately lead to therapeutic strategies (Cairns, 2001). The rapid pace at which this field is progressing means that we can all look forward to hearing about many more significant advances in the next Genes and Cancer conference at Warwick in 2002.

Acknowledgements

Major sponsors of this conference were the Cancer Research Campaign, Imperial Cancer Research Fund, British Association for Cancer Research, AstraZeneca Central Toxicology Laboratory, University of Wales College of Medicine and Boehringer Ingelheim.

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DOI: 10.1093/embo-reports/kvf100