Unravelling novel intracellular pathways in cell-based assays

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The pharmaceutical industry is currently facing several challenges to identify and develop novel drug targets. Traditional drug discovery focussed on a small number of well-characterized gene products. Recently, this picture has changed with the completion of the draft sequence of the human genome, which has led to the identification of thousands of novel genes with unknown or poorly understood function. To cope with this overwhelming number of potential drug target candidates, new strategies for the elucidation of gene function, as well as their involvement in intracellular pathways, are required.

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▼ With R&D costs soaring at >20% per year and an average development cost of US$500 million to bring a new drug to the market, pharmaceutical companies are facing an unprecedented pressure to find better and more efficient drug candidates. In the past, drug discovery was based on a small number of scientifically well-understood targets for which the academic community performed much of the basic science, and pharmaceutical companies performed most of the developmental work to move drugs through clinical testing into the market place. This approach has led to the identification of ~500 therapeutic drugs [1]. The completion of the draft sequence of the human genome is likely to increase the speed with which novel drug targets will be identified and developed [2]. Based on estimates, the number of potential new drug targets could be in the order of 5000–10,000 [3]. However, to profit from this new genomic information, it will be crucial to place these novel genes into their functional and therapeutic relevant pathways as quickly as possible.

Signal transduction

Many distinct intracellular signal transduction pathways enable a cell to receive, process and respond to information. For decades, scientists have extensively studied biological and biochemical pathways that control the mechanisms of gene expression. At present, we have a general understanding of how transcription (the transfer of sequence information from DNA to RNA) and translation (the synthesis of proteins from messenger RNA at ribosomes) are controlled. We can follow how the activity of certain proteins is regulated by modifications, such as phosphorylation, glycosylation, acetylation, methylation or degradation [4–6]. For some human diseases, such as diabetes or haemophilia, the respective defects have been traced to single genes [7,8]. However, little is known about the majority of diseases in which multiple genes could be affected.

Intracellular pathways – a network of interconnecting signals

Whenever information is transmitted from the plasma membrane to the nucleus, or from one compartment within the cell to another, specific intracellular signal transduction pathways are involved. A conceptual view of signal transduction pathways is depicted in Figure 1. In this simplistic diagram, which we could describe as a three-dimensional pyramid, genes and their products are depicted as 'circuits' or control stations, which make decisions about the route of a signal generated. The stimulation (input signal) of, for example, a specific receptor (top of the pyramid), will lead to the induction of a signalling cascade with signal transmission being controlled at each circuit. This stimulation results in a specific output of signals. We assume that each circuit can accept at least three different functional states: (1) Stop: prevents the signal from being further transmitted; (2) Allow: allows the signal to go to the next circuit; and

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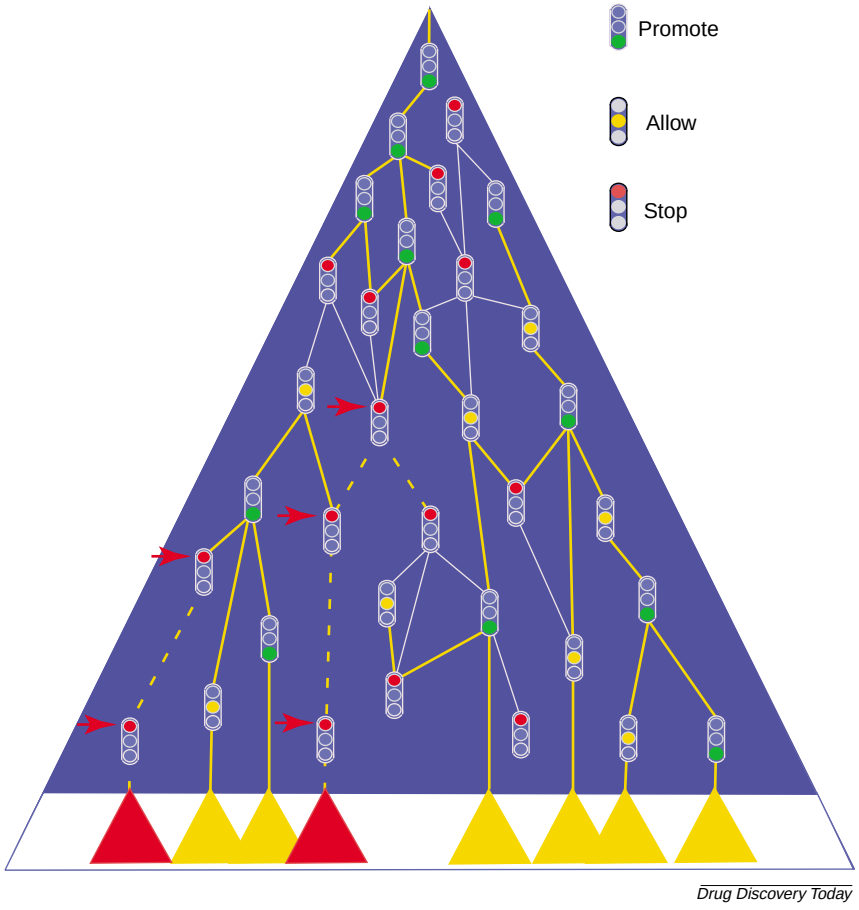

Figure 1. Schematic representation of a signal transduction cascade. Members of the signal transduction cascade are shown as circuits or control stations in a hierarchical network. Each circuit represents a protein (e.g. kinase) receiving an input signal and transmitting an output signal, and thereby influencing the signal flow (Promote, Allow or Stop). Solid lines indicate the signal transduction in a normal cell, whereas broken lines indicate a pathological signal flow. Arrows indicate putative points of therapeutic intervention to prevent the pathological signal flow. Triangles indicate normal and pathological gene expression.

(3) Promote: enhances or amplifies signal transduction. The loss of a specific control station (e.g. an inactivating mutation in a tumour-suppressor gene), will abolish the negative control signal (Stop) and eventually lead to an increased signal flow with possible adverse effects, such as tumour growth (broken lines).

In the examples discussed here, the respective points of control represent signals that regulate cell growth and proliferation. Normally, a finely tuned balance between regulatory signals, activators and inhibitors ensures that proliferation is a tightly controlled process. Deregulation of controlling circuits within the pathway will interfere with the proper homeostasis of positive and negative signals. During tumour development or metastasis the balance is shifted towards activation of proliferation, which can be accomplished either by inhibition of negative regulatory signals or by hyperactivation of positive regulatory signals. For example, mutations that inactivate the tumour suppressor PTEN (phosphatase and tensin homologue deleted on chromosome 10), a phospholipid phosphatase that downregulates proliferative signals induced by phosphatidylinositol 3-kinase (PI 3-kinase) (Box 1), have a high incidence in glioblastomas, endometrial cancer and prostate cancer [9–11]. Similarly, amplifications of the gene for the catalytic subunit of PI 3-kinase, p110, or its downstream effector, Akt, have been identified in a subset of human tumours [12,13]. This shows that this circuit can turn awry, either through inhibition of the negative regulator PTEN or through activation of the positive regulators PI 3-kinase or Akt. In each case, the result is the chronic activation of the pathway that promotes proliferation and inhibits apoptosis. Human cancer cells that have contracted either loss of PTEN function or p110 amplification are characterized by their increased invasive growth potential [10,12]. The identification of the molecular changes that accompany the development of this phenotype is likely to provide novel targets for therapeutic intervention.

Although this view of signal conversion (as shown in Fig. 1) looks relatively simplistic, reality has taught us that mediators of signal transduction do not interact in a linear fashion but, rather, within complex biochemical networks. For example, as illustrated in Figure 2, depending on the cellular context, PI 3-kinase controls intracellular pathways that regulate diverse cellular responses, such as proliferation, survival, migration, vesicle transport, differentiation or glucose uptake in response to treatment with growth factors [14,15]. This complex crosstalk between signalling networks imposes challenges for the discovery of optimal therapeutic intervention points. By inhibiting the PI 3-kinase pathway to interfere with cancer-cell growth one could, for example, also affect glucose transport and cause a secondary diabetic response. In one cell type, growth factor receptors, as well as insulin receptors, have been shown to

recruit PI 3-kinase either for proliferation or glucose transport [16–18]. Similarly, MAP kinase, a signalling molecule that is activated by the same subset of stimuli and acts in a parallel pathway, appears to regulate proliferation and survival, but also differentiation [19]. Therefore, it could be important to identify more specific effectors that act further downstream in this signal transduction network. Ideally, these downstream effectors would specifically regulate cell growth or survival, but not glucose metabolism or differentiation, respectively.

The lack of more detailed knowledge about the interplay between signalling networks makes it difficult to develop drugs that interfere with disease-causing gene functions and do not cause side effects. As shown schematically in Figure 1, complex signalling pathways contribute to both normal, as well as pathological, conditions. Therefore, the ultimate challenge will be to unravel the signalling cascades that contribute to the onset of disease and identify and analyze individual key regulatory genes within these pathways. Although a disease might be initiated by the malfunction or defect of a single gene, the actual onset of the disease could be caused by a series of molecular and genetic changes [20]. The completion of the draft sequence of the human genome, in combination with new technologies in the fields of genomics, proteomics, bioinformatics and pharmacogenomics, will facilitate the analysis of gene activities that can contribute to disease.

Box 1. The PI 3-kinase/PTEN signalling pathway

Phosphatidylinositide 3-kinase (PI 3-kinase) is a heterodimeric signalling molecule, mediating signal transduction that is induced by activated growth-factor receptors and tumour antigens. PI 3-kinase regulates intracellular processes, such as proliferation, survival, differentiation, migration, vesicle transport and insulin signalling [a,b]. PI 3-kinase phosphorylates phosphoinositide type phospholipids at the 3′ position of the inositol ring, to generate the phospholipid second messengers PI 3,4-P_2 and PI 3,4,5-P_3. PTEN, also called MMAC or TEP-1, is a tumour suppressor that is frequently inactivated in late-stage aggressive human tumours [c]. PTEN antagonizes PI 3-kinase signalling by reversing the PI 3-kinase reaction. PTEN is essential for early development; mouse embryos lacking both copies of the PTEN gene die *in utero*. Mice lacking one PTEN allele develop metastatic tumours in different organs [d,e].

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Genomic and genetic approaches

Following completion of the Human Genome Project, the next challenge will be to develop approaches for the systematic determination of gene function. In the past, studies in model organisms, such as *Drosophila melanogaster* and *Caenorhabditis elegans*, have made important contributions to the basic understanding of gene function. In these experiments, so-called orthologues (homologous sequences between species) or paralogues (homologous sequences within one organism, for example, gene families that have originated from gene duplication) have been tested for their function. Forced expression of the catalytic subunit of the fly PI 3-kinase, Dp110, in *D. melanogaster*, for example, resulted in the appearance of enlarged wings and eyes, associating this gene with the regulation of cell growth not only in the mammalian but also in the fly system [21]. Genes involved in apoptosis, or programmed cell death, have been identified using similar studies in *C. elegans* [22,23]. Although these genetic model systems serve as valuable tools, many pathways or genes are too distinct from their human counterparts to draw conclusions about the function of the respective human gene. In addition, because of the higher complexity of the human genome, gene families typically contain more members, making direct extrapolation from lower organisms difficult. Because of a high degree of similarity in gene function, and even in chromosomal organization, between the human and the mouse genome, it is probable that mouse models for human diseases will aid in further identifying novel gene functions and their roles in disease. One of the most challenging approaches in this context is the genome-wide mutagenesis project, in which mutant mice are randomly generated using alkylating agents to induce germ-line mutations [24–26]. The offspring of these mice are subjected to multi-parameter testing for paradigms of morphology, development and clinical or behavioural abnormalities.

Although a phenotype-driven mutagenesis approach has produced, and further will produce, thousands of interesting phenotypes, the screen will, in most cases, cause mutations in only one of the two alleles and, thus, will probably miss those genes for which a more substantial inhibition of gene function is required for causing phenotypical changes. Therefore, to complete the genome analysis, conventional transgenic and knock-out approaches will still be necessary. In various instances, these classical approaches

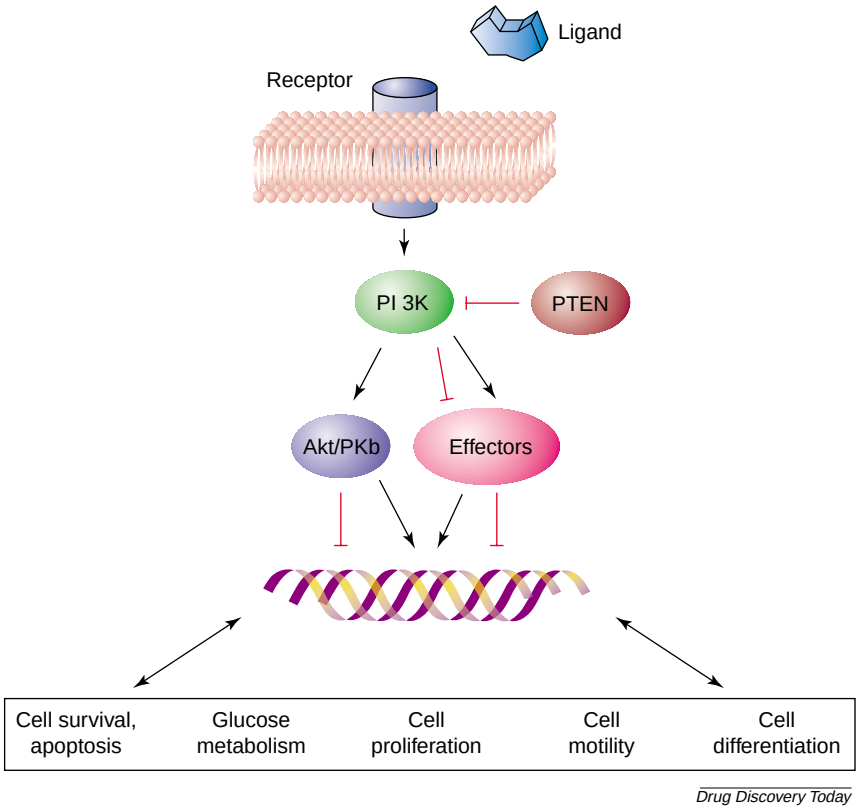

Figure 2. Simplified pictorial representation of the phosphatidylinositol (PI) 3-kinase signalling pathway. Ligand (e.g. growth hormone) binding to a cell-surface receptor induces PI 3-kinase activation. Some of the molecules that are known to regulate or mediate signalling responses induced by PI 3-kinase are shown. Arrows indicate activating interactions, whereas bars indicate inhibiting interactions.

have already proven to be able to establish gene function relationships [27,28]. However, a sizeable number of all knock-out mouse experiments show embryonic lethality or no obvious phenotype because of redundancy (where related genes can compensate for each other in function), limiting the possibility of studying gene function in living mammals [29,30]. Recent developments, using regulatable gene-expression systems or conditional gene knock-outs that change gene expression at various timepoints or in a tissue-specific manner, will improve success rates [31–33].

Another important outcome of the Human Genome Project is the identification of millions of nucleotide sequence variants between different human genomes. The majority of these variants are so-called single nucleotide polymorphisms (SNPs), which represent natural genetic variations in a defined genetic location in a given population [34,35]. SNPs are considered to be the major cause of phenotypic variability that distinguishes individuals within a given species. SNPs can occur in non-coding regions, as well as in coding regions (cSNPs). Variations in the coding region could affect protein expression or function directly, as shown for cystic fibrosis or Huntington's disease [36,37]. Other SNPs might constitute individual susceptibility loci that have already been identified to predispose individuals to, for example, Alzheimer's disease [38]. SNPs will probably be used as diagnostic tools in the future to determine an individuals' susceptibility to a disease or even sensitivity to a therapeutic drug regimen.

Array technologies

A recent popular approach for associating genes with specific signalling pathways is the use of nucleic acid microarrays, which contain immobilized cDNA fragments or short oligonucleotide probes. Microarrays can comprise the entire human genome and novel techniques enable the detection of a specific target within several thousand transcripts [39]. Using these tools, scientists are now in a position to identify genes that are specifically up- or downregulated in response to a defined stimulus [40,41]. Microarrays have also successfully been used to find differentially expressed genes by analyzing large sets of patient samples, or by comparing normal with diseased tissues [42,43]. Recently, protein arrays have also been developed that have the potential to detect posttranslational changes in proteins, such as phosphorylation, acetylation, glycosylation, ubiquitination or other covalent protein modifications [44–46]. These modifications, which do not require *de novo* transcription, would be missed by analyzing changes exclusively at the mRNA level.

Other types of arrays, such as sensor arrays, directly translate molecular interactions into optical or electrical signals [47]. Aptamers, which are small nucleic acid molecules, and antibodies can function as biosensors for numerous diagnostic and screening applications [48]. Both capture tools can recognize targets ranging from small molecules to complex multimeric structures with high specificity *in vitro* and *in vivo* [49]. A systematic screen for potent aptamer-DNA sequences yielded, for example, high affinity biosensors for anthrax spores [50]. Although all of the tools described earlier are powerful tools to associate genes and their products with certain pathways or processes, in most cases this association remains correlative. Further

studies will be required to determine whether the role of the gene of interest is also causal in, for instance, the progression of a disease.

Cell-based assays to unravel gene function

Although the approaches described previously have provided valuable information in deciphering gene function and the role of specific genes in pathological processes, the enormous complexity of the human genome requires additional strategies to elucidate the contribution of individual genes to the function of interconnecting signalling networks. Cell-based assays provide a possible solution to resolve this issue. A step-wise approach is likely to facilitate the identification of the respective biological context, such as a specific signalling cascade, to study a previously uncharacterized candidate gene. This type of approach allows the study of a candidate gene and its role under normal versus pathological conditions.

Two-hybrid screen and antibody libraries

A cell-based assay to map protein–protein interactions is provided by the so-called two-hybrid screen or interaction trap cloning [51]. This approach represents a relatively simple but sensitive assay to detect an interaction between two proteins in living cells. Although this technology has resulted in the identification of protein–protein interactions that were proven to be relevant for therapeutic intervention [52], additional work is required to verify that the interactions are specific and that they actually do occur in biological systems. The reasons are intrinsic to the screen, which is in most cases performed in yeast and based on the overexpression of two sets of proteins. The artificial host environment, temperature and assay conditions might not mimic the situation in, for example, a human cell. Moreover, the screen does not allow for ternary interactions of other factors that might only be present in the relevant human cell-type.

An alternative protein-based approach to unravel gene function is the use of antibody libraries. Scientists have generated large libraries representing millions of different humanized antibodies [53,54]. In fact, functional antibodies can be used directly as therapeutics; this was demonstrated with the development of antibodies against a specific receptor involved in breast cancer [55].

High content screening approaches using cell-based labelling techniques

Many cellular responses regulate the activation status of a protein not only, for example, by phosphorylation, but also by relocalization to different intracellular compartments (e.g. cytoplasm to nucleus). To follow the translocation of proteins in response to a stimulus, tags based on fluorescent proteins, such as green fluorescent protein (GFP) or red fluorescent protein (RFP), can be employed [56]. Because of its high resolution, the fluorescent resonance energy transfer technology (FRET) is a preferred method for this type of analysis [57,58]. This technique detects the proximity of fluorescently labelled molecules over a distance of 10–100 Å [59]. Because of its non-invasive mode of detection, this imaging technology enables a rapid screen to identify inhibitors or activators of specific protein–protein interactions or even entire pathways in cell-based assays. For example, the translocation of the GLUT-4 glucose transporter, or the signalling kinase Akt, from the cytoplasm to the membrane after stimulation with insulin or growth factors, can be followed [60,61]. Recently, fluorescent labelling techniques have even been applied in a cell-based screen to identify tumour-specific small-molecule inhibitors [62]. This study compared a pair of isogenic colon-cancer cell lines, which differ in the type of K-Ras protein expressed. One of these colon cancer cell

Box 2. Gene expression knockdown or silencing technologies

- Ribozymes: ribozymes are auto-catalytic RNA molecules, so-called molecular scissors, which cut RNA at specific sites [f].
- Antisense molecules: the inhibition of gene expression by antisense molecules is based on the ability of an oligonucleotide to bind to a complementary RNA sequence. Upon binding of the antisense molecule, processing or translation of the RNA is inhibited [g].
- RNAi: RNA interference (RNAi), or posttranscriptional gene silencing, is mediated by a double-stranded RNA molecule. A sequence-specific multi-component nuclease destroys RNAs that are homologous to the RNAi molecule [h].

The described nucleic-acid-based gene-expression inhibition approaches provide a useful means of studying gene function. By inhibiting RNA translation, genes and their products can be effectively turned off, thus facilitating gene function studies.

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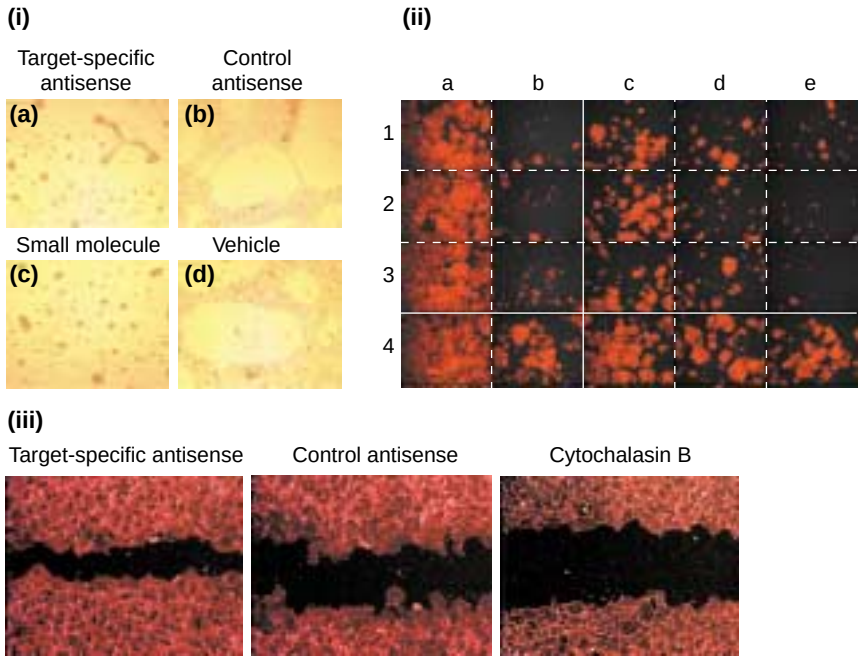

Figure 3. Cell-based assays to unravel gene function. **(i)** Cell motility. Human cells were seeded on an extracellular matrix after either transfection with an antisense molecule (a and b) or treatment with a small-molecule compound (c and d) and the phenotypic changes were analyzed. The picture was taken 24 hours post-incubation. **(ii)** Cell proliferation. Cells were seeded in a 96-well format and transfected with different antisense molecules directed against specific target mRNAs. Cell viability and proliferation rate were analyzed five days after treatment by detecting metabolic activity and by nuclear staining, respectively. Rows a1 to a4, untreated cells. Rows b1 to b3, positive control antisense molecule targeting a known gene involved in cell proliferation. Rows c, d and e, specific antisense molecule directed against a candidate gene (15nM, 30nM and 60nM, respectively). Lane 4 indicates control antisense molecules having four mismatches of antisense molecule used in b and c, and e, respectively. **(iii)** Cell migration. Cells were seeded and treated with antisense molecules or controls. 48 hours later the cell monolayer was wounded with a 0.2 mm wounding tool. Control-treated cells (middle panel) were allowed to migrate for 8 hrs. Cells treated with a small molecule (Cytochalasin B) (right panel) do not migrate because of disruption of the actin cytoskeleton. Cells treated before wounding with an antisense molecule directed against a specific candidate gene show a significant increase in migration. Figures 3(i) and (ii) are 4 × magnification; Figure 3(iii) is 20 × magnification.

lines, which expressed an oncogenic form of K-Ras, was labelled by introducing a vector directing expression of yellow fluorescent protein. The second cell line, in which the K-Ras allele had been deleted, was transfected with a vector expressing blue fluorescent protein. The co-culture of both cell lines enabled the screening of compounds with selective toxicity towards the oncogenic Ras genotype.

Overexpression approaches

Another way to unravel the function of key regulatory components of signal transduction pathways is to overexpress a gene of interest in cells and to follow the molecular and phenotypic changes of these cells over time. Scientists have used constitutively active and dominant-negative mutants to elucidate gene function in specific biochemical pathways [63,64]. However, for this approach, substantial previous knowledge regarding the function of the genes and/or proteins and their biological context is required. Such detailed information will not be available for the majority of newly identified genes. Although computer-aided comparisons might identify specific motifs, which could aid in their classification as, for instance, kinases or phosphatases, there are still questions remaining. For example, it is often not obvious which amino acid residues need to be changed to produce either a dys-functional and interfering protein mutant or to induce a hyperactivated molecule. Without the knowledge of additional functional domains or modules within a molecule, the overexpressed protein can easily perturb the overall cellular machinery by titrating out ('squelching') other cellular components, which might be present in limiting concentrations [65]. In some cases, it has been shown that the stringent selection process of cells expressing a transgene might already have changed the cellular physiology, and thus yields non-conclusive results.

Antisense and RNA interference

A more direct approach to discover genes that can serve as targets for therapeutic intervention is blocking or knocking-down their function by inhibiting gene expression. In many 'knockdown' approaches, the mRNA level of a specific gene product is reduced by molecules such as ribozymes, antisense molecules or RNA interference (RNAi) molecules (Box 2). In these cases, the inhibition of mRNA expression will result in a significantly reduced protein level. The knockdown approach of gene function is transient and, therefore, different from the chronic and complete gene knockout technology discussed previously. Unlike the knockout technology, the knockdown approach is acute and does not require any selection process, which avoids problems such as the induction of compensatory effects during the selection procedure. Furthermore,

it mimics more closely the action of small-molecule drugs on target inhibition because it induces a state of diminished, but not complete loss, of gene function. Figure 3(i) shows an experiment in which cells have been treated with an antisense molecule (a and b), and the phenotypic effect was compared to the action of a small-molecule compound (c and d). Both the antisense and the small-molecule compound successfully blocked the function of a specific candidate gene, which was suspected to be involved in cell migration. The small-molecule compound inhibits gene function at the protein level, whereas the antisense molecule targets the mRNA level (translation). Based on the mode of action, antisense molecules can be used to perform dose-response studies, which facilitates the decision of whether the respective gene should be developed further or be terminated as a target. For instance, if a nearly 100% knockdown of the protein is required to induce a phenotypic change, such as inhibition of tumour cell growth, it will be extremely difficult to develop a small-molecule compound with the appropriate potency. Figures 3(ii) and (iii) show cell-based assays in which certain candidate genes are analyzed for their involvement in cell proliferation or cell migration. Targeting the mRNA, rather than the protein itself, offers another unique feature of antisense molecules for target validation studies in cell-based assays, namely the dissection of gene families. Many protein kinases, which are attractive targets for pharmaceutical and biotechnology companies, display a high degree of similarity at the amino acid level in their catalytic domain. Therefore, small molecules that were selected for inhibiting the function of a specific kinase molecule could cause unwanted side-effects by blocking more than one family member. By contrast, antisense molecules act in a sequence-specific manner and, therefore, can be designed to specifically target only one kinase molecule by selecting nucleotide sequences that are unique to the kinase of interest. After a successful antisense-based identification of a target molecule that is crucial for the maintenance of a phenotype, HTS of small-molecule compound libraries can be run with better success for specificity. In addition, the antisense technology can be used to confirm the target specificity of small-molecule leads. In the course of such a 'lead validation' program, changes in gene expression patterns obtained after antisense treatment are compared with those obtained after small-molecule lead treatment. Similarities and differences in the resulting gene expression patterns are then carefully analyzed. It has to be stressed at this point that knockdown experiments require careful analysis and the use of at least, for example, two antisense molecules, including proper controls, to rule out non-specific oligonucleotide-mediated side-effects.

Concluding remarks

Cell-based assays provide a means of studying gene function in a biological context, and enable verification of candidate targets for therapeutic intervention. We believe that the use of cell-based assays will support the paradigm shift initiated by the completion of the draft sequence of the human genome. Inhibition of candidate gene function might not, in all instances, result in an obvious phenotypic change and, therefore, it might be relevant to first identify the proper biological context (i.e. in which specific signalling pathway the particular gene of interest functions). Recent developments have substantially improved the level of assay sensitivity and assay throughput, for studying gene function in cell-based assays. Cell-based assays will probably be the method of choice to elucidate the molecular changes that occur over time and that lead to the manifestation of a pathological phenotype. Step-wise analysis of biological processes could be required to dissect signalling pathways and find novel and better points of therapeutic interventions.

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