General Discussion
Genetic screens in mammalian cells

A central paradigm in biomedical research is that understanding of the (mal-) functioning of biological systems will enable the design of rational intervention strategies. Intensive research over the past decades into the molecular basis of life, in particular the complete DNA sequence of the human genome, has yielded detailed mechanistic insight into health and disease[1,2]. Despite the generation of these enormous amounts of data, most of the therapies applied in the medical clinic today cannot be classified as rational. This certainly holds true for the treatment of cancer, for which most of the protocols are based on therapies invented long before our detailed knowledge on cell cycle regulation, DNA-repair and apoptosis. Paradoxically, our increased understanding of cancer biology has made us realize that we are only just starting to uncover the complexities of this disease.

In recent years, a few modest successes have been accomplished with new treatments based on detailed knowledge of the underlying defects. A translocation product, known as the Philadelphia chromosome, results in the oncogenic fusion product BCR-ABL, a tyrosine kinase that causes Chronic Myeloid Leukemia (CML). A drug, called imatinib (Gleevec), inhibits the ABL kinase to physiological levels inducing apoptosis of leukemic but not normal cells[3]. Other rationally designed drugs are likely to be clinically tested within the next few years. For instance, small compounds inhibiting HDM2 binding to p53 appear to induce apoptosis of cancerous but not healthy cells[4-6].

Clearly, an important success factor for the design of rational therapies includes knowledge on "druggable targets" involved in the relevant signaling network(s). Preferably, these targets are proteins with enzymatic activity (like the ubiquitin E3 HDM2, or ABL) that can be inhibited by systemic delivery of small chemical compounds and act specifically on cancer cells, thereby minimalizing side effects. Although reasonably accurate sequence information about all proteins coded in the human genome is now available, the function of most of these proteins is unknown. Furthermore, the fact that many proteins are involved in more than one defined cell-biological process makes their functional annotation an even more daunting task. Indeed, it is very likely that many proteins involved in signaling networks deregulated in cancer, including potential drug targets, remain to be identified.

Genetic screens in model organisms, such as yeast, Drosophila and C. elegans, have yielded many new players and invaluable insights into signaling networks and will likely continue to do so[7-9]. In addition, bioinformatics approaches based on transcriptional co-regulation or co-evolution are starting to make valuable contributions[10,11]. However, since some important signaling pathways and processes in humans are not well conserved and others simply do not exist in lower organisms, model organisms have limitations for studying human diseases. Furthermore, most bioinformatics approaches are based on transcriptional regulation, limiting insight into proteins that are primarily post-transcriptionally regulated. Genetic screens in human cells can partially compensate for these shortcomings. To study their function, proteins can either be over-expressed or endogenous protein can be inhibited. Over-expression screens are generally referred to as gain-of-function screens. Only recently, tools have been developed that allow efficient loss-of-function screens (i.e. inhibition of endogenous proteins)[12]. Indeed, the recent discovery that short interfering RNAs (siRNAs) can suppress the expression of proteins in mammalian cells, a process known as RNA interference, has received special attention. In the next few paragraphs, some of the general principles and examples of both types of screens will be given, placing the genetic screens described in the previous chapters into context.

Gain-of-function screens

Due to the polyclonal format of most cDNA over-expression libraries, gain-of-function screens generally rely on positive selection for a rare event from a large population of cells. Consequently, screening systems usually select for genes that confer resistance to a strong growth-inhibitory signal. In addition, due to the high complexity of cDNA libraries, the growth inhibition needs to be stringent, meaning that only very few cells spontaneously acquire the phenotype that is selected for. For instance, screening systems to identify genes that confer resistance to apoptosis or bypass senescence adhere to these conditions and have been used successfully[13-17]. Other screens

have relied on complementation of a genetic defect resulting in a cellular phenotype. For example, screening for genes that give resistance to mitomycin C in Fanconi anemia cells led to the identification of the FANCC gene[18]. Genetic screens to identify critical target genes by complementing the growth defect of c-MYC-deficient cells, initially suggested that only MYC itself could restore normal proliferation[19,20]. However, by creating cDNA libraries lacking c-MYC and N-MYC, mitochondrial serine hydroxy-methyltransferase (SHMT) was identified as a direct MYC-target, suggesting that MYC has a direct function in regulating central metabolic processes[21].

Yet other screening systems have relied on activity of a reporter construct driving expression of a selectable marker. An elegant example involves the identification of the transcription factor TFE3 as a SMAD co-regulator[22]. Cells deficient of HPRT are sensitive to medium containing hypoxanthine but resistant to 6-thioguanine (6-TG), a highly toxic purine analogue. By coupling a TGF-β responsive promoter fragment to the expression of a bacterial HPRT homologue, a conditional HPRT positive cell line was created that in response to TGF-β became hypoxanthine resistant but 6-TG sensitive. By screening a cDNA library in the absence of TGF-β and presence of hypoxanthine, TFE3 was found to activate the artificial promoter.

In Chapter 5 of this thesis, we describe the development of a cell system that allows screening for genes that confer resistance to a retinoblastoma protein (pRB) dependent growth arrest. In contrast to the previously described cell systems, this screen relies on the ectopic over-expression of a potent growth inhibitory protein (i.e. pRB-NPC). Interestingly, by screening a high-complexity cDNA library in these cells we identify TFE3 as a gene regulating the pathway. Furthermore, we provide evidence that at least part of the mechanism whereby TFE3 renders cells insensitive for pRB-dependent growth inhibition involves activation of *CYCLIN E* transcription.

Loss-of-function screens

During the last decades, yeast has been the geneticists preferred tool for loss-of-function screens [9]. Mutant yeast strains can be produced in various ways including chemical and insertional mutagenesis. Importantly, since yeast can proliferate with a haploid genome, recessive mutations are easily recovered. Furthermore, diploid mating cycles allow complementation and epistatic analysis. Obviously these approaches cannot be used to perform genetic screens in higher eukaryotes. Until recently, efficient and effective approaches to perform loss-of-function genetics in mammalian cells were virtually non-existent. Although genetic-suppressor-element-(GSE), ribozyme-, and anti-sense libraries, have been used successfully, their wide-scale use has been restricted due to various practical limitations [12]. The recent discovery of RNA interference in mammalian cells, a gene suppression phenomenon pioneered in C. elegans, promises large-scale loss-of-function screens in mammalian cells.

RNAi is an evolutionary conserved gene silencing response, triggered by the introduction of double stranded RNA (dsRNA) into a cell[23,24]. The silencing occurs at the level of the target mRNA and is highly specific, resulting only in degradation of transcripts with high homology to the double stranded siRNA. The components and mechanisms involved in RNAi is the topic of intensive research and are currently unfolding. A critical step in RNAi involves the incorporation of one of the siRNA strands into a multi-protein complex named RISC (RNA-induced silencing complex). This complex somehow finds the complementary cellular mRNA, binds and subsequently cleaves it, resulting in mRNA degradation.

The siRNAs used to trigger RNAi, can be delivered into mammalian cells in various ways[12]. Initially, in vitro synthesized dsRNA was transiently transfected resulting in a temporary “knockdown” of the target gene. In addition, more usable and cost-effective methods have been developed that also allow stable knockdown of target genes[25,26]. For example, short-hairpin RNAs with siRNA properties have been expressed from various retroviral vectors[27,28]. Indeed, the high degree of usability and specificity has made RNAi a standard technique in virtually all molecular biology laboratories in record time.

RNAi-based screens are now emerging as a powerful tool to analyze loss-of-function phenotypes in mammalian cells. The first RNAi screens were based on gene families, groups of proteins with shared functional domains (e.g. kinases or deubiquitinating enzymes), resulting in the identification of, for example, the CYLD gene in NF-κB signaling reported in this thesis[29,30]. Typically, these studies have analyzed gene-function in a “single-well single-gene” format. Also, the identification of USP1 as a regulator of the DNA-repair protein FANCD2 is an example of this type of loss-of-function screen (Chapter 4). Single-well approaches allow the fast identification of knockdown phenotypes with limited penetrance and are therefore very powerful. However, the number of genes that can be analyzed by hand is limited by the number of available PhD students,

and automated systems are generally expensive. An alternative to single well assays are experimental set-ups analogous to the polyclonal cDNA screens. For instance, a senescence by-pass screen using a large RNAi library targeting approximately 8000 genes led to the identification of five new players in the p53 tumor suppressor pathway[31].

Inspired by screens pioneered in yeast, a technique referred to as siRNA barcode screens has been developed[12,31-34] (Brummelkamp et al., submitted). In short, bar-coding allows the identification of positively or negatively selected hairpin vectors in a complex mixture by microarray analysis. The integrated pro-viruses containing the library-derived unique shRNA sequences, function as molecular identifiers (i.e. barcodes). Alternatively, additional (external) barcode sequences can be inserted into the knockdown vectors[34]. Importantly, the relative abundance of these barcodes in a population of cells is directly linked to the phenotype induced by the hairpin vector. For example, the number of cells in a population that contain a knockdown vector that inhibits proliferation will decrease over time. The barcodes can be amplified by PCR and hybridized to a customized microarray containing the complementary hairpin or external barcode sequences[31]. Thus, by comparing the amount of specific barcodes in a population of interest to a control population, a fast and quantitative analysis can be made of the effects of individual knockdown vectors. siRNA barcode screens will probably significantly contribute to the functional annotation of the human genome.

A particularly appealing prospect of loss-of-function screens in mammalian cells is the ability to identify synthetic lethal genetic interactions. Not only do we need to identify the functions of individual proteins, we must also understand the complex genetic interactions between the various components. For instance, protein redundancy cannot be revealed by classical genetics since mutation of one of the redundant genes by definition, will not result in a phenotype. Synthetic lethality screens can also reveal crosstalk between signaling networks. In conclusion, the hallmark of synthetic lethality is that only double mutants display a (lethal) phenotype, whereas either single mutant is functional. In yeast, synthetic lethal screens are now being performed in a bar-coded format, allowing fast identification of drug-gene interactions[35]. Similar screens, based on RNAi bar-coding, will possibly become available for mammalian cells in the near future. Since cancer cells display many genetic alterations that distinguish them from their normal counterparts, they likely display (synthetic) vulnerabilities not observed in healthy cells. Therefore, synthetic lethality screens may prove to be an invaluable addition to the geneticists' toolbox for the identification of new anti-cancer drug targets.

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