Large scale Loss-of-Function Genetic Screens in Mammalian Cells using RNA Interference.

René Bernards. Division of Molecular Carcinogenesis, The Netherlands Cancer Institute, Plesmanlaan 121 1066 CX Amsterdam, The Netherlands.

One of the major remaining deficits in our understanding of the human genome is that information regarding gene function is available for only one quarter of the approximately 30,000 genes. Many of these hitherto anonymous genes are potential targets for the development of new anti-cancer drugs. It is therefore important to functionally annotate the tens of thousands of genes for which this information is currently lacking. My laboratory has developed functional genetic approaches to obtain information regarding gene function using high-throughput screens in mammalian cells. In general, genetic screens represent an unbiased approach to identify genes that act in specific cellular pathways. We have developed both gain-of-function genetic screens (using retroviral cDNA expression libraries) and loss-of-function genetic screens (using vector-based RNA interference libraries) to carry out large-scale genetic screens in mammalian cells. We focus on the central growth-regulatory pathways that are most frequently deregulated in cancer, such as the $p16^{INK4A}$-cyclin D-RB pathway, the $p19^{ARF}$-MDM2-p53 pathway and the NF-κB pathway.

Gain-of-function genetic screens.

We use retroviral cDNA expression libraries to identify genes that act in pathways, which are frequently deregulated in human cancer. In short, these genetic screens involve the infection of a cell population with a high-complexity retroviral cDNA expression library, selection of cells with altered phenotype, followed by identification of the cDNA responsible for the phenotype. In the past years, we have used gain-of-function genetic screens to identify genes that that confer resistance the anti-proliferative effects of the p53-dependent senescence response in primary murine cells. Using this approach, we identified more than ten genes that allow escape from senescence, including *BCL6*. This gene was originally identified as the target of an oncogenic translocation in non-Hodgkin's lymphoma. Importantly, *BCL6* also extends the life span of primary human B cells in culture, indicating that *BCL6* has a similar mode of action in rodent fibroblasts and in human B cells (Shvarts et al., 2002).

Using a similar approach, we have identified *DRIL1*, the human orthologue of the *Drosophila Dead Ringer* transcriptional regulator, as a gene that allows escape from oncogenic *RAS*-induced premature senescence (Peeper et al., 2002).

Loss-of-function genetic screens in mammalian cells using RNA interference.

RNA interference (RNAi) is an ancient defense mechanism to protect cells from invasion by viruses and transposable elements. This response is triggered by double stranded RNAs generated by these parasitic nucleic acids, which are processed into short interfering RNAs (siRNAs) by specialized nucleases, such as dicer. These siRNAs target homologous RNAs

for destruction through recruitment of a RNA-Induced Silencing Complex (Hannon, 2002). In *C. elegans* and *Drosophila*, the use of large-scale RNAi screens has proven to be powerful for the identification of loss-of-function phenotypes in several developmental pathways. In most mammalian cells the use of long double-stranded RNA provokes an interferon response, leading to a general shut off of protein synthesis. This response can be bypassed by using *in vitro* synthesized 21 base pair double-stranded siRNAs, which can cause strong, but transient, inhibition of gene expression in nearly all mammalian cells (Elbashir et al., 2001). Such RNA molecules are short enough to stay below the radar screen of the interferon response system, yet are powerful inducers of RNA interference. However, the use of these siRNAs is limited by the transient inhibition of gene expression, the difficulty to use these siRNAs in a polyclonal screening format and their high cost. To circumvent these limitations of siRNAs, we have developed a mammalian expression vector (named pSUPER) that directs the synthesis of short hairpin RNAs (shRNAs), which are processed *in vivo* into siRNA-like molecules that can suppress gene expression over prolonged periods of time and are highly target-specific (Brummelkamp et al., 2002). We have recently shown the feasibility of using shRNA vectors to identify loss-of-function phenotypes in mammalian cells by creating a set of vectors to suppress most members of the de-ubiquitinating enzyme gene family. This allowed us to identify the cylindromatosis tumour suppressor gene as a regulator of the transcription factor NF-κB (Brummelkamp et al., 2003).

A collection of siRNA vectors for large-scale loss of function genetic screens.

We used a retroviral derivative of the pSUPER siRNA vector to generate a large collection of siRNA vectors that each target a single gene for suppression. Three knockdown vectors were designed for each transcript to obtain more efficient suppression of gene expression (Fig. 1).

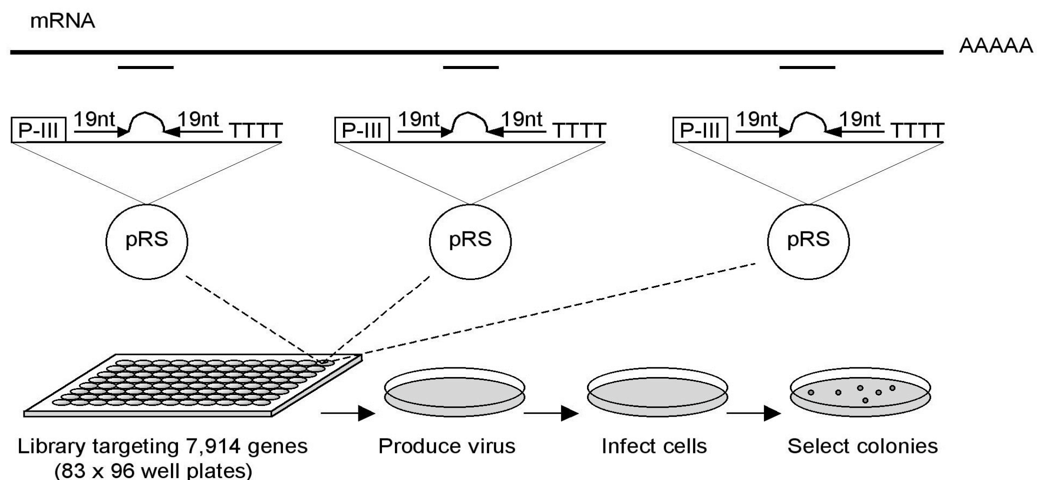

Figure 1 The NKi RNAi library.

For each transcript three19 nucleotide (nt) sequences were selected. These were converted into pairs of complementary 59-mer oligonucleotides designed to direct the synthesis a hairpin transcript and cloned into pRETRO-SUPER. Three vectors targeting one gene were pooled in a single well of a 96 well plate. From each 96 well plate high titer polyclonal retroviral supernatant can be produced, which can subsequently be used to infect cells. Colonies of phenotypically distinct cells can then be selected.

In total, we constructed a set of 23,742 siRNA vectors that together target 7,914 human genes for suppression by RNA interference. In first instance, we have used this collection of siRNA vectors to perform large-scale screens for loss-of-function phenotypes in the p53 tumor suppressor pathway. We selected this pathway because its integrity is crucial to maintain genome stability due to the fact that it transmits both anti-proliferative and pro-apoptotic signals in response to a variety of stress signals (Sherr, 2001). Consequently, the *p53* gene is mutated in over half of all human cancers. In addition, the other major components of the p53 pathway such as $p19^{ARF}$ in mouse ($p14^{ARF}$ in man) and *MDM2* (*HDM2* in man) are mutated in many forms of cancer. This raises the possibility that additional, yet to be discovered, components of this pathway may also be major players in oncogenesis. The results of these large-scale loss-of-function genetic screens in mammalian cells will be presented.

References.

Brummelkamp, T. R., Bernards, R., and Agami, R. (2002). A System for Stable Expression of Short Interfering RNAs in Mammalian Cells. Science *296*, 550-553.

Brummelkamp, T. R., Nijman, S. M., Dirac, A. M., and Bernards, R. (2003). Loss of the cylindromatosis tumour suppressor inhibits apoptosis by activating NF-kB. Nature *424*, 797-801.

Elbashir, S. M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., and Tuschl, T. (2001). Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature *411*, 494-498.

Hannon, G. J. (2002). RNA interference. Nature *418*, 244-251.

Peeper, D. S., Shvarts, A., Brummelkamp, T., Douma, S., Koh, E. Y., Daley, G. Q., and Bernards, R. (2002). A functional screen identifies hDRIL1 as an oncogene that rescues RAS-induced senescence. Nat Cell Biol *4*, 148-153.

Sherr, C. J. (2001). The ink4a/arf network in tumour suppression. Nat Rev Mol Cell Biol *2*, 731-737.

Shvarts, A., Brummelkamp, T. R., Scheeren, F., Koh, E., Daley, G. Q., Spits, H., and Bernards, R. (2002). A senescence rescue screen identifies BCL6 as an inhibitor of anti-proliferative p19(ARF)-p53 signaling. Genes Dev *16*, 681-686.