Genetic cell culture screens reveal mitochondrial apoptosis control

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1. ABSTRACT

Recent years have witnessed an exponential increase in our knowledge about the cellular suicide programme of apoptosis. Historically, genetic screens in model organisms such as *C.elegans* and *D. melanogaster* were among the experiments that initiated this field. While mammalian cell culture systems did not seem to be amenable for screening, recent developments in high-throughput assays such as robotic instrumentation, the annotation of complete mammalian genomes and novel genetic tools such as RNA interference have led to a number of genetic screens in mammalian culture cells. Some of these screens were focussed on cell death and resulted in a considerable extension of our knowledge on apoptosis. Here we summarize the underlying concepts and the data that these genetic screens generated so far. The results indicate a complex range of signalling pathways in mammals. In particular, numerous signalling components in mitochondria have been discovered in this way in accordance with the prominent role of this organelle for cell death regulation.

2. INTRODUCTION

Pivotal for discoveries in genetics is the mutagenesis of the genome for each biological entity, be it an organism such as *D. melanogaster*, *C.elegans* or mammalian cells in culture. Subsequently, the consequences on the phenotype are detected. Two fundamentally different ways can be discerned that allow find a relevant phenotype change. In a genetic selection the targeted biological effect is enriched from a background of "noise", i.e. irrelevant phenotype changes. In contrast, in a screen the consequences of the genetic manipulation are monitored on an individual basis. This makes screens more laborious but also, importantly, renders them more sensitive (Figure 1). Conceptually, two principal versions of genetic screens can be distinguished (Figure 2). "Reverse" genetic screens start with a known gene that is altered and re-introduced into the organism to test its effect. This approach is, for example, a popular experiment to analyse deletion mutants of a known gene in order to map functional domains in its protein. The second version,

Table 1. Overview of screening programmes for cell death regulators

Technology	Features	Reference
cDNA screen	Test of large cDNA pools	(22)
RISCI	Robotic test of individual cDNAs	(12)
RISCI	Robotic test of individual cDNAs in a commercial screen	(44)
Reverse transfection	Plasmid arrays overlaid with cells	(45)
Reverse transfection	Plasmid arrays overlaid with cells	(46)
cDNA screen	Transfections in combination with imaging technology	(47)
RISCI	Individual robotic cDNA tests for cell death inhibitors by company	(48)

See text for a more detailed description of the features and technologies used.
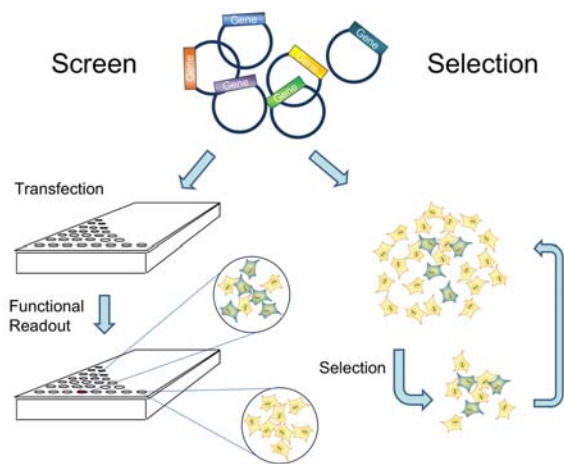

Figure 1. Comparison between selection and screening strategies. In genetics terminology, a selection (right) involves the use of techniques that specifically enrich for those cells that display a phenotype of interest. Multiple cycles of mutagenesis and selection results in a cell pool consisting predominantly of the desired cells. In contrast, in a screen (left), cells are not subjected to selective pressure, but individual experiments are used to monitor the phenotype of interest using various types of assays.

genetic "forward" screens, start with a comprehensive mutagenesis step such as transfection of a complex gene library followed by the detection of a particular phenotype. The genetic alteration(s) that are causing this phenotype are then detected and correlated with a molecular function. Such screens have the advantage that they focus on a predefined function and are unbiased since no prior assumption is made about the genes to be detected.

So, why are genetic screens so suitable for apoptosis research? Apoptosis is a degenerative process at the end of which the dying cells disappear and as such it is necessarily transient. Moreover, apoptosis generates a very pronounced phenotype in cells that was the basis of its first description and allows easy detection in screens (1). Hence, a screen, in contrast to a selection, is the best way to detect genes that impact on this cellular response. In a previous

review (2) we presented screens in mammalian cells in general. Here we will focus on screens tailored to detect genes involved in apoptosis. While we will see that a wide array of genes have been isolated in this way, a recurrent theme is the determination of mitochondrial factors that impact on apoptosis. Research over the last years has proven that mitochondria are not only the "powerhouse of the cells" but also the organelle that is chiefly involved in dismantling the cell. Hence, the isolating of numerous mitochondrial factors in genetic screens is proof that a plethora of factors and signalling pathways exist in mitochondria to regulate apoptosis.

3. GENETIC SCREENS IN MAMMALIAN CELLS

The massive amount of data generated by the various sequencing project (3, 4) and the urgent need to assign functions to those genes is one of the drivers underlying the recent activity in genetic screens in mammalian cells in general and for apoptosis screens in particular. This data avalanche was accompanied by the development of novel tools such as the annotation of complete genomes (5) and comprehensive gene libraries such as the cDNA assembly from the Mammalian Genome Collection (6) or RNAi expression compendiums (7, 8). In the wake of further developments in sequencing tools (9), such as pyrosequencing or nanopore sequencing, other high-throughput approaches for functional read-outs such as automatic image detection for the analysis of cellular phenotypes have made great progress (10). This has also led to a reduction of the costs for such screens, which makes it affordable for academic laboratories to adopt assays that were formerly confined to industrial laboratories. Moreover, the novel method of RNA interference has for the first time allowed a targeted disruption of genes in culture cells without the tedious deletion of the endogenous alleles (which is difficult in most cell lines as they are polyploid). This genetic technique notably contributed to the recent surge in screening activities in mammalian culture cells. Importantly, a paradigm shift has taken place over the last couple of years as systems biology gained widespread recognition: scientists realised that a complex range of cellular parameters have to be monitored in order to explain how a signal (a perturbation in systems biology jargon) leads to a given cellular response (11). This has resulted in an increase in efforts to screen the genome for predefined functions and the induction and inhibition of the apoptosis signal is one of them (see table 1 for an overview).

4. SCREENS FOR DOMINANT GENE ACTIVITIES

"Dominant" in genetics terminology refers to an allele of a gene whose activity that can be detected in a heterozygous state, while "recessive" gene functions can only be assessed in a homozygous state. Overexpression of a gene reveals its dominant activity as its protein is expressed at a higher level than the endogenous protein. Hence, transfecting and overexpressing a gene is one of the easiest ways to reveal the dominant trait of a gene. Moreover, overexpression is an immensely useful tool to
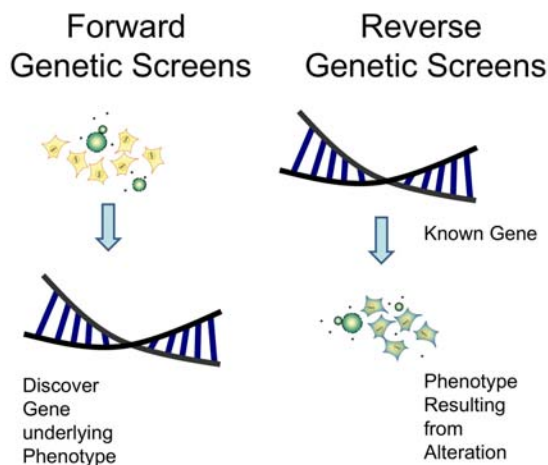
Figure 2. Comparison between "forward" and "reverse" genetic screens. A reverse genetic screen studies the phenotype effect of a know (and sometimes altered) gene sequence. In contrast, a forward screen is the classical screen in genetics that seeks to determinate the gene (s) that cause a specific phenotype (here indicated by apoptotic cells).

circumvent the functional redundancy of mammalian genomes.

So, what is the rational for using gene overexpression in apoptosis screens? An overview of mediators of apoptosis shows that most of them, with very few exceptions, can also induce apoptosis upon ectopic overexpression (12). This holds true for adaptor proteins such as FADD (13) and TRADD (14) but also for membrane receptors such as dependence receptors (15). This might be due to the fact that in most cases the signalling for apoptosis is mediated by protein complexes and that they are assembled through protein-protein interactions once one of the components is overexpressed. The apoptosome, for example, is a multi-protein that forms upon apoptosis induction (16), the DISC complex is initiated by the ligand of the Fas receptor with adaptor proteins such as FADD mediating the recruitment of other pro-apoptotic proteins (17). Moreover, caspases, the executioners of apoptosis, are activated by close proximity, which is achieved by a host of different adaptor protein domains (18). While this correlation between apoptosis induction upon overexpression and the ability to relay an endogenous signal for apoptosis might constitute a justifiable underpinning for dominant screens in apoptosis, concerns have been raised. Could we, for example, be misled to pursue artefacts, as overexpression of genes might lead to damage of the cells and to unspecific effects? Are the genes from such screens only inducing apoptosis when they are overexpressed or do their endogenous counterparts also mediate a signal for apoptosis induction? These are legitimate questions that arise when designing a screen for dominant apoptosis genes. We have addressed this in a couple of experiments (12). First, we transfected several oncogenes and dominant-negative mutants of known genes into cells to generate

unphysiological signals. However, we have not detected any signs of apoptosis (12). In addition, compounds were titrated into HEK293 cells and apoptosis was monitored. We found that only a minority of those signals that induce apoptosis in other cellular contexts could also generate this response in HEK293 cells (12). Importantly, we have detected that the mitochondrial ANT1 transporter is a very potent apoptosis inducer (see below), whereas its 90% homologous gene ANT2 is not causing apoptosis (19). Hence, we concluded that at least in our cell system one needs a defined signal for apoptosis induction and that unspecific damage of the cells does not suffice to cause apoptosis.

Nevertheless, one has to acknowledge that a gene that causes apoptosis upon overexpression is not necessarily a mediator of apoptosis at its endogenous expression level. Our results so far, however, indicate that genes from the screen also relay a signal for apoptosis. This is true for KAI1 (20), cybL (12), and Spike (21). Moreover, results from other groups led to similar conclusions (see below). Furthermore, one should keep in mind that even in screens for recessive gene functions if the inactivation of a gene leads to a reduced apoptosis, this does not necessarily indicate that this gene is mediating a signal for apoptosis. Other, indirect effects could also be responsible such as a cellular compensation to replace the missing gene product. Hence, overexpresssion screens can, if properly interpreted and controlled, lead to equally valid genetic information.

Alcala *et al.* performed one of the most recent screens for apoptosis inducing genes. They used large pools comprising 96 cDNA clones each from one library of activated T cells and co-transfected them together with GFP to assess the morphological changes during apoptosis as a read-out (22). A total of 135,000 cDNAs were screened in this way leading to 90 genes that could induce apoptosis. Interestingly, a number of these genes encode mitochondrial proteins. The group investigated one gene in more detail, the mitochondrial phosphate carrier. They found that it can interact with ANT-1, a component of the PT pore (see below) and that this interaction was enhanced upon apoptosis induction by staurosporine. Importantly, downregulation of the mitochondrial phosphate carrier by RNAi reduced apoptosis as measured by cytochrome c release, caspase activation and quantification of apoptotic nuclei. Hence, the mitochondrial phosphate carrier gene is another example for a gene that induces apoptosis upon overexpression but also mediates a pro-apoptotic signal at its endogenous expression level.

Our laboratory has used a different approach. While we started this project some years ago with a manual screen that was using small plasmid pools (23), in order to increase the sensitivity we further developed this into a robotic high-throughput assay in a 96 well plate format that tests individual plasmids for apoptosis induction (12). This assay relies on the co-transfection of a reporter plasmid and the detection of its enzymatic activity (24, 25). The high-throughput DNA isolation in our screen is accomplished by a special protocol that does not use expensive columns but centrifugation steps to yield high purity plasmid DNA (26).

Only this enables an academic laboratory to perform a sufficiently high number of plasmid DNA isolations. A number of positive controls were isolated from the screen. These are known genes, also known as inducers of apoptosis such as FADD (13), an adaptor protein in the TNF receptor complex; calcineurin, a phosphatase that dephosphorylates and thereby activates BAD (27); NIP3, one of the proapoptotic Bcl-2 family members (28), CIDE-A and CIDE-B (29); ZIP kinase (30); and the p53 target PERP (31). Interestingly and surprisingly we have detected hemoglobin alpha as an apoptosis inducer in this screen (12). While at the time this was regarded as an overexpression artefact, later it was found that this gene is upregulated in many culture cells upon treatment with pro-apoptotic signals and its inhibition reduced apoptosis (32). Ectopic expression of hemoglobin alpha accelerates Bax recruitment to mitochondria and cytochrome c release from this organelle suggesting that a mitochondrial signalling pathway is engaged (32). We have observed that genes whose proteins localise to mitochondria are especially efficient in this screen and produce a particular phenotype of apoptosis (12, 33). Two mitochondrial protein complexes were investigated in more detail as their components were isolated in our screen: the PT pore and complex II of the respiratory chain. The SDHC and SDHD subunits of complex II are both inner mitochondrial membrane proteins that anchor the remaining subunits of complex II. Interestingly, both genes not only cause cell death in our screen but have been found to function as tumour suppressor genes in paragangliomas, which are benign head and neck tumours (34, 35). Our studies indicated that complex II mediates a number of diverse pro-apoptotic signals such as cisplatin, etoposide, and doxorubicin. Of note, during apoptosis complex II is specifically inhibited, while complex I remains unaffected (12). An inhibition of respiratory chain complexes has been shown to generate ROIs and both compounds and enzymes that scavenge ROIs were shown to reduce apoptosis caused by complex II inhibition. Hence, we believe that complex II of the respiratory chain is an important sensor for apoptosis induction and is shut down to generate ROIs for apoptosis induction.

The second mitochondrial protein aggregate defined by isolates from the screen and further investigated in our laboratory is the permeability transition (PT) pore. This protein complex interconnects the inner with the outer mitochondrial membrane and is converted into an unspecific pore upon apoptosis induction. This leads to a breakdown of the respiratory chain, to the formation of reactive oxygen intermediates (ROIs), and to apoptosis induction. While the exact composition of the PT-pore is still subject to discussions (36-39), the PT pore has been known for some time to be causative for cell death induction (40). Many signals both in physiological and pathological circumstances such as heart disease and tumourigenesis cause the activation of the PT pore (41, 42). Our own results show that the PT pore component ANT1 (adenosine-nucleotide-translocase-1), which was detected in our screen, can potently induce apoptosis (19). Additional data suggest that this is not mediated by its transport activity but rather through protein-protein interactions, possibly by titrating out inhibitors of the PT pore (19, 43).

A similar screen for apoptosis inducers in a commercial organization also isolated the mitochondrial ANT1 as well as a number of other genes that the authors present as tumour suppressor candidates (44).

Other innovative approaches have been employed for screening of apoptosis mediators. Mannherz *et al.* used reverse transfection cell array technology (45) where the transfection mix, each containing a gene-of-interest, was arrayed and spotted onto glass slides, after which the cells were overlaid. This approach produced a good distribution of the readout signal within each spot, allowing for scalability of the screen to automated high-throughput level. These workers screened a collection of 382 human sequence-verified, full-length open reading frames for proapoptotic genes and identified seven genes, one of which is again the mitochondrial ANT1, five genes have yet to be annotated with function (FLJ20551, CXorf12, FAM105A, TMEM66, C19orf4) while one, ST6 beta-galactosamide alpha-2,6-sialyltransferase 2 (ST6GAL2) had no known apoptotic function at the time. A similar screen was performed using cell arrays fabricated with 1959 mammalian open reading frames from the Mammalian Genome Collection. 79 genes (4%) were initially considered positive and a re-screen verified a total of 10 proapoptotic genes but one of them had a mitochondrial localisation or an obvious link to this organelle (46).

In comparison, Park *et al.* screened a collection of 938 hypothetical genes using standard transfections with a combination of technology such as high-throughput subcellular imaging with traditional essays like western blotting and DNA fragmentation ELISA. Out of these 938 genes three were identified as apoptosis inducing (C10orf61, MGC 26717 and FLJ13855) (47). Bioinformatics analysis associated C10orf61 with involvement in cell cycle regulation mediated by hedgehog signalling, while FLJ13855 contains an ubiquitin-conjugating enzyme E2 domain predicted to mediate apoptosis through the degradation of other factors. MGC 26717 represents another novel apoptosis mediator due to the lack of homologous domains.

Few screens for dominant genes that inhibit apoptosis have been conducted as protection against cell death can be easier addressed by selection schemes. However, Zitzler *et al.* took advantage of the superior sensitivity of screens and searched for inhibitors of oxidative stress-induced apoptosis in neurons (48). Starting with 5,000 cDNAs five know inhibitors were determined (catalase, glutathione peroxidase-1, peroxiredoxin-1, peroxiredoxin-5, and nuclear factor erythroid-derived 2-like 2) and also the novel inhibitor glutamine-fructose-6-phosphate transaminase (GFPT) 2, a rate-limiting enzyme in hexosamine biosynthesis.

5. SCREENS FOR RECESSIVE GENE ACTIVITIES

RNAi screens have recently become the method of choice for inactivating a gene. Using this genetic technique the Blenis group screened for kinases and
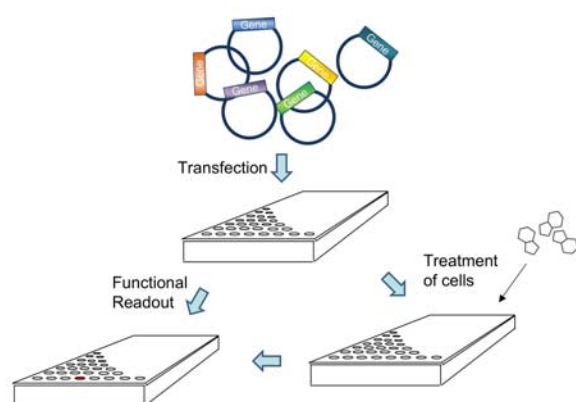
Figure 3. Screens targeting genes that exert a specific effect or genes that modify an exogenous signal for apoptosis. Upon transfecting a cDNA or RNAi library a genetic screen for apoptosis regulators can be performed in two principal ways, either by screening directly for genes that exert an effect on the survival of the cells, or by first exposing the cells to compounds or conditions to simulate cell death prior to the functional readout. The screen is then usually continued with further experimental studies to verify the hits or bioinformatics analysis to elucidate the signalling pathways of the genes.

phosphatases that mediate cell survival (49). Hela cells were transfected in 96 well plates with siRNAs that cover a substantial part of the kinome and phosphatome and those constructs that induced apoptosis were determined. These authors also screened for genes that sensitize cells for apoptosis by downregulating them and screening for those constructs that led to resistance to apoptosis induced by etoposide, taxol, and cisplatin. MacKeigan *et al.* generated a siRNA library targeting a mix of 510 human genes with both known and unknown functions and used phenotype screening for mediators of TRAIL-induced apoptosis (50). While many mediators of apoptosis were identified by the authors, FLJ32312, named DOBI (DOwnstream of BId), was of particular interest. The inhibition of DOBI produced a similar effect as BID inhibition by preventing caspase-9 processing while retaining caspase-3 or caspase-8 cleavage. After TRAIL induction, BID is processed such that the activated BID results in the release of cytochrome c from mitochondria and subsequent formation of the apoptosome. RNAi experiments targeting DOBI prevented TRAIL-induced release of cytochrome c, thus suggest that DOBI is responsible for progression of a signal from the TRAIL receptor through BID to the mitochondrial route of apoptosis. While most of the screens presented here focussed on genes that exert an effect on their own, the two screens from this chapter are examples of an experiment that targets genes that modify an exogenous apoptosis signal (Figure 3). Another example for this approach is a screen that looked at genes that sensitize cells to sub-lethal concentrations of paclitaxel. 87 genes were determined in this way (51). Since taxol has its target in the cytoskeleton mostly cytosolic proteins were determined in this screen.

6. PROSPECTS

Screens for apoptosis genes in mammalian culture cells are increasingly used to reveal the signalling for cell death. The fact that we are still isolating so far unknown factors indicates that the signalling pathways are much more complicated than anticipated. We believe that most, if not all of the components of signalling pathways are meanwhile discovered in more primitive organisms such as C.elegans and that the additional complexity of mammalian apoptosis signalling (52) is best addressed by screens in cell culture. Importantly, many mitochondrial proteins were detected, at least in those screens that covered enough candidate genes, and this despite the fact that genetic screens are unbiased and focus solely on functions. This underscores the importance of this organelle for apoptosis induction. Hence, no coherent picture of apoptosis signalling has emerged from these screening efforts so far but the screens provide a framework to completely elucidate the apoptosis signalling pathways. They could lead to the identification of all genes involved in apoptosis. Consequently, we envision great contributions coming from genetic screens in the very near future.

7. ACKNOWLEDGEMENT

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