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Identification of Candidate Genes with Pro-apoptotic Properties by Functional Screening of Randomly Fragmented cDNA Libraries

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Abstract
The sequences of many genomes are available; therefore, relevant methods are needed for rapid and efficient identification of functional genes. The ability of tumour cells to resist apoptosis induced by anticancer agents may decide the success of failure of tumour elimination. Although the CD95-signalling pathway is functional in tumour cells, the increased resistance of tumour cells to CD95-mediated apoptosis has been widely reported. In order to identify genes that might determine the response of tumour cells to CD95-mediated apoptosis, we modified the conventional technical knock out (TKO) strategy for isolation of genes that function in CD95-mediated apoptosis. Due to the fact that multiple different plasmids are usually introduced into the same cells, the effectiveness of the conventional TKO strategies is low. To overcome this obstacle, we replaced the conventional TKO strategy (based on stably expressed randomly fragmented cDNA libraries) with a multi-cycle selection procedure (based on transiently expressed randomly fragmented cDNA libraries with multi-cycle selection). Using this approach we could rapidly and significantly identify small numbers of antisense mRNA molecules, whose re-introduction into different tumour types confirmed their ability to block the pro-apoptotic function of their cognate genes. Thus, our modified TKO strategy provides a generally applicable procedure for the identification of functional genes with pro-apoptotic properties that may be clinically relevant to tumor therapy.

Introduction

Alteration in the control of apoptosis mediated through the CD95 system contributes to the pathogenesis of a number of disorders, such as cancer. Decreased sensitivity to CD95-mediated apoptosis is a common trait shared by many cancer cells, which provides them with critical survival advantages ultimately promoting malignancy [1]. Apoptosis can be triggered by a vast variety of signals, including the activation of different death receptors of the tumour necrosis receptor (TNF-R) family, γ-irradiation and various chemical agents. The signalling pathways activated by various apoptotic stimuli converge into a common death pathway either at the mitochondrial level or at the level of effector caspase activation [2, 3]. The sensitivity to apoptosis induction in any cell, however, critically depends on the expression and activity of multiple apoptosis-regulatory proteins [4, 5].

In recent investigations we have demonstrated that the CD95 (APO-1/Fas) -mediated signalling pathways are functional in many tumour cell lines [6, 7, 8].

Although the components of the CD95 signalling pathway have extensively been characterized, there might still be additional, yet to be identified functionally relevant gene products that decisively determine the response of human cancer cells to CD95-mediated apoptosis.

The results of the human genome project (HGP) now provide complete sequence information of the human genome, allowing the identification of functional human genes and to elucidate their functions that are involved in particular signal transduction pathways such as a cell cycle, growth factor-mediated pathways or apoptosis. Herein, the efficacy of the screening procedure applied has a major impact on the anticipated results. Both yeast two-hybrid system and DNA microarrays have been widely used for the identification of functional genes. The yeast two-hybrid system is a powerful method for the detection of direct interactions between proteins in vivo and for identification of genes whose products interacts with protein derived from a cDNA library. The method is limited, by the fact that the interaction does not always reflect a role in phenomenon of interest and false-positive results are frequent. The DNA microarray technique is based on observation of changes in gene expression during various intracellular processes [9, 10]. However, using this method is difficult to identify functional genes associated with qualitative alterations, such as phosphorylation, that are responsible for alterations in a phenotype without any change in the expression of specific genes.

In this report, we describe the modification of a gene discovery system based on the conventional technical knock out (TKO) strategy [11, 12] and our published functional screening [8] for rapid and efficient identification of functional genes in CD95-mediated apoptosis pathways, using randomly fragmented cDNA libraries. However, as the conventional TKO strategy requires weeks for each selection cycle, we here used an approach that capitalizes on the rapidity of the appearance of specific phenotypes, that tran-

siently express randomly fragmented cDNA libraries. We here demonstrate that this modified TKO strategy is in fact useful to identify rapid and functional genes in the post- genome era. In summary, our modified TKO strategy provides a generally applicable procedure for the identification of functional genes with pro-apoptotic properties that may be clinically relevant for tumor therapy.

Experimental Protocol

Construction of randomly fragmented cDNA libraries: The cDNA libraries were generated from a mixture of RNAs harvested from cell lines derived from melanoma (A375), keratocanthoma (HS 892-T), squamous cell carcinoma (CLS-354), renal cell carcinoma (ACHN), and human breast adenocarcinoma (MCF-7) before and after treatment with the agonistic anti-CD95 antibody CH11 (500 ng/ml) for 1, 2, 3, 6, 12 hours, so that both constitutive and CH11-induced mRNA transcripts were included. Double stranded cDNA was generated using the cDNA Synthesis Kit according to the manufacturer's protocol (Clontech, USA). The provided primers had been modified by integration of the restriction sites Hind III and Bgl II so that the entire cDNA population could be inserted into the pTKO2 vector [12] between the Hind III-Bgl II restriction sites in the antisense orientation.

Amplification and representative control of the library: The anti-sense cDNA library consisting of approximately $1x10^6$ independent clones was washed off from agar by thoroughly scraping the surface using 1 ml LB-medium containing 100 μg/ml ampicilline. The resulting bacterial suspension was allowed to grow in LB-medium containing 100μg/ml ampicilline for 3- 4 h. The isolation and purification of the DNA plasmids were performed by using the plasmid purification kit according to the manufacturer's protocol (Qiagen, Germany). The representative control of cDNA library was based on the analysis of a PCR- generated population of cDNA inserts using pairs of primers corresponding to the flanking regions of the vector. In addition, we used several pairs of primers, which correspond to internal sequences of certain cDNA sequences that are expected to be present in our cDNA library.

Cell culture and transfection procedures: The different tumour cell lines A375, HS892-T, CLS-354, HepG2, ACHN and MCF-7 expressing the human gene for CD95 were grown in DMEM medium supplemented with 10 % FCS (Sigma, Germany) 2 mM L-glutamine and 100 units/ml penicillin/streptomycin (GIBCO, Germany). Transfection was performed using the nucleofector™ method according to the established protocol modified with the nucleofector optimization kit (Amaxa, Germany) as described previously [13], [8]. Briefly, A375, HS892-T, CLS-354, HepG2, ACHN and MCF-7 cells were plated a day before transfection so that the confluence of the cell culture was under 80% at the day of transfection. Following the washing with cold PBS, cells were detached from dishes and washed twice in PBS. A $1x10^6$ cells were used for nucleofector transfection with 2 μg of the plasmid carrying the library DNA. After transfection, the cells were plated in two culture dishes and allowed to grow for 24 hour before starting with the selection.

Identification of pro-apoptotic genes using randomly fragmented cDNA libraries: The randomly fragmented cDNA libraries were introduced into A375, HS892-T, CLS-354, HepG2, ACHN and MCF-7 cells by the nucleofector transfection kit (Amaxa, Germany). After 24h, cells that had been transfected with randomly fragmented cDNA library were treated with the agonistic anti-CD95 antibody CH11 (500 ng/ml) after transient transfection (modified TKO methods with multi-cycle selection) or with CH11 (500 ng/ml) and hygromycin (300 μg/ml) (conventional TKO procedure with one cycle selection). After 5 days, surviving clones were picked manually and plasmids were purified from transfected cells as described [8]. Then, we introduced isolated plasmids into *E. coli* for the amplification. After purification of amplified plasmids we re-introduced these plasmids into the target cells and repeated these cycles five times. Finally, isolated plasmids were introduced into *E. coli* and transformed *E.coli* were plated on a LB agar plate. Each independent plasmid was isolated from clones of *E. coli* cells. The sequences of the antisense cDNAs included in these plasmids were analyzed with an automated DNA sequencer. Finally, we searched for the target genes and identified them as previously described [8].

Assessment of cell survival: A375, HS892-T, CLS-354, HepG2, ACHN and MCF-7 cells in exponential growth phase were transfected with selected antisense cDNAs clones using the nucleofector transfection protocol (Amaxa Biosystem). Transfected cells were then transferred to 96-microwell plates (Gibco, Germany) at $1x10^4$ cells per well in a 0.1 ml standard growth medium at 37°C and 5% (v/v) CO2. After 24 h transfected cells were exposed to the anti-CD95 antibody CH11 (500 μg/ml) in standard growth medium. Subsequently, plates were incubated for 12 hours. The percentage of viable cells was then determined using the colorimetric MTT assay (Roche, Germany) as described previously [8].

Detection of apoptosis using Annexin V/propidium iodide (PI): Cells transfected either with pTKO2 empty vector or with antisense cDNAs directed against newly identified genes were cultured for 24h before exposure to anti-CD95 antibody CH11 (250 ng/ml) for 24 hours. Cells were then washed with PBS twice, stained with Annexin V/PI as described [13], [6] and subjected to flow cytometry analysis using a FACSCalibur (Becton Dickinson Biosciences, Heidelberg, Germany).

Results

Preparation of Antisense cDNA Libraries

We started with the preparation of several libraries of ~$1x10^6$ different randomly fragmented cDNAs from A375 (Melanoma), HS892.T (Keratoacanthoma), CLS-

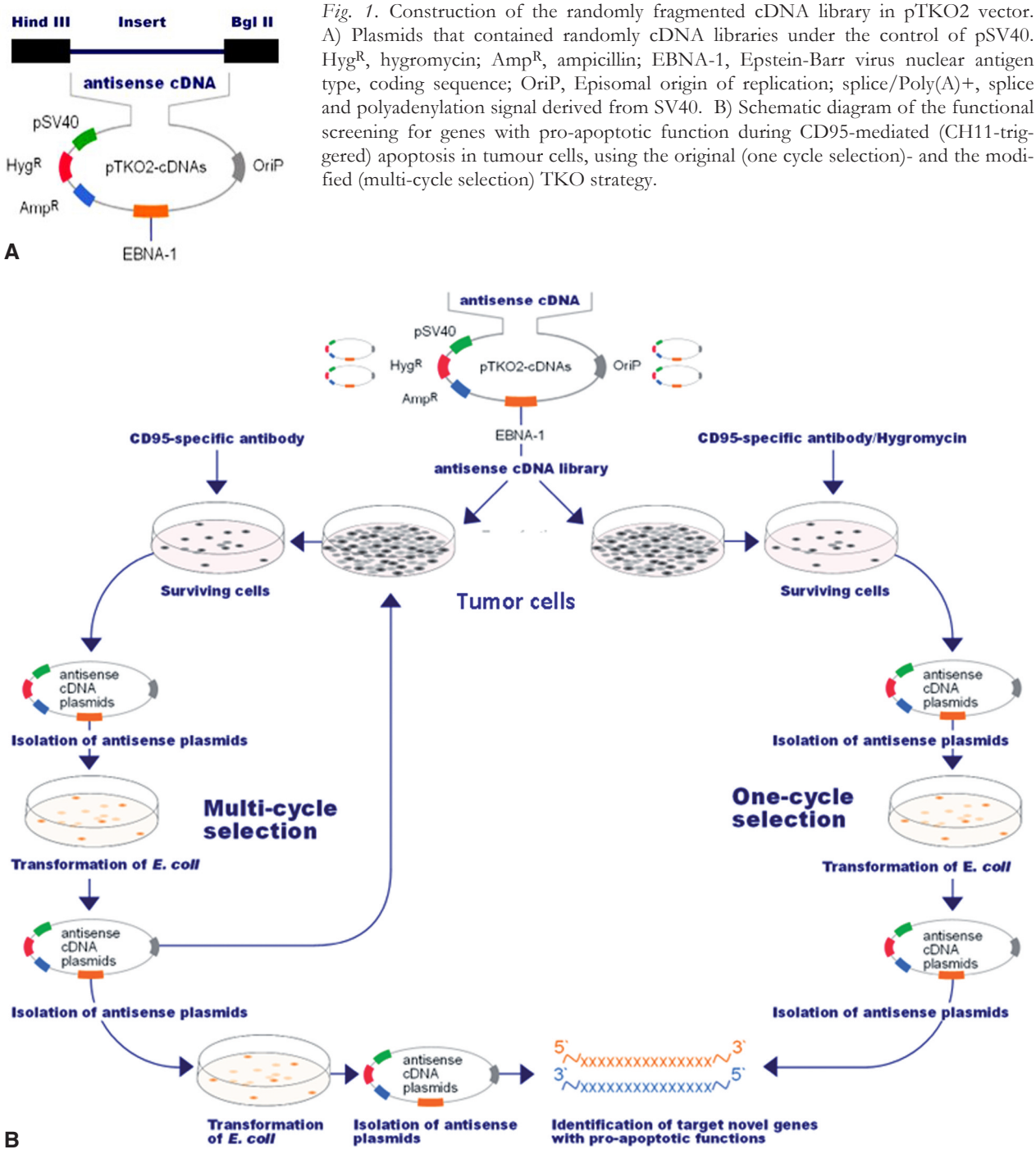

Fig. 1. Construction of the randomly fragmented cDNA library in pTKO2 vector. A) Plasmids that contained randomly cDNA libraries under the control of pSV40. Hyg^R, hygromycin; Amp^R, ampicillin; EBNA-1, Epstein-Barr virus nuclear antigen type, coding sequence; OriP, Episomal origin of replication; splice/Poly(A)+, splice and polyadenylation signal derived from SV40. B) Schematic diagram of the functional screening for genes with pro-apoptotic function during CD95-mediated (CH11-triggered) apoptosis in tumour cells, using the original (one cycle selection)- and the modified (multi-cycle selection) TKO strategy.

354 (head and neck squamous cell carcinoma), ACHN (Renal cell carcinoma), MCF-7 (Breast adenocarcinoma), HepG2 (Hepatocellular carcinoma) cells during CH11-triggered (i.e.CD95-mediated) apoptosis, using the pTKO2 EBV-based vector as detailed (Fig. 1A).

The Original Technical Knock Out (TKO) Protocol and Its Modification

A schematic diagram of functional genetic screening for pro-apoptotic regulation of CD95-mediated apoptosis with the TKO method is presented in Fig. 1B. According to the original TKO protocol, the tumour cell lines were transfected with randomly fragmented cDNA libraries and then treated simultaneously with both the agonistic anti-CD95 antibody CH11 and hygromycin. After four weeks, surviving clones stably expressing antisense cDNAs were picked manually to purify the plasmid cDNA for further amplification in *E. coli*. This TKO procedure functioned optimally with the delivery of a single cDNA-encoding plasmid per cell. However, multiple different plasmids were usually introduced into the same cell, which then may contain the specific antisense cDNA of pro-apoptotic gene together with other unrelated background, randomly fragmented cDNAs (Data not shown). Therefore, we extended the original TKO protocol (based on stably expressed anti-sense cDNAs) by a modifica-

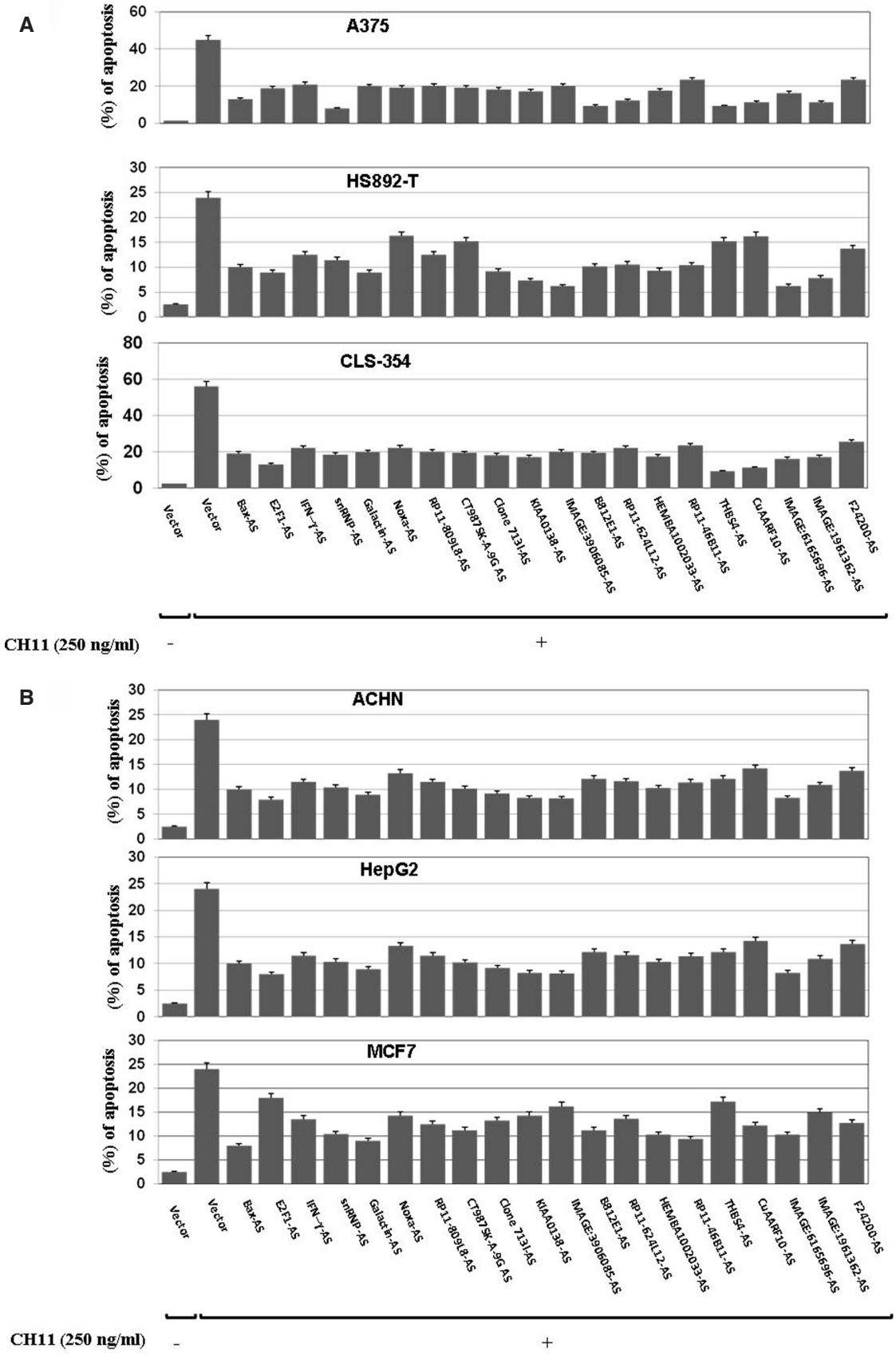

Fig. 2. Apoptotic function of target genes. Extent of apoptosis 96 h after treatment with CH11 in: A) populations of A375 (Melanoma), HS892-T (Kerathokanthoma), CLS-354 (Head and neck squamous cell carcinoma). B) Populations of ACHN (renal cell carcinoma), HepG2 (hepatocellular carcinoma), MCF-7 (breast adenocarcinoma). The cells were transiently transfected with either mock-vector (pTKO2) or with the different isolated antisense cDNA construct (encoding for anti-sense RNA molecules). After 48h, the transected cells were exposed to CH11 for a further 48h, followed by flow cytometric analysis using annexin V/PI. Data are the mean ± SD of three independent experiments performed separately.

Table 1. Functional genes, whose cognate antisense RNA molecules conferring resistance to the agonistic anti-CD95 antibody (CH11)-induced apoptosis in different tumour cell types.

Gene/clone name	GenBankno
Bax	U19599
E2F1	BC050369
Interferon-γ	X62468
snRNP	XM_292062
Galectin-1	BC020675
PMAIP1 (Noxa)	NM_021127
RP11-809L8	AC120051
CIT987SK-A-9G	AC004131
Ets-like protein (clone 713)	Z49980
KlAA0138/689	D50928
IMAGE:3906085	BF47568
B812E1	AP001172
Clone RP11-624L12	AL365364
HEMBA1002033	AU144472
Clone RP11-46B11	AL355796
THBS4	AY566253
Clone CuAARF 1 0	AV710703
IMAGE:6165696	BU161481
IMAGE:1961362	AI354965
F24200	AC004611

tion using a multi-cycle selection procedure of transiently expressed antisense libraries.

In the modified TKO method (Fig. 1B), tumour cell lines (A375, HS892-T, CLS-354, ACHN, HepG2, MCF-7) were transfected with randomly fragmented cDNA libraries first, and then exposed to agonistic anti-CD95 antibody (CH11) for five days. Surviving clones transiently expressing randomly fragmented cDNA libraries were picked manually and the plasmid DNA was purified from these surviving cells. The isolated plasmids were pooled and introduced into *E. coli* for limited amplification. After purification, the amplified plasmids were re-introduced into A375, CLS-354, HS892-T, ACHN, HepG2, and MCF-7 cells. The cycles were repeated five times. Finally, we introduced isolated plasmids into *E. coli* and transformed *E.coli* cells were plated on an LB agar plate. Each independent plasmid was isolated from clones of *E. coli*. The sequences of cDNAs included in these plasmids were sequenced and the target genes were identified as described [8].

Using the modified technical knock out (TKO) strategy (Fig. 1B) several genes (with apoptotic properties) were identified. These include IFN-γ [14], E2F-1 [15]snRNP [16, 17], bax [18], Galectin-1 [19] as well as phorbol-12-myristate-13-acetate-induced protein 1, MAIP1(Noxa) [13], ets-like protein [20], Fer-1-like3, myoferlin [21] and Homo sapiens thrombospondin 4 [22]. However, inhibition of CD95-mediated pathways to apoptosis was noted in A375, HS892-T, CLS-354 (Fig. 2A) as well as in ACHN, HepG2 and MCF-7 cells (Fig. 2B) that expressed antisense RNA molecules targeting 11 unknown and 9 known genes (Table 1), eventually demonstrating the validity of the modified TKO strategy for rapid and efficient identification of apoptotic mediators. Moreover, the inhibition of CD95-mediated apoptosis pathways in tumour cells by the knockout of known and unknown identified genes by their cognate antisense RNA molecules confirmed the importance of the identified genes for the modulation of CD95-mediated apoptosis pathways in tumour cells.

Discussion

In this study, we demonstrated the validity of the modified TKO procedure for the rapid and efficient identification of genes that function in CD95-mediated apoptosis pathways. We examined two procedures for the expression of antisense cDNA libraries in our tumour model: the original TKO procedure (based on stably expressed antisense cDNAs) and the modified TKO procedure (based on transiently expressed cDNA libraries with multi-cycle selection). Using the modified TKO procedure we could rapidly and efficiently identify functional genes with very low background. Although previous studies identified several genes by TKO strategies using antisense cDNA libraries [8, 23, 24, 25, 12], the modified TKO procedure is the first transient procedure that is successfully used to identify functional genes involved in the CD95-mediated apoptosis pathway.

Notably, the modified TKO procedure may ideally function with the delivery of single plasmid encoding efficient inhibitory antisense RNA molecule per cell. However, in all transfection systems frequently multiple plasmids are introduced into the same cell. Thus, background clones exhibiting CD95-mediated apoptosis pathways can be observed when a cell contains an inhibitory antisense RNA molecule and other unrelated background antisense RNA molecules. To overcome this problem, we performed multi-cycle selection and determined numbers of surviving clones after each cycle. We also compared the validity of conventional and the modified TKO procedure in order to confirm the efficiency of the modified TKO procedure. Taken together, the modified TKO procedure can be successfully used when a change in phenotype appears within a short period of time (up to one week). Since plasmids are gradually lost from transfected cells one week after transfection, it is not advisable to use the modified TKO procedure when a change in phenotype appears in this time period. In the case of CD95-mediated apoptosis pathways the change in phenotype appears 96 h after the start of treatment with anti CD95 antibody (CH11).

Besides the identification of functional genes based on the downregulation of gene expression and loss of gene function using a gene-silencing tool (accumulation of antisense RNA molecules) as it was performed in this study, there are also alternative strategies such as differential display and microarray technologies that can be used to identify genes that are turned on or turned off in association with a particular phenotype. Yet, genes identified using these strategies might prove difficult to identify genes, which are directly related to a specific change in phenotype. Notably, this was pos-

sible using the modified TKO procedure. More importantly, we successfully identified candidate genes with pro-apoptotic function during CD95-mediated apoptosis in tumour cells.

It should be noted that the inhibition of apoptosis by antisense RNA molecules is not necessarily complete, as due to the presence of multiple apoptotic pathways, the disruption of one factor (pathway) usually causes only a reduction in apoptosis rather than complete inhibition.

Our results furthermore illustrate that the phenotypes of cells harbouring the antisense RNA molecules of Bax, IFN-γ, E2F-1, snRNP, or Galectin-1 are correlated with the phenotype of positive clones obtained by the modified TKO procedure. Moreover, harbouring the antisense RNA molecules of the corresponding cognate unknown genes showed a significant reduction of apoptosis after the treatment with CH11 (Fig. 2), confirming the usefulness and validity of the modified TKO procedure. Although the pro-apoptotic functions of the identified genes have been confirmed in the CD95-mediated apoptosis, the mechanisms of their active role in apoptosis have not yet been assessed in detail.

Taken together, the successful isolation and identification of known and unknown pro-apoptotic genes convincingly confirms that the modified TKO strategy provides a powerful tool for rapid and efficient identification of functional genes in post-translational events in the cell suffering apoptosis under certain circumstances. Further studies, are needed to elucidate the exact role of the newly identified pro-apoptotic genes in the apoptotic signalling cascade.

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