Network: **Combating Cancer through Integrated Functional Genomic Research**

Project: Characterization of Survival Genes Recovered from a Functional Gene Trap Screen in Human Breast Cancer Cells

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Introduction

Breast cancer is a common neoplasm with a high mortality rate among woman with advanced stage disease. As is currently believed for most cancers, one of the major factors contributing to cellular transformation and, ultimately, to invasive cancer is the cancer cell's ability to evade apoptosis. Although much has been learned about the molecules involved in apoptotic signal transduction and its regulation, the identification and validation of molecular targets for apoptosis-based cancer therapy still poses a major challenge. Strategies addressing this challenge in the postgenomic era are genomewide screens for transformation related survival genes. The validity of such screens has been recently underscored by a SAGE analysis of primary breast cancer material that led to the identification of survival factors with oncogenic potential (1).

In line with this, we have developed a genomewide screen for survival genes based on the cellular response to tumor necrosis factor alpha (TNF-alpha). As has been previously shown, TNF-alpha induces two intracellular protein complexes with opposite effects on cell survival (2). While one complex engages NF-κB and upregulates survival genes, the other initiates apoptosis by recruiting FADD and caspase-8. By specifically blocking the assembly of the apoptotic complex, we have identified several putative survival genes of which some might be useful as diagnostic markers and/or molecular targets for anti-breast cancer drug development.

Project Status

Experimental strategy

By combining gene trap mutagenesis with site specific recombination (3), we have developed a strategy that enriches for survival genes induced by TNF-alpha in the human breast cancer cell line MCF-7. The strategy relies on a one way gene expression switch, which is activated by gene trap insertions into TNF-alpha inducible genes. As activation entails the expression of dominant negative FADD (dnFADD), a protein that blocks the assembly of apoptotic complexes (4), the strategy enables the recovery of genes induced by the TNF-alpha dependent survival (NF-κB) complex (Figure 1).

Identification of TNF-alpha regulated genes

We have established a MCF-7 reporter cell line expressing the CMVlx*tkneo*lx*dnFADD* switch cassette and used this cell line to construct an integration library consisting of $2x10^6$ insertions of the retroviral gene trap vector U3Cre (5). From this library, we have isolated 66 cell lines with U3Cre insertions in TNF-alpha inducible genes. PCR amplification and sequencing of the genomic regions adjacent to the gene trap insertion sites (gene trap sequence tags; GTSTs) revealed that 28 GTSTs belonged to known genes, 12 corresponded to full length cDNAs with unknown function and 12 were part of expressed sequence tags (ESTs). Five GTSTs matched GeneScan predicted, transcribed regions and 9 corresponded to not yet annotated genomic regions. Among the known genes, some have been shown previously to be upregulated in various cancers, e.g. *erb*B3, *ctnnd*2, *spata*5 and *S*100*A*10. Moreover, the protein EMSY, encoded by one of the recovered genes, is directly linked to the development of sporadic breast cancers. As has been recently shown, the gene is amplified in more than 10% of sporadic breast and ovarian cancers whereby ist product EMSY inhibits the BRCA2 tumor suppressor protein (6). Accordingly, is believed that EMSY

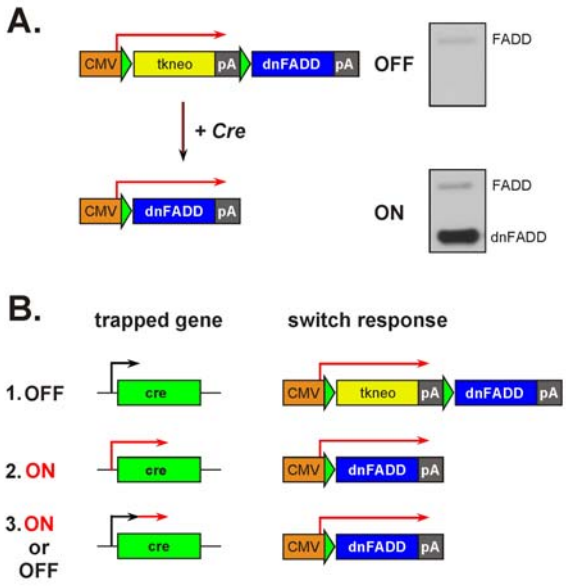

Fig 1: *Strategy for the recovery of inducible genes in MCF-7 cells. (A) Anatomy of the switch cassette. A HSV-thymidine-kinase/neomycinphosphotransferase cassette (tkneo), flanked by loxP sites (green triangles) in direct orientation relative to each other, is expressed from a constitutively active CMV promoter and simultaneously blocks the expression of the downstream dominant negative FADD (dnFADD) gene by premature polyadenylation (pA). MCF-7 cells expressing the switch do not express dnFADD (OFF) and die in presence of TNF-alpha. Cre expression deletes the tkneo cassette, which induces dnFADD expression (ON). Cells expressing the recombined switch survive in presence of TNF-alpha, because dnFADD blocks apoptotic signaling. (B) Use of the one-way switch in a genetic screen for TNF-alpha inducible genes. MCF7 cells with the CMVlxtkneolxdnFADD switch cassette inserted into their genome are transduced with a Cre gene trap vector. Insertions into active genes induce Cre expression which recombines the switch. Thus, selection in G418 eliminates all insertions into expressed "housekeeping" genes leaving insertions into silent genes behind (1. OFF). Exposure to TNF-alpha induces the expression of gene traps inserted in TNF-alpha regulated genes, which activates the switch (2. ON). As a result, the cells express dnFADD and survive in TNF-alpha. Since the switch recombination is irreversible, once activated, dnFADD expression becomes independent of the trapped cellular promoter (3. ON or OFF), This enables the recovery of genes that are only transiently expressed.*

overexpression is the functional equivalent to BRCA2 mutations typical for one inherited form of breast cancer. Collectively, these results clearly underscore the validity of the gene trap approach.

Validation of TNF-alpha induction of the trapped genes by Northern blotting revealed that the majority of them is regulated. Figure 2 shows two examples including JUNB, a known NF-κB target gene, and the human homolog of the mouse BCL9-2 gene, which thus far has not been associated with TNF-alpha signalling.

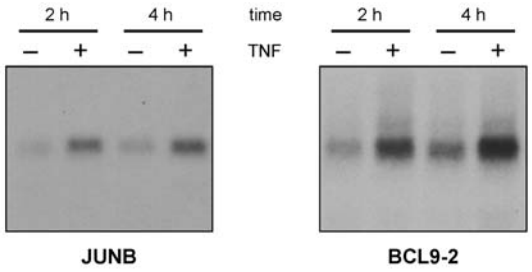

Fig 2: *Examples of trapped genes regulated by TNF-alpha. Total RNA isolated from MCF-7 cells treated with 25 ng/ml TNF-alpha for the indicated times was fractionated on formaldehyde/agarose gels, blotted onto nylon filters and hybridized to ^{32}P labeled JUNB or BCL9-2 specific probes.*

Identification of antisense transcripts

Closer inspection of the GTSTs revealed that the majority gene trap insertions occurred into the 5'-ends of genes, reflecting the well known integration preference of retroviruses for promoter proximal regions. Interestingly, more than 40% of the proviral insertions occurred in opposite transcriptional orientation relative to the trapped gene, strongly suggesting the presence of naturally occurring antisense transcripts (NATs) initiated by Pol II promoters residing on the minus strand.

An in silico analysis of these antisense insertions using three different, publicly available promoter prediction programs, identified putative promoters on the minus strands in almost 50% of the cases. This indicates that the gene trapping strategy employed here enables the recovery of non-coding, regulatory RNAs in addition to protein coding mRNAs that are regulated by a biological stimulus.

Outlook

Functional properties of candidate proteins

We have selected several TNF-alpha regulated candidate genes for more detailed functions analysis. Studies aimed at determining putative transforming and/or anti-apoptotic functions of the recovered proteins are in progress. Experiments involve protein overexpression exercises coupled with the analysis of intracellular protein distribution (Figure 3) as well as knock down approaches using RNAinterference (RNAi).

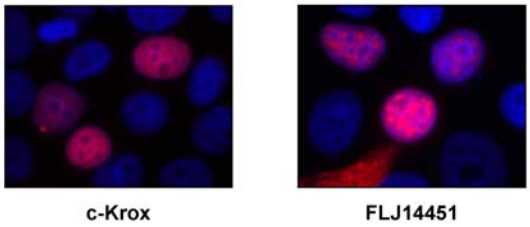

Fig 3: *Subcellular localization of candidate proteins. MCF-7 cells transiently transfected with vectors expressing the V5-epitope-tagged proteins c-Krox and FLJ14451 were stained with an anti-V5-antibody (red) and counterstained with DAPI.*

Analysis of antisense transcripts

Promoters predicted on the antisense strands of the trapped genes will be verified experimentally by cloning the respective genomic upstream sequences into luciferase reporter vectors. Promoter activity will be assessed in transient transfection asssays of MCF7 cells in the absence and presence of TNF-alpha. In addition, we will isolate the full length antisense transcripts and subject these to an in depth structural and functional analysis. Experiments will focus on their potential role in regulating gene expression. Finally, making sense of the antisense transcripts will greatly assist the ongoing annotation of the human genome.

Lit.: ***1.*** *Porter et al. A neural survival factor is a candidate oncogene in breast cancer. Proc Natl Acad Sci USA. 2003 Sep 16;100(19):10931-6.* ***2.*** *Micheau and Tschopp. Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes. Cell. 2003 Jul 25;114(2):181-90.* ***3.*** *Russ et al. Identification of genes induced by factor deprivation in hematopoietic cells undergoing apoptosis using gene-trap mutagenesis and site-specific recombination. Proc Natl Acad Sci USA. 1996 Dec 24;93(26):15279-84.* ***4.*** *Hsu et al. TRADD-TRAF2 and TRADD-FADD interactions define two distinct TNF receptor 1 signal transduction pathways. Cell. 1996 Jan 26;84(2):299-308.* ***5.*** *Wempe et al. Gene trapping identifies transiently induced survival genes during programmed cell death. Genome Biol. 2001 Jun 27;2(7): research 0023.1-0023.10.* ***6.*** *Hughes-Davies et al. EMSY links the BRCA2 pathway to sporadic breast and ovarian cancer. Cell. 2003 Nov 26;115(5):523-35.*