

Translational and functional oncogenomics. From cancer-oriented genomic screenings to new diagnostic tools and improved cancer treatment

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ABSTRACT

We present here an experimental pipeline for the systematic identification and functional characterization of genes with high potential diagnostic and therapeutic value in human cancer. Complementary competences and resources have been brought together in the TRANSFOG Consortium to reach the following integrated research objectives: 1) execution of cancer-oriented genomic screenings on tumor tissues and experimental models and merging of the results to generate a prioritized panel of candidate genes involved in cancer progression and metastasis; 2) setup of systems for high-throughput delivery of full-length cDNAs, for gain-of-function analysis of the prioritized candidate genes; 3) collection of vectors and oligonucleotides for systematic, RNA interference-mediated down-regulation of the candidate genes; 4) adaptation of existing cell-based and model organism assays to a systematic analysis of gain and loss of function of the candidate genes, for identification and preliminary validation of novel potential therapeutic targets; 5) proteomic analysis of signal transduction and protein-protein interaction for better dissection of aberrant cancer signaling pathways; 6) validation of the diagnostic potential of the identified cancer genes towards the clinical use of diagnostic molecular signatures; 7) generation of a shared informatics platform for data handling and gene functional annotation. The results of the first three years of activity of the TRANSFOG Consortium are also briefly presented and discussed.

Introduction

Sooner or later during the development of most types of human cancer, primary tumor masses spawn pioneer cells that move out, infiltrate adjacent tissues, to then travel to distant sites to colonize new terrain in the body where, at least initially, nutrients and space are not limiting. These distant settlements of tumor cells, that is, metastases, are the most life-threatening aspects of the oncogenic process and account for 90% of human cancer deaths. Like the formation of the primary tumor mass, successful invasion and metastasis depend on a critical balance between deregulated proliferation and inhibition of apoptosis. However, these alterations must act in concert with more subtle operational strategies, involving changes in the physical coupling of cells to their microenvironment and activation of proteases that degrade the extracellular matrix. The integration of all these cellular behaviors defines a complex, multi-step program of tumor-host interactions that is conventionally termed "invasive growth"¹.

Several classes of proteins involved in the tethering of cells to their surroundings in a tissue have already been found altered in cells possessing invasive or metastatic capabilities. These include cadherins, which mediate cell-cell contacts and thus must be quantitatively or qualitatively down-regulated in order for cells to abandon the primary

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tumor², integrins, which link cells to extracellular matrix substrates³, and extracellular matrix proteases⁴. Whereas cadherins, integrins and matrix proteases are physical effectors of cell invasion and metastasis, soluble factors acting through signaling-competent receptors are the functional mediators and coordinators of this process.

It is well known that many growth factors and cytokines can stimulate cell proliferation, dissociation, locomotion, and survival. For instance, full execution of the various steps that make the invasive growth program possible is specifically controlled by a family of growth factors called scatter factors, together with their receptors, and by their phylogenetically correlated cousins semaphorins and plexins⁵.

Formation of new blood vessels, or angiogenesis, in addition to supplementing the tumor with oxygen and nutrients, facilitates local shedding of cancer cells into the tumor venous drainage and thus initiation of the metastatic process, which is also eased by the immature nature of the newly formed vessels. Interestingly, angiogenesis itself recapitulates the process of invasive growth (modification of endothelial cell-cell and cell-matrix adhesion, motility, matrix degradation). The implications of this model are that anti-invasion compounds specifically targeted at key players of the invasive growth process should also be anti-angiogenic and therefore block metastasis growth with a doubled impact. Therefore, identification of new genes involved in the invasive growth program and in-depth characterization of the complex networks governing embryonal epithelial morphogenesis and cancer metastatic progression are likely to provide new tools for personalized diagnosis and for targeted therapeutic approaches.

Although extensive analysis over the last two decades has led to a deep insight into the control of cell proliferation and survival and their alterations during cancer onset, much remains to be clarified about the genetic lesions and alterations of cell signaling that lead to aberrant activation of invasive growth, cancer progression and metastasis. To this aim, much advantage may come from the historical changes in perspectives and modality of gaining information that biomedical research has been facing since the inception of the new century. Indeed, genome-sequencing projects have been completed for many organisms, including *Homo sapiens* (<http://www.ncbi.nlm.nih.gov/genome/guide/human/>) and *Mus musculus* (<http://www.ncbi.nlm.nih.gov/genome/guide/mouse/>). This reversed the conventional approach to biomedical discovery, in which understanding a certain biological function required identification of one or more genes involved in that function. The current situation is that thousands of genes have been sequenced but still wait for any functional information to be assigned to them. The fact that genes of unknown function represent over 70% of all genes suggests that current comprehension of most biological and pathological processes is by far incomplete. This is particular-

ly true in the case of cancer progression, where systematic exploration of gene function is likely to yield a huge amount of information in the next years.

There are several ways of obtaining information about gene function, some of which have been evolving at an incredibly high pace. For example, the DNA microarray technology currently enables mRNA expression analysis in parallel for thousands of genes. Indeed, being expressed (at least at the RNA level) is an essential prerequisite for a gene to exert its function, and by studying the sites and pathways of regulation of a certain gene expression it is possible to putatively assign it to a broad functional group. In this view, genes with restricted, tissue-specific expression are likely to play key roles in the biochemical and biological processes specifically occurring at the expression sites.

Another powerful approach to gene functional characterization is exploration of the consequences of gene loss of function in various model organisms, ranging from unicellular microorganisms to invertebrates, vertebrates and mammals. In particular, generation of mutations in murine embryonic stem (ES) cells by targeted and random approaches offers a powerful tool for loss-of-function studies in the mouse. ES cells can be grown *in vitro* as a continuous cell line, genetically modified and subsequently returned to the embryo, where they can generate chimeric mice and eventually contribute to the germ line. Mouse ES cells are now widely used for gene disruption by homologous recombination or chemically induced mutagenesis, to create mutant mice that lack or express an altered form of a specific gene. Indeed, an International Mouse Mutagenesis Consortium has been established, with the long-term goal of producing at least one heritable mutation, in either ES cells or mice, in every gene in the mouse genome⁶.

In many cases however, functional redundancy or subtle phenotypes may impair functional characterization of the targeted genes. Moreover, this approach is aimed at defining gene function in the context of the organism but is hard to direct to exploring basic biological and biochemical functions at the cellular level. This latter type of information can be achieved by systematic screenings exploring features of the gene protein product, like subcellular localization, biochemical activity, interactions, and others.

Recently, development of small interfering RNA (siRNA)-based approaches rendered loss-of-function studies more easily practicable in cell lines and higher organisms⁷. As a complementary approach, genes can be characterized by gain-of-function approaches, relying on overexpression of cloned genes in cells and organisms⁸ or on random activation of gene expression⁹. Finally, detailed analysis of post-translational modifications and protein-protein interactions by innovative proteomics approaches is crucial to correctly place the gene protein products within the dynamic and complex network of the normal and neoplastic living cell¹⁰.

From this brief outline of the major strategies for gene functional characterization, it clearly emerges that a crucial issue in functional genomics is the development of technologies for high-throughput functional analysis. Towards this aim, development of large-scale functional screens focused on cancer requires a coordinated approach involving complementary competences and establishment of dedicated facilities, for which the TRANSFOG (Translational and Functional Onco-Genomics) initiative intends to provide a European-level framework. The members of the TRANSFOG consortium are listed in Table 1. Further information is available on the TRANSFOG website: <http://www.transfog.org>.

Results and discussion

The TRANSFOG experimental pipeline consists of seven research components that synergistically enable streamlined translation of large-scale genomic screenings into high-impact contributions to cancer diagnosis and therapy. The seven activities, illustrated in Figure 1, are described in the following paragraphs together with a brief outline of the most significant results obtained during the first three years of activity.

1. Cancer-oriented genomic screenings in tumors and cell lines Genome-wide screenings by DNA microarrays, array-CGH, epigenetic and proteomics have been carried out by 14 partners and finally merged with the particular aim of identifying and prioritizing genes with a potential role in cancer metastasis, the “candidate genes”. Recent works have shown that it is possible to exploit gene expression profiling of tumor samples to

define sets of genes (signatures) whose expression correlates, positively or negatively, with metastasis-free survival, e.g., in breast cancer¹¹. It has also been found that a general signature associated with metastatic behavior can be shared between solid tumors of different organs¹², which indicates that common alterations of basic cellular functions and signaling pathways trigger metastatic progression of cancer. The TRANSFOG screenings concentrated on breast, lung and colon cancer, which altogether account for most cancer deaths in the general population. Apart from tumors, screenings have also included cancer-oriented experimental models, like serine and tyrosine kinase receptor-driven transcriptional and proteomic responses^{13,14}, transcriptional responses to oncogenic Ras mutation¹⁵, ligand-induced *in vitro* epithelial morphogenesis and invasive growth, and *in vitro* angiogenesis of endothelial cells. The aim was to obtain a genome-wide exploration of the basic mechanisms of cancer progression. By merging the results of the screenings, we could find “common” genes, i.e., genes emerging from more than one screening as associated to invasion and metastasis, and “specific” genes, whose expression is only altered in small subgroups or subtypes of tumors/metastases or cellular models. The relevant genes have been ranked for priority towards functional characterization and/or diagnostic validation, with the main priority criterion being their emergence in more than one screening.

2. Development of enabling technologies for systematic gene gain-of-function One approach for functional characterization of candidate genes identified by Activity 1 is based on enabling the expression of their full-length cDNAs in cells of interest. This required the as-

Table 1 - The TRANSFOG Consortium

Organization name	Acronym	Nation	Principal investigator
Organisation of European Cancer Institutes	OECI	Belgium	G Storme
Institute for Cancer Research and Treatment	IRCC	Italy	PM Comoglio
Centro Nacional de Investigaciones Oncologicas	CNIO	Spain	M Barbacid
Deutsches Krebsforschungszentrum	DKFZ	Germany	A Poustka
Nederlands Kanker Instituut/Antoni van Leeuwenhoek ziekenhuis	NKI	Netherlands	R Bernards
University Medical Center Utrecht	UMCU	Netherlands	JL Bos
Istituto FIRC di Oncologia Molecolare	IFOM	Italy	M Pierotti
European Bioinformatics Institute	EMBL-EBI	Germany	R Apweiler
Biomedical Sciences Research Centre “Alexander Fleming”	FLEMING	Greece	G Panayotou
Friedrich Miescher Institute for Biomedical Research	FMI	Switzerland	N Hynes
Agendia BV	AGENDIA	Netherlands	L van 't Veer
Medical University of Innsbruck (MUI), Institute of Pathophysiology	IPP	Austria	S Geley
Karolinska Institutet	KI	Sweden	Cl Smith
Kings College London	KCL	United Kingdom	AJ Ridley
Ludwig Institute for Cancer Research, Uppsala branch	LICR-UPP	Sweden	CH Heldin
Consorzio Interuniversitario Biotecnologie	LNCIB	Italy	C Schneider
Max-Delbrück-Centrum für Molekulare Medizin (MDC) Berlin-Buch	MDC	Germany	W Birchmeier
The Weizmann Institute of Science	WIS	Israel	Y Yarden
ALTA Ricerca e Sviluppo in Biotecnologie Srl	ALTA	Italy	A Tagliabue

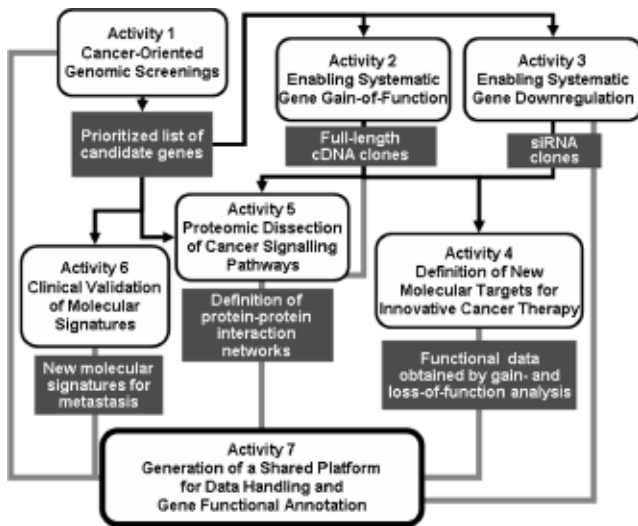


Figure 1 - Flowchart of the seven research activities of the TRANSFOG pipeline (light gray boxes), of their outputs (dark gray boxes), dependencies (black arrows) and interconnections (gray lines). The main outcome of Activity 1, the list of candidate genes resulting from cancer-oriented screenings, informs Activities 2 and 3 for the production of tools for specific gene gain and loss of function, respectively. These tools are in turn essential for Activities 4 and 5, which carry out systematic analysis of gene function and signal transduction. An important feed-back will come to Activity 1 from Activity 5, which will identify new binding partners for the proteins studied and therefore add them up to the novel candidate cancer gene list. Activity 1 also instructs Activity 6, with the definition of molecular signatures of potential clinical value to be validated. A key element to enable data standardization, sharing and mining is Activity 7, which will provide the TRANSFOG bioinformatics backbone.

sembly of a core full-length cDNA collection, exploiting the expertise and resources of the Partner DKFZ¹⁶. Over 200 full-length cDNAs have been delivered to the various partners for their studies of functional characterization.

3. Generation or acquisition of tools for RNA interference-based systematic gene loss-of-function analysis A second way to analyze the function of genes is by inducing loss of function via RNA interference. In this view, a key effort of TRANSFOG has been the generation of a shared collection of hundreds of human shRNA constructs in a plasmid/retroviral expression system (which allows easy further transfer of the construct in the target cells of choice), mainly targeting genes of unknown function that emerge from TRANSFOG cancer-oriented genomic explorations described in Activity 1. The technology has been made available by Partner NKI, who set up a methodology for systematic generation of retroviral shRNA vectors for functional screenings¹⁷. This approach is being flanked by the use of double-stranded siRNA oligonucleotides for the 100 top genes from the

TRANSFOG prioritized gene list. Such siRNAs represent a high-quality reagent that can be used in standard transfection assays by all consortium members, allowing optimal comparison of data between laboratories. The use of single gene-silencing RNA species will allow the identification of individual gene functions, whereas combinatorial approaches will allow the characterization of polypeptides active in the same cellular pathways. Finally, regulatable RNAi vector systems have also been developed to avoid cell vitality problems deriving from stable silencing of essential genes¹⁸.

4. Gene functional characterization by cell-based assays and analysis in model organisms Modulation of growth, motility, survival, invasion, adhesion, morphogenesis, senescence, and other basic biological functions altered during tumor progression and metastasis have been and are being analyzed by transduction of cultured cells with full-length cDNAs, shRNAs or siRNAs. Some of the proposed assays reached an adequate throughput for systematic gene functional analysis, allowing the identification of genes modulating the SRC proto-oncogene¹⁹ or the analysis of complex and combinatorial effects of multiple gene modulation²⁰. In other cases, low-throughput studies allowed detailed characterization of only a few genes. As an example, a transcriptional switch between two EGF transcriptional targets, the actin-binding proteins tensin and cten, was found to be essential for EGF-driven mammary cell migration²¹.

Specific animal models were exploited to assess the therapeutic potential of targeting the MET proto-oncogene²², the cooperation between oncogenic KRAS activation and chronic pancreatitis to promote pancreatic cancer²³, or the surprising dispensability of the cell cycle proteins Cdk2 and Cdk4 for mouse viability²⁴. Finally, systematic gene functional analysis is also being conducted in lower vertebrates like zebrafish and drosophila²⁵, with particular care on accurate annotation of the results, for optimal cross-species comparison²⁶.

5. Proteomic approaches to the study of signal transduction and protein-protein interactions Protein post-translational modifications and interactions with other proteins play a key role in many biological processes related to cancer progression. However, a comprehensive view of the networks of interactions and of their dynamics in normal and cancer cells is still lacking and technically challenging. To shed light on the candidates of interest from this point of view, the TRANSFOG partners have exploited mass spectrometry, Biacore biosensor and cell-based analysis. Much work is still ongoing, but the research activity led to: *i*) a detailed characterization of the role of the c-Jun N-terminal kinase in the PDGF receptor pathway promoting cell migration²⁷; *ii*) the definition of post-translational modifications essential for the activity of the FOXO4 transcription factor²⁸, and

iii) modulation of the activity and localization hypoxia-inducible factor-1 alpha via MAPK-mediated phosphorylation²⁹. These results add a further level of complexity to the simplistic view of the signal transduction “pathway” and rather highlight the real “network” status of the intracellular signaling system.

6. Preliminary diagnostic validation of molecular cancer signatures Converting a molecular signature emerged from a cancer genomic screening into a validated tool of potential clinical utility is a demanding task. For instance, the platform originally used to define the signature (e.g., a certain type of microarray) may not be the most adequate for subsequent capillary diffusion of the signature assay. A translational research phase has therefore been initiated to re-assess the signatures of interest on new tumor samples and with other platforms (e.g., real-time PCR, Tissue microarrays, immunohistochemistry) and to make cross-comparisons between platforms available at different sites. Standardized procedures have been defined for the various platforms and for the management of clinical and experimental data. The main diagnostic problem that TRANSFOG is addressing is the prediction of the probability with which a primary carcinoma will give rise to metastasis. In the first phases of the project, careful assessment of the optimal data treatment and cross-comparison has been performed, dealing in particular with data clustering³⁰, analysis and optimization of the predictive models³¹, and projection of data clusters across independent experimental datasets³². In addition to complex multigene classifier validation, single gene analyzes are also being conducted to explore new potentially useful diagnostic tools. As an example, specific mutations of the EGFR gene are associated to impaired ubiquitination and down-regulation, potentially leading not only to oncogenic activation, but also to refractoriness to targeted anticancer treatments aimed at EGFR down-regulation³³.

7. Generation of a common platform for data handling and gene functional annotation The TRANSFOG pipeline has to deal with a large variety of data coming from the various activities: Activity 1 - microarray data from different platforms and different organisms and related biological/clinical information, epigenetic data and differential proteomics data; Activities 2-3 - availability of vectors or reagents for specific gene overexpression and down modulation; Activity 4 - cell-based functional assays (from various organisms), phenotype descriptions (model organisms), clinical descriptions (preclinical proof-of-concept experiments with mouse xenografts and other mouse models); Activity 5 - protein-protein interaction data and networks from various organisms; Activity 6 - experimental and clinical data from signature validation experiments and from tissue microarrays. Capturing and representing all this infor-

mation in a format appropriate for data mining is a complex task. Moreover, these data come from different laboratories and different organisms; therefore we needed to develop data communication standards and systematic orthologue analysis. All the data have been standardized and integrated to reach a comprehensive human/mouse/other organism genome annotation system, exploiting the Distributed Annotation System³⁴ (DAS, <http://www.biodas.org>), originally developed by Lincoln Stein, to provide a simple, flexible bioinformatics backbone.

DAS requires a reference server that provides the framework to which all annotation will be anchored (usually the genomic sequence). In this project, the reference system is made by all the explored genes anchored to their position in the genomic sequence. Relative to the reference framework, multiple annotation servers can provide annotation for the reference objects. We adapted the system to include additional data types of our interest, e.g., the availability of full-length cDNAs, results of genomic screenings, conditional expression changes derived from microarray results, and functional or proteomic assays. DAS clients can connect to the reference server and any number of annotation servers and present an integrated view of the annotation of the reference objects. Figure 2 shows how single bits of annotation anchored to each gene can be added in multiple layers to provide a high-level, integrated view of experimental results from the project partners. Experimental details remain accessible through hyperlinks to the database systems of the project partners or to the submitted data in public repositories.

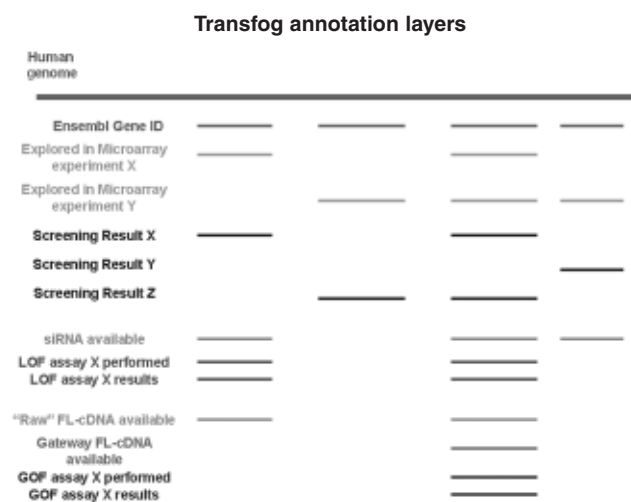


Figure 2 - Graphic representation of the various annotation layers of the TRANSFOG DAS system. All the data and results available on a single gene are mapped to a common backbone, the Ensemble Gene IDs mapped on the Ensemble human genome. LOF, loss of function; GOF, gain of function.

A significant effort within this activity is also devoted to standardization of the research output. To this aim, the Partner EBI leads or participates in international standardization initiatives, such as the definition of a “Functional Genomics Experiment model”³⁵, of the “minimum information required for reporting a molecular interaction experiment”³⁶, or of the “minimum information about a proteomics experiment”³⁷.

Final remarks

The goal of the TRANSFOG Consortium and research pipeline is dual: *i*) to develop innovative cancer-specific molecular signatures based on in-depth analysis and understanding at the genomic level of tumor development and metastatic spread, as a novel approach for the diagnosis and treatment of breast, lung, colon and possibly other epithelial cancers; *ii*) to identify key genes controlling basic biological functions involved in cancer progression and potentially exploitable as new molecular targets for innovative therapies. Over the first three years of activity, several technical challenges have been faced, but the results obtained to date are encouraging and likely to open new perspectives in the diagnosis and treatment of cancer. A key challenge of the near future will be to optimally integrate the new knowledge and tools made available by the post-genomic era with the existing golden standards for cancer diagnosis and treatment.

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