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Translational Oncogenomics and Human Cancer Interactomics: Advanced Techniques and Complex System Dynamic Approaches

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1. Introduction

An overview of translational, human oncogenomics, transcriptomics and cancer interactomic networks is presented together with basic concepts and potential, new applications to Oncology and Integrative Cancer Biology. Novel translational oncogenomics research is rapidly expanding through the application of advanced technology, research findings and computational tools/models to both pharmaceutical and clinical problems. A self-contained presentation is adopted that covers both fundamental concepts and the most recent biomedical, as well as clinical, applications. Sample analyses in recent clinical studies have shown that gene expression data can be employed to distinguish between tumor types as well as to predict outcomes. Potentially important applications of such results are *individualized* human cancer therapies or, in general, 'personalized medicine'. Several cancer detection techniques are currently under development both in the direction of improved detection sensitivity and increased time resolution of cellular events, with the limits of single molecule detection and picosecond time resolution already reached. The urgency for the complete mapping of a human cancer interactome with the help of such novel, high-efficiency, low-cost and ultra-sensitive techniques is also pointed out.

1.1 Current status in translational genomics and interactome networks

Upon completion of the maps for several genomes, including the human genome, there are several major post-genomic tasks lying ahead such as the translation of the mapped genomes and the correct interpretation of huge amounts of data that are being rapidly generated, or the important task of applying these fundamental results to derive major benefits in various medical and agricultural biotechnology areas. Translational genomics is at the center of these tasks that are running from *transcription* through *translation* to *proteomics* and *interactomics*. The *transcriptome* is defined as the set of all 'transcripts' or messenger RNA (mRNA) molecules produced through transcription from DNA sequences by a single cell or a cell population. This concept is also extended to a multi-cellular organism as the set of all its transcripts. The transcriptome thus reflects the active part of the genome at a given instant of time. *Transcriptomics* involves the determination of mRNAs

expression level in a selected cell population. For example, an improved understanding of cell differentiation involves the determination of the stem cell transcriptome; understanding carcinogenesis requires the comparison between the transcriptomes of cancer cells and untransformed ('normal) cells. However, because the levels of mRNA are not directly proportional to the expression levels of the proteins they are encoding, the protein complement of a cell or a multi-cellular organism needs to be determined by other techniques, or combination of techniques; the complete protein complement of a cell or organism is defined as the *proteome*. When the network (or networks) of complex protein-protein interactions (PPIs) in a cell or organism is (are) reconstructed, the result is called an *interactome*. This complete network of PPIs is now thought to form the 'backbone' of the signaling pathways, metabolic pathways and cellular processes that are required for all key cell functions and, therefore, cell survival. Such a complete knowledge of cellular pathways and processes in the cell is essential for understanding how many diseases -- such as cancer (and also ageing) —originate and progress through mutation or alteration of individual pathway components. Furthermore, determining human cancer cell interactomes of therapy-resistant tumors will undoubtedly allow for rational clinical trials and save patients' lives through individualized cancer therapy. Since the global gene expression studies of DeRisi et al in 1997, translational genomics is very rapidly advancing through the detection in parallel of mRNA levels for large numbers of molecules, as well as through progress made with miniaturization and high density synthesis of nucleic acids on microarray solid supports. Gene expression studies with microarrays permit an integrated approach to biology in terms of network biodynamics, signaling pathways, protein-protein interactions, and ultimately, the cell interactome. An important emerging principle of gene expression is the *temporally coordinated regulation* of genes as an extremely efficient mechanism (Wen *et al* 1998) required for complex processes in which all the components of multi-subunit complexes must be present/available in defined ratios at the same time whenever such complexes are needed by the cell. The gene expression profile can be thought of either as a 'signature/ fingerprint' or as *a molecular definition of the cell in a specified state* (Young, 2000). Cellular phenotypes can then be inferred from such gene expression profiles. Success has been achieved in several projects that profile a large number of biological samples and then utilize pattern matching to predict the function of either new drug targets or previously uncharacterized genes; this '*compendium approach*' has been demonstrated in yeast (Gray *et al* 1998; Hughes *et al* 2000), and has also been applied in databases integrating gene expression data from pharmacologically characterized human cancer lines (NCI60, http://dtp.nci.nih.gov), or to classify cell lines in relation to their tissue of origin and predict their drug resistance or chemosensitivity (Weinstein *et al*, 1997; Ross *et al* 2000, Staunton *et al* 2001). Furthermore, sample analyses in clinical studies have shown that gene expression data can be employed to distinguish between tumor types as well as to predict outcomes (Golub *et al* 1999; Bittner *et al*, 2000; Shipp et al 2002; Furteal et al., 2004). The latter approach seems to lead to important applications such as individualized cancer therapy and 'personalised medicine'. On the other hand, such approaches are complemented by studies of protein-protein interactions in the area called *proteomics*, preferably under physiological conditions, or more generally still, in *cell interactomics.* Several technologies in this area are still developing both in the direction of improved detection sensitivity and time resolution of cellular events, with the limits of single molecule detection and picosecond time resolution already attained. In order to enable the development of new applications such techniques will be briefly described in the next section, together with relevant examples of their recent applications.

1.2 Basic concepts in transcription, translation and interactome networks

The analysis of bionetwork dynamics of protein synthesis considered as a channel of information operates through the formation of protein amino acid sequences of polypeptides via *translation* of the corresponding polynucleotide sequences of (usually single –stranded, **messenger**) ribonucleic acid, that is:

DNA (gene) *transcription* → **mRNA** → *translation* **into a** polypeptide's aminoacid sequence → **protein** (quaternary) *assembly* from polypeptide subunits.

Although not shown in this scheme, several key enzymes make such processes both efficient and precise through highly-selective catalysis; moreover, the protein assembly involves both specific enzymes and ribosome 'assembly lines'. Furthermore, such processes are compartmented in the mammalian cells by selective intracellular membranes; this seems to be also important for cell cycling and the control of cell division. On the other hand, the *reverse transcription*, **RNA→ DNA,** does also occur (under certain conditions), catalized by a reverse transcriptase that contains both polypeptide chains and an RNA (master) strand. If error free, the first of these two sequence of processes —which are of fundamental biological importance-- generates true replicas of the information contained in the *sense codons* of the genes that are transcribed into mRNA *anti-codons*. Recall also that DNA stores information in the neucleotide bases A **(**Adenine**)**, C **(**Cytosin**)**, G **(**Guanine**)** and T **(**Thymine**)**, and that a triplet of such nucleotides in the DNA sequence is called a *codon,* which may encode unambiguously just the information necessary to specify a single amino acid. Moreover, the genetic code is quasi-universal, and capable of 'reverse transcription' from certain types of RNA back into DNA. Notably also, not all nucleotide or codon sequences present in the genome (DNA) are transcribed *in vivo.* Typically only a small percentage is transcribed. The transcribed (mRNA) sequences form what is naturally called the *transcriptome;* the protein--encoded version of the transcriptome is called the *proteome,* and upon including all protein--protein interactions for various cellular states one obtains the (global) *interactome* network. More generally, biological interactive networks as a class of complex bionetworks consist of local cellular communities **(**or *'organismic sets'***)** that are organized and managed by their characteristic selection procedures. Thus, in any partitioning of the organismal, or cell, structure, it is often necessary to regulate the *local* properties of the organism rather than the *global* mechanism, which explains an organism's need for specialized, 'modular constructions'. Such a modular, complex system biology approach to modeling signaling pathways and modifications of cell-cycling regulatory mechanisms in cancer cells was recently reported (Baianu, 2004); several consequences of this approach were also considered for the proteome and interactome networks in a 'prototype' cancer cell model (Prisecaru and Baianu, 2005). Note, on the other hand, that there seem to be also present in the living cell certain proteins and enzymes that are involved in *global* intra-cellular interactions which are thought to be essential to the cell survival and cell's flexible adaptation to stresses or challenge. Recent modeling techniques draw from a variety of mathematical sources, such as: topology (including graph theory), biostatistics, stochastic differential equations, Boolean networks, and qualitative system dynamics (Baianu, 1971a; de Jong *et al* 2000; 2003, 2004). Non--boolean network models of genetic networks and the interactome were also developed and compared with the results of Boolean ones (Baianu, 1977, 1984, 1987; Georgescu, 2006; Baianu, 2005; Baianu *et al. 2006).* The traditional use of comparatively rigid Boolean networks (reviewed extensively, for example in Baianu, 1987) can be thus extended through flexible, multi--valued (non--Boolean) logic algebra bionetworks with complex, *non-linear dynamic* behaviors that mimic complex systems biology (Rosen, 2000).

The results obtained with such non--random genetic network models have several important consequences for understanding the operation of cellular networks and the formation, transformation and growth of neoplastic network structures. Non--boolean models can also be extended to include *epigenetic* controls discussed in **Section 6**, as well as to mimic the coupling of the genome to the rest of the cell through specific signaling pathways that are involved in the modulation of both translation and transcription control processes. The latter may also provide novel approaches to cancer studies and, indeed, to developing 'individualized' cancer therapy strategies and novel anti-cancer medicines targeted at specific signaling pathways involved in malignant tumors resistance to other therapies.

2. Techniques and application examples

2.1 DNA microarrays

DNA microarray technology is widely employed to monitor in a single experiment the gene expression levels of all genes of a cell or an organism. This includes the identification of genes that are expressed in different cell types as well as the changes in gene expression levels caused, for example, by differentiation or disease. The terabytes of data thus obtained can provide valuable clues about the interactions among genes and also about the interaction networks of gene products. It has been reported that cDNA arrays were pioneered by the Brown Laboratory at Stanford University (Brown and Botstein, 1999; URL: http://cmgm.stanford.edu/pbrown/mguide/index.html). Several quantitative and high-density DNA array applications were then reported in rapid succession (Schena et al 1995; Chee et al 1996; Brown and Botstein, 1999). Such microarrays are generated by automatically printing double-stranded cDNA onto a solid support that may be either glass silicon or nylon. The essential technologies involved are robotics and devlopment/selection of sequence-verified and array-formatted cDNA clones. The latter ensures that both the location and the identity of each cDNA on the array is known. Sequence-verified and array-formatted cDNA clone sets are now available from companies such as Incyte Genomics (Palo Alto, CA; URL: http://www.synteni.com/) and Research Genetics (Huntsville, AL; URL: http://www.resgen.com/). In cDNA-based gene expression profiling experiments, the total RNA is extracted from the selected experimental samples and the RNA is fluorescently labeled with either cye3- or cye5-dUTP in a single round of reverse transcription. The latter have several advantages: they are readily incorporated into cDNA by reverse transcription, they exhibit widely separated excitation and emission spectra, and also they possess good photostability. Such fluorescently--labeled cDNA probes are then hybridized to a single array through a competitive hybridization reaction. Detection of hybridized probes is achieved by laser excitation of the individual fluorescent markers, followed by scanning using a confocal scanning laser microscope. The raw data obtained with a laser scanning systems is represented as a normalized ratio of cye3: cye5 and automatically color coded; thus, red color is conventionally selected to represent those genes that are transcriptionally upregulated in the test versus the reference, whereas green color represents genes that are downregulated; those genes that exhibit no difference between test and reference samples are shown in yellow. The analysis of the gene expression data obtained by such a high throughput microarray technology is quite complex and requires advanced computational/bioinformatics tools as already discussed in **Section 1.2**. Other aspects related to interactomics are discussed in **Section 3**. An alternative technology to cDNA microarrays is discussed in the next section.

2.2 Oligonucleotide arrays

By combining oligonucleotide synthesis with photolithography it was possible to synthesize specific oligonucleotides with a selected orientation onto the solid surface of glass or silicon chips (Lockhart *et al* 1996; Wodicka L, *et al* 1997), thus forming oligonucleotides arrays. The expression monitoring was then carried out by hybridization to high-density oligonucleotide arrays (Lockhart *et al* 1996; Wodicka L, *et al* 1997). Commercially available oligonucleotides array products include human, mouse and several other organisms. Each gene included on the oligonucleotides array is represented by up to 20 different oligonucleotides that span the entire length of the coding region of that gene. To reduce substantially the rate of false positives, each of these oligonucleotides is paired with a second mismatch oligonucleotide in which the central base in the sequence has been replaced by a different base. As in the cDNA approach, fluorescently labeled probes are generated from test and reference samples in order to carry out comparative gene expression profiling. After cDNA amplification, the differential fluorescent signal is detected with a laser scanning system and provides a map of the alterations in the transcriptional profile between the test and reference samples that are being compared. Dynamic analysis and further sophistication is added to such oligonucleotides array capabilities by the techniques briefly discussed in **Section 2.6.** The molecular classification of cancers is of immediate importance to both cancer diagnosis and therapy. Tumors with similar histologic appearance quite often have markedly different clinical response to therapy. Such variability is a reflection of the underlying cell line and molecular heterogeneity of almost any tumor. Gene expression profiling has been successfully employed for molecular classification of cancers. It would seem from available data that each patient has her/his own molecular identity signature or fingerprint (Mohr et al 2002). Thus, Ross *et al*. (2000) reported the gene expression analysis in 60 cancer cell lines utilized in the Developmental Therapeutics Program by the National Cancer Institute (NCI) at NIH (Bethesda, MD, USA); the report also stated that cell lines could be grouped together according with the organ type and specific expression profiles corresponded to *clusters of genes*. Similar findings were reported for ovarian and breast cancers; in the latter case, Perou *et al*. (2000) reported that specific epithelial cell line genes clustered together and are relevant in breast cancer subdivision into the basal- like and luminal groups. On the other hand, the eventual use of microarray technologies for clinical applications will involve the utilization of proteome and tissue arrays in addition to gene expression profiling by cDNA microarrays and oligonucleotides arrays. Thus, tissue markers revealed unexpected relationships, as in the case of gene expression analysis of small-cell lung carcinoma, pulmonary carcinoid tissue and bronchial epithelial tissue culture (Anbazhagan *et al* 1999). Because a single biomarker has serious limitations for clinical applications there is a need for a battery of disease biomarkers that would provide a much more accurate classification of cancers. High-density screening with microarray technologies is therefore valuable in pharmacogenomic (individualized therapy), toxicogenomic, as well as in clinical - diagnostic investigations.

2.3 Proteome arrays

In a manner similar to the transcriptome, the proteome does undergo both qualitative and quantitative changes during pathogenesis, and this is also true in carcinogenesis. Proteome array-based methodologies involve either proteins or protein-binding particles (DNA, RNAs, antibody, or other ligands). Utilizing such proteome arrays one can respectively

study either differential protein expression profiling or protein-ligand interaction screening under specified, or selected, physiopathological conditions. According to Kodadek (2001), these two classes of practical applications of proteome arrays are respectively defined as protein function and protein-detecting arrays. A protein-detecting array may consist of an arrayed set of protein ligands that are employed to profile gene expression and therefore make visible 'proteosignatures' characterizing a selected cellular state or phase. In view of the potential clinical importance of a proteomic survey of cancers, the 'hunt' is now on for such proteosignatures of cancer cells but the amount of data reported to date is still quite limited. Already, the coupling of proteome arrays with high-resolution chromatography techniques followed by mass spectrometry has provided powerful analytical tools with which one can profile the protein expression in cancer cells. For example, a ProteinChip™ (Ciphergen Inc, Fremont, CA, USA) was successfully utilized to investigate the proteome of prostate, ovarian, head and neck cancer cells (von Eggeling et al 2000). Such methods identified protein fingerprints from which cancer biomarkers can also be obtained. A reverse proteome array was also reported in which many extracted proteins from a patient sample are 'printed' onto a flat, solid support (Paweletz et al 2001); this reverse system was then utilized to carry out a biochemical screening investigation of the signaling pathways in prostate cancer. Through such investigations it was found that the carcinoma progression was positively correlated with the phosphorylation state of Akt and negatively correlated with ERK pathways; furthermore, the carcinoma progression was positively correlated with the suppression of the apoptotic pathways, a finding which is consistent with the more detailed, recent reports on cyclin CDK2 and transcriptional factors affected by CDK2 that will be discussed in **Section 4.** Immunophentotyping of leukemias with antibody microarrays was also reported (Belov, de la Vega, dos Remedios, et al 2001), and does provide an increased antigen differentiation (CD) in leukemia processing.

2.4 Tissue arrays

The logical step after the identification of potential cancer markers through genomic and/or proteomic array analysis is the evaluation of such cancer markers by tissue arrays/ tissue chips for diagnostic, prognostic, toxicogenomic and therapeutic relevance. Such tissue microarrays (TMAs) were often designed to contain up to 1000 sections of 5micron thick sections, usually chemically--fixed and arrayed upon a glass slide. TMAs allow large-scale screening of tissue specimens and can be utilized, for example, for the pathological evaluation of molecular irreversible changes that are important for cancer research and treatment. Therefore, they can speed up the process of translating experimental, or fundamental, discoveries into clinical practice and improved cancer treatments.

TMAs have been utilized in cancer research in conjunction with **fluorescence** *in situ* **hybridization** (FISH), to analyze in parallel the gene amplification in multiple tissue sections thus allowing the researchers to map the distribution of gene amplification throughout an entire tumor. This also allowed the monitoring of changes in gene amplification during the cancer progression (Bubendorf et al 1999). Furthermore, utilizing immunohistochemical staining of tissue arrays it was possible to measure the protein levels in tumor specimens. Thus, topoisomerase II alpha was reported to be highly expressed in patients with the poorest prognosis in oligodendrogliomas (Miettinen et al 2000). TMAs may become a clinical validation, as well as a 'global' tool; thus, recent studies reported this technique to be highly efficient for the identification of molecular (irreversible) alterations

during cancer initiation and progression (Lassus et al 2001). A pathologist might, however, object that the tissue microarray provides only a partial analysis of the tumor. The array-based technologies briefly described here provide powerful means for functional analyses of cancer and other complex diseases. Undoubtedly, much more can, and will be, done with proteome or tissue arrays combined with other state-of-the-science spectroscopic techniques as suggested in the following **Sections 2.5, 2.6, 4 and 6.**2.

The following three **Sections 2.5 and 2.6 and 6.2** will illustrate how advanced, ultra-fast and super-sensitive techniques can be used in conjunction with either nucleic acids or proteome arrays to both speed up thousand-fold the microarray data collection (for nucleic acids, proteins, ligand-binding, etc.) and also increase sensitivity to its possible limit--that of single molecule detection.

2.5 Fluorescence correlation spectroscopy and fluorescence cross—correlation spectroscopy: applications to DNA hybridization, PCR and DNA binding

In the bioanalytical and biochemical sciences Fluorescence Correlation Spectroscopy (FCS) techniques can be utilized to determine various thermodynamic and kinetic properties, such as association and dissociation constants of intermolecular reactions in solution (Thompson, 1991; Schwille, Bieschke and Oehlenschläger, 1997). Examples of this are specific hybridization and renaturation processes between complementary DNA or RNA strands, as well as antigene-antibody or receptor-ligand recognition. Although of significant functional relevance in biochemical systems, the hybridization mechanism of short oligonucleotide DNA primers to a native RNA target sequence could not be investigated in detail prior to the FCS/FCCS application to these problems. Most published models agree that the process can be divided into two steps: a reversible first initiating step, where few base pairs are formed, and a second irreversible phase described as a rapid zippering of the entire sequence. By competing with the internal binding mechanisms of the target molecule such as secondary structure formation, the rate-determining initial step is of crucial relevance for the entire binding process. Increased accessibility of binding sites, attributable to single-stranded open regions of the RNA structure at loops and bulges, can be quantified using kinetic measurements (Schwille, Oehlenschläger and Walter, 1996).

The measurement principle for nearly all FCS/FCCS applications is based so far upon the change in diffusion characteristics when a small labeled reaction partner (eg, a short nucleic acid probe) associates with a larger, unlabeled one (target DNA/RNA). The average diffusion time of the labeled molecules through the illuminated focal volume element is inversely related to the diffusion coefficient, and increases during the association process. By calibrating the diffusion characteristics of free and bound fluorescent partner, the binding fraction can be easily evaluated from the correlation curve for any time of the reaction. This principle has been employed to investigate and compare the hybridization efficiency of six labeled DNA oligonucleotides with different binding sites to an RNA target in a native secondary structure (Schwille, Oehlenschläger and Walter, 1996). Hybridization kinetics was examined by binding six fluorescently labeled oligonucleotide probes of different sequence, length and binding sites to a 101-nucleotide-long native RNA target sequence with a known secondary structure. The hybridization kinetics was monitored and quantified by FCS, in order to investigate the overall reaction mechanism. At the measurement temperature of 40°C the probes are mostly denatured, whereas the target retains its native structure. The binding process could be directly monitored through diffusional FCS analysis, via the change in translational diffusion time of the labeled 17-mer to 37-mer oligonucleotide probes HS1 to HS6 upon specific

hybridization with the larger RNA target. The characteristic diffusion time through the laser-illuminated focal spot of the 0.5 µm-diameter objective increased from 0.13 to 0.20 ms for the free probe, and from 0.37 to 0.50 ms for the bound probe within 60 min. The increase in diffusion time from measurement to measurement over the 60 min could be followed on a PC monitor and varied strongly from probe to probe. HS6 showed the fastest association, while the reaction of HS2 could not be detected at all for the first 60 min. Thus, FCS diffusional analysis provides an easy and comparably fast determination of the hybridization time course of reactions between complementary DNA/RNA strands in the concentration range from 10^{-10} to 10^{-8} M. The FCS-based methodology also permits rapid screening for suitable anti-sense nucleic acids directed against important targets like HIV-1 RNA with low consumption of probes and target. Because of the high sensitivity of FCS detection, the same principle can be exploited to simplify the diagnostics for extremely low concentrations of infectious agents like bacterial or viral DNA/RNA. By combining confocal FCS with biochemical amplification reactions like PCR or 3SR, the detection threshold of infectious RNA in human sera could be dropped to concentrations of 10^{-18} M (Walter, Schwille and Eigen, 1996; Oehlenschläger, Schwille and Eigen, 1996). The method allows for simple quantification of initial infectious units in the observed samples. The isothermal Nucleic Acid Sequence-Based Amplification (NASBA) technique enables the detection of HIV-1 RNA in human blood-plasma (Winkler, Bieschke and Schwille, 1997). The threshold of detection is presently down to 100 initial RNA molecules per milliliter by amplifying a short sequence of the RNA template (Schwille, Oehlenschläger and Walter, 1997). The NASBA method was combined with FCS, thus allowing the online detection of the HIV-1 RNA molecules amplified by NASBA (Oehlenschläger, Schwille and Eigen, 1996). The combination of FCS with the NASBA reaction was performed by introducing a fluorescently labeled DNA probe into the NASBA reaction mixture *at nanomolar concentrations*, hybridizing to a distinct sequence of the amplified RNA molecule. After having reached a critical concentration on the order of 0.1 to 1.0 nM (the threshold for single-photon excitation / FCS detection is ~0.1 nm), the number of amplified RNA molecules could be determined as the reaction continued its course. Evaluation of the hybridization/extension kinetics allowed an estimation of the initial HIV-1 RNA concentration present at the beginning of amplification. The value of the initial HIV-1 RNA number enables discrimination between positive and false-positive samples (caused, for instance, by carryover contamination). This possibility of sharp discrimination is essential for all diagnostic methods using amplification systems (PCR as well as NASBA). The quantification of HIV-1 RNA in plasma by combining NASBA with FCS may be useful in assessing the efficacy of anti-HIV agents, especially in the early infection stage when standard ELISA antibody tests often display negative results. Furthermore, the combination of NASBA with FCS is not restricted only to the detection of HIV-1 RNA in plasma.

On the one hand, the diagnosis of Hepatitis (both B and C) remains much more challenging. On the other hand, the number of HIV, or HBV, infected subjects worldwide is increasing at an alarming rate, with up to 20% of the population in parts of Africa and Asia being infected with HBV. In contrast to HIV, HBV infection is not particularly restricted to the high-risk groups.

Multi-photon (MPE) NIR excitation of fluorophores--attached as labels to biopolymers like proteins and nucleic acids, or bound at specific biomembrane sites-- is one of the most attractive options in biological applications of FCS. Many of the serious problems encountered in spectroscopic measurements of living tissue, such as photodamage, light scattering and auto-fluorescence, can be reduced or even eliminated. FCS can therefore

provide accurate *in vivo* and *in vitro* measurements of diffusion rates, "mobility" parameters, molecular concentrations, chemical kinetics, aggregation processes, labeled nucleic acid hybridization kinetics and fluorescence photophysics/ photochemistry. Several photophysical properties of fluorophores that are required for quantitative analysis of FCS in tissues have already been widely reported. Molecular "mobilities" can be measured by FCS over a wide range of characteristic time constants from $\sim 10^{-3}$ to 10^3 ms.

Novel, two-photon NIR excitation fluorescence correlation spectroscopy tests and preliminary results were obtained for concentrated suspensions of live cells and membranes (Baianu et al, 2007). Especially promising are further developments employing multi-photon NIR excitation that could lead, for example, to the reliable detection of cancers using NIR-excited fluorescence. Other related developments are the applications of Fluorescence Cross-Correlation Spectroscopy (FCCS) detection to monitoring *DNA- telomerase interactions*, DNA hybridization kinetics, ligand-receptor interactions and HIV-HBV testing. Very detailed, automated chemical analyses of biomolecules in cell cultures are now also becoming possible by FT-NIR spectroscopy of single cells, both *in vitro* and *in vivo*. Such rapid analyses have potentially important applications in cancer research, pharmacology and clinical diagnosis.

2.6 Near infrared microspectroscopy, fluorescence microspectroscopy and infrared chemical imaging of single cells

Novel methodologies are currently being evaluated for the chemical analysis of embryos and single cells by Fourier Transform Infrared (FT-IR), Fourier Transform Near Infrared (FT-NIR) Microspectroscopy, Fluorescence Microspectroscopy. The first FT-NIR chemical images of biological systems approaching 1micron (1μm) resolution were reported (Baianu, 2004; Baianu et al 2004), and FT-NIR spectra of oil and proteins were obtained under physiological conditions for volumes as small as 2μm³. Related, HR-NMR analyses of oil contents in somatic embryos were presented with nanoliter precision. Therefore, developmental changes may be monitored by FT-NIR with a precision approaching the *picogram* level when adequately calibrated by a suitable primary analytical method.

Indeed, detailed chemical analyses are now becoming possible by FT-NIR Chemical Imaging and Microspectroscopy of single cells. The cost, speed and analytical requirements are fully satisfied by FT-NIR spectroscopy and microspectroscopy for a wide range of biological specimens. These techniques were also suggested to be potentially important in functional genomics and proteomics research (Baianu et al 2004) through the rapid and accurate detection of high-content microarrays (HCMA). Multi-photon (MP), pulsed femtosecond laser NIR Fluorescence Excitation techniques were shown to be capable of *single molecule detection* (SMD). Thus, microspectroscopic techniques allow for most sensitive and reliable quantitative analyses to be carried out both *in vitro* and *in vivo.* In particular, MP NIR excitation in FCS allows not only *single molecule detection*, but also non-invasive monitoring of molecular dynamics and the acquisition of high-resolution, *submicron* imaging of *femtoliter* volumes inside functional cells and tissues. Such ultra-sensitive and rapid NIR-FCS analyses have therefore numerous potential applications in biomedical research areas, clinical diagnosis of viral diseases, cancers and also in cancer therapy.

3. Mapping interactome networks

Mapping protein-protein interaction networks, or charting the global interaction maps, that correspond through translation to entire genomes is undoubtedly useful for understanding

cellular functions, especially when such databases can be integrated into a wide collection of biologically relevant data. A prerequisite for any 'ab initio' determination of a selected protein interactome network is to clone the open reading frames (ORFs) that encode each protein present in the selected network. Note, however, that all current analyses involve the assumption of a *model* together with some 'hidden', or implicit, *assumptions* about sampling, 'noise' levels, or uniformity/ accuracy in the database, and therefore, the 'ab initio' claim is subject to the restrictions imposed by such additional assumptions. More than 20,000 of publicly accessible, full ORF clones have been already collected for human and mouse protein-coding genes in the Mammalian Genome Collection (MGC; http://mgc.nci.nih.gov). This community resource enables the next stages of human interactome analysis that will be directed at obtaining a reliable map of the entire human protein interactome. An additional, 12,500 ORFs are now available from the Dana Farber Cancer Institute in Boston (USA) from high-throughput, yeast two-hybrid (Y2H) analyses. A disconcerting aspect of the latest human (partial) interactome studies by different methods is the little apparent overlap of the new human interaction datasets with each other and/or with previously reported data. This aspect will be further addressed later in this section; the principal cause for the lack of overlap is likely to be caused by the low (<20%) overall coverage of the protein-protein interactions selected in such studies. A possible solution to this problem has been suggested (Warner et al 2006): several groups cooperating to produce 'networks of networks', constructed from separate—but coordinated—interaction mapping projects, 'each of which would target a specific functionality related subset of proteins and interactions'. A more effective solution would be, however, to increase the throughput, accuracy and reliability of PPI data through improved technologies (such as FCCS, or other techniques already proposed in Section 2.5, for example), reduce significantly the cost of such analyses, as well as improve the models employed for data analysis. Examples of improved modeling tools for this purpose, such as logical, ontological genetics and categorical ones, that are also appropriate for assembling the '*networks of networks…*' as in the previous approach suggested by Warner et al. (2006), were presented above in Section 1, and are described in further detail in a recent report (Baianu et al 2006) and also in two forthcoming publications (Baianu and Poli, 2011; Baianu et al 2010). Interactome network studies are currently undertaken by a number of international research teams in the US, Europe and Japan (CSH/WT, 2006; Warner et al 2006). These studies are currently undertaken only for interactome subnetworks because of both technique and funding limitations. The organisms studied were: yeast (*Saccharomyces cerevisiae*), worm (*Caenorhabditis elegans*), fruitfly (*Drosophila melanogaster*) and humans. Proteome networks were investigated for several, specific, biological processes such as: DNA degradation, ubiquitin conjugation, multivesicular formation, intracellular membrane traffick, signal transduction/ TNF☐ tumor necrosis and NF☐B mediated pathways, and early stages of T-cell signaling (for a brief summary note the recent review by Warner *et al* 2006, and references cited therein). Such challenging studies face both methodological problems such as limited sampling (Han *et al* 2006) and consideration of only pairwise ('binary') protein-protein interactions, and also the more serious technical problem of false-positive interactions in the presence of a significant 'noise' levels associated with the experimental technologies and design currently employed in such studies. Such limitations should be borne in mind (Han et al 2006) when global topology predictions are made for the whole interactome based on partial, incomplete data obtained for subnetworks that may contain less than 20% of the entire interactome network. On a more optimistic note are the recent attempts at comparing the

cancer protein, human interactome (sub) networks with normal human interactome networks that involve multiple protein-protein interactions (Jonsson and Bates, 2006). The latter studies reduced the 'noise' level in the human protein interaction data by employing an orthology-based method described previously by Jonsson et al. (2006). This method claims to reduce the 'noise' level in protein-interaction (PPI) data by identifying putative interactions based on homology to experimentally determined interactions in a range of different species; both the DIP (Salwinsky et al 2004) and the MIPS, Mammalian Protein — Protein Interaction (Pagel et al 2005) databases were utilized. Furthermore, the complete interactome data set that was employed is available as Supplementary Material from *loc. cit.* The conclusions was drawn that cancer proteins have an increased frequency of protein-protein interactions in comparison with the proteins that were studied in normal cells, and this was interpreted as evidence "*indicating an underlying evolutionary pressure to which cancer genes, as genes of central importance are subjected.*" It remains to be seen, however, if human interactome studies-- which occur with increasing frequency-- have indeed overcome the sampling objections raised by Han et al. (2006). The more extensive interactome data and analysis — though still quite limited- that has been reported to date is readily available and includes the following: Y2H (partial data-based) interactome maps for *C. elegans* (Li et al 2004) and *Drosophila melanogaster* (Giot et al 2003; Formstecher et al 2005), and also proteome maps obtained by co-affinity purification followed by mass spectrometry analysis in yeast-*Saccharomyces cerevisiae* (co-AP/MS: Gavin et al 2002; Ho et al 2002; Han et al 2004). The reports on the microbial transcriptional regulation network of *Escherichia coli* (Shen-Orr et al 2002) and on *Helicobacter pylori* protein complexes in the proteome map (Terradot et al 2004) are also worthile mentioning in this context. A first-draft of the human interactome has also been reported (Lehner and Fraser, 2004); although this human interactome map does not seem to have been included in the computational investigations of Han et al. (2006), it remains to be verified, or validated, by further extensive studies with improved technology and adequate models for a more comprehensive data analysis. The comprehensive two-hybrid analysis for exploring the protein interactome network was previously reported by Ito et al. (2001). Alternative interaction mapping strategies have also been developed over the last five years. An example is the tandem affinity purification (TAP) in conjunction with liquid chromatography tandem mass spectrometry (LC-MS/MS; see, for example, Gavin et al 2006). Such methods have, however, both advantages and limitations. An interesting, new approach to the determination of protein complexes has been developed that involves a combination of fluorescence spectroscopy with peptide microarrays (Stoevesandt, *cited in* Warner 2006); this methodology was then applied to investigate T-cell signaling.

4. Cell cyclins expression and modular cancer interactome networks

Carcinogenesis is a complex process that involves dynamically inter-connected biomolecules in the intercellular, membrane, cytosolic, nuclear and nucleolar compartments that form numerous inter-related pathways referred to as networks. One such family of pathways contains the cell cyclins. Cyclins are often overexpressed in cancerous cells (Dobashi et al 2004).

Our novel theoretical analysis based on recently published studies of cyclin signaling, with special emphasis placed on the roles of cyclins D1 and E, suggests novel clinical trials and rational therapies of cancer through re-establishment of cell cycling inhibition in metastatic cancer cells.

4.1 Cyclins

Cyclins are proteins that link several critical pro-apoptotic and other cell cycling/division components, including the tumor suppressor gene TP53 and its product, the Thomsen-Friedenreich antigen (T antigen), Rb, mdm2, c-Myc, p21, p27, Bax), which all play major roles in carcinogenesis of many cancers. Cyclin-dependent kinases (CDK), their respective cyclins, and inhibitors of CDKs (CKIs) were identified as instrumental components of the cell cycle-regulating machinery. CDKs are enzymes that phosphorylate several cellular proteins thus 'fueling' the sequential transitions through the cell division cycle. In mammalian cells the complexes of cyclins D1, D2, D3, A and E with CDKs are considered motors that drive cells to enter and pass through the **"S"** phase. Cell cycle regulation is a critical mechanism governing cell division and proliferation, and is finely regulated by the interaction of cyclins with CDKs and CKIs, among other molecules (Morgan et al 1995).

It was also reported that CDKs have another key role –the coordination of cell cycle progression with responses to possible DNA-damage that could, if unchecked or unfixed, lead to a lack of genomic integrity marking the onset of cell disease including cancers (Huang et al 2006 in *Science*). The **S**-phase is thought to be the most vulnerable interval of the cell cycle because during this interval all of 3 billion DNA bases of the human genome must be replicated precisely in the sense of 'carbon copies' being made of the existing DNA strands, without any breaks in the sequence or base substitutions of the copied/replicated strands. Therefore, this correct replication process controls the cell's survival, especially under genotoxic conditions such as those caused for example by mutagens or X-ray and gamma-radiation. Furthermore, Huang et al. (2006) reported that CDK mediated the phosphorylation of the FOXO1 transcriptional activator of the proapoptotic genes during the **S**-phase; when DNA damage occurs either before or during the **S**-phase, a complex network is activated in the cell which 'silences' CDK thereby either delaying or stopping/arresting the cell cycle progression. This may allow the cell to repair the DNA damage by recombination involving BRCA2 and survive. However, if this is not possible because the DNA damage was too great/irreparable, then FOXO1 would trigger apoptosis (cell death). It was proposed that during the unperturbed (normal) **S**-phase CDK2 phosphorylates FOXO1 at the Serine[249] residue in the cell nucleus, which then results in the transfer and sequestering of the FOXO1 in the cytoplasm, where it is well--separated from the proapoptotic genes, the 'target' of FOXO1 action.

Moreover, the CDK-mediated phosphorylation of BRCA2 during the unperturbed **S**-phase renders inactive the DNA recombination. On the other hand, when DNA becomes damaged, CDK2 is inhibited through the Cdc25A pathway, with the consequence of a dephosphorylated FOXO1 which then remains in the cell nucleus and is able to activate the proapoptotic genes, unless BRCA2 is able to induce DNA recombination and repair in time to prevent apoptosis. The steps that follow are then as explained above: either DNA repair and continued cell cycling, or apoptosis induced by FOXO1. There are still several important questions regarding the entire process that need to be answered before the FOXO1 and CDK2 mechanisms of action can be translated into successful clinical trials based on such knowledge.

A positive correlation has been noticed between over-expression of several cell--cycle proteins and unfavorable prognoses and outcomes in several different cancer types (van Diest et al 1995; Fukuse et al 2000). In human lung tumors and soft tissue sarcomas, it was discovered that cyclin A/cdk2 complex expression and kinase activity were reliable predictors of proliferation and unfavorable prognosis, thereby further substantiating the epidemiological factors of cyclin signaling (Dobashi et al 2003; Noguchi et al 2000).
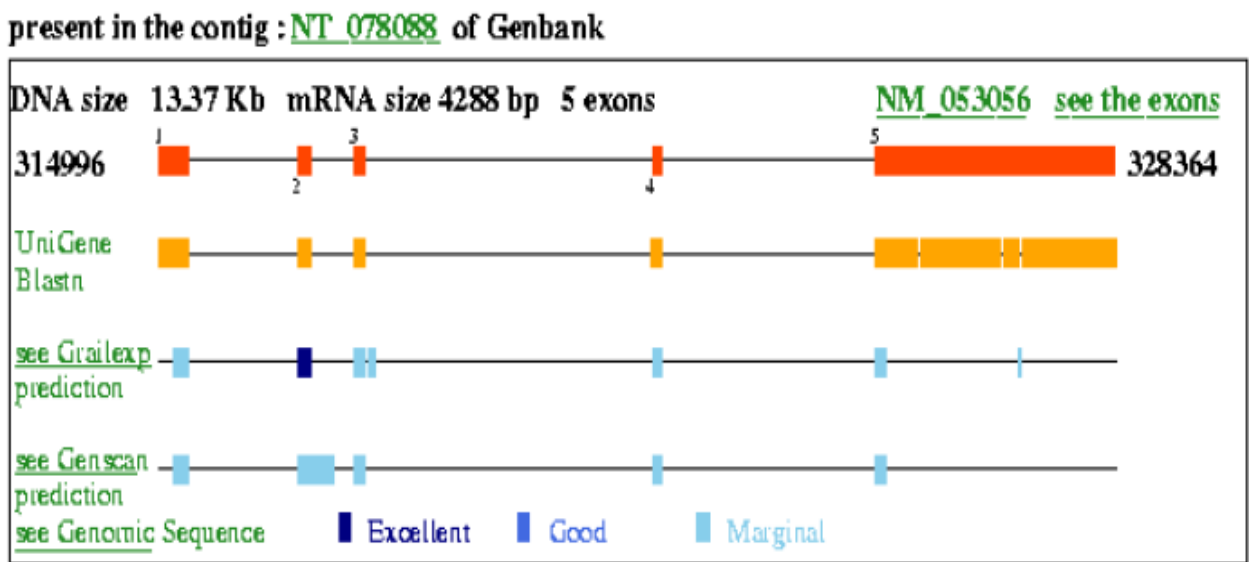
Fig. 1. Gene database of Cyclin-D1; *Source:* PBD website:
http://www.dsi.univ-paris5.fr/genatlas/fiche.php?symbol=CCND1

4.2 The p27 and p21 proteins

The proteins p27 and p21 were reported to be implicated in cyclin regulation and cancer development (**Fig. 2**). Mouse embryonic fibroblasts that were deficient for p27 and p21 were found to contain less cyclin D1 (Hashemolhosseini S, Nagamine Y, Morley SJ, et al., 1998). and D2 (Cheng et al 1999) as well as cyclin D3 (Bagui et al 2000) than controls. Similarly, mammary glands of p27-deficient mice were shown to possess decreased cyclin D1 levels (Muraoka et al 2001). It has been demonstrated *in vivo* that p27 is necessary for maintaining proper levels of cyclins D2 and D3, and this dependency on p27 is common to a wide variety of cells/tissues *in vivo*. Regarding the molecular interaction between p27 and D-cyclin, CDK4 is a clear candidate as a mediating molecule (Bryja et al 2004). Cells employ CDK4/6– cyclin D complexes to flexibly titrate p27 from the complexes containing CDK2, and thereby they control their proliferation. However, mutual dependency between cyclin D and p27 serves also some yet unidentified function in differentiation-related processes. Thus, loss of p27 not only causes unrestricted growth due to inefficient inhibition of CDK2– cyclin E/A, but may also elicit a decrease in levels of D-type cyclins, resulting in differentiation defects. Upon ablation of cyclin D, cells lose their ability to titrate p27 from CDK2–cyclin A/E complexes and proliferation is suppressed. However, defects in differentiation caused by the absence of D-cyclin are reminiscent to defects produced by the absence of p27 (Bryja et al 2004). When the changes in levels of p27 and/or D-type cyclins occur, an equilibrium alteration could result between proliferation/differentiation processes that may in the end result in tumorigenesis (Bryja et al 2004).

4.3 D1 vs. E- cyclins

The D-type and E-type cyclins control the G1 → S phase transition during normal cell cycling and are important components of steroid- and growth factor-induced mitogenesis in breast epithelial cells (Sutherland and Musgrove, 2004). Cyclin D1 null mice are resistant to breast cancer that is induced by the *neu* and *ras* oncogenes, which suggests a pivotal role for

cyclin D1 in the development of some mammary carcinomas (Sutherland and Musgrove, 2004). Cyclin D1 and E1 are usually overexpressed in breast cancer, with some association with adverse outcomes, which is likely due in part to their ability to confer resistance to endocrine therapies. The consequences of cyclin E overexpression in breast cancer are related to cyclin E's role in cell cycle progression, and that of cyclin D1 may also be a consequence of a role in transcriptional regulation (Sutherland and Musgrove, 2004). One critical pathway determining cell cycle transition rates of **G1 → S** phase is the cyclin/cyclin-dependent kinase (Cdk)/ p16Ink4A/ retinoblastoma protein (pRb) pathway (Sutherland and Musgrove, 2004). Alterations of different components of this particular pathway are very ubiquitous in human cancer (Malumbres and Barbacid, 2001). There appears to be a certain degree of tissue specificity in the genetic abnormalities within the Rb pathway. A model relating Rb to cyclin control in the overall scheme of pro-apoptotic behavior is shown in **Fig. 2.**

In breast cancer these abnormalities include the over-expression of cyclins D1, D3 and E1, the decreased expression of the p27Kip1 CKI and p16Ink4A gene silencing through promoter methylation. These aberrations occur with high frequency in breast cancer, as each abnormality occurs in ~40% of primary tumors. This fact implicates a major role for the loss of function of the Rb pathway in breast cancer. Cyclin D1 is the product of the *CCND1* gene and was first connected to breast cancer after localization of the gene to chromosome 11q13, a region commonly amplified in several human carcinomas, including ~15% of breast cancers (Ormandy et al 2003). The fact that cyclin D1 was overexpressed at the mRNA and protein levels in 50% of primary breast cancers have caused cyclin D1 to be considered one of the most commonly over-expressed breast cancer oncogenes (Gillett et al 1994). Although cyclin E1 locus amplification is rare in breast cancer, the protein product is overexpressed in over 40% of breast carcinomas (Loden et al 2002). Cyclin D1 is pre-dominantly overexpressed in ERC tumors, and cyclin E overexpression is confined to ER¡ tumors (Gillett et al 1994; Loden et al 2002). The overexpression of several cell cycle regulators has been strongly associated with apoptotic-like behavior, as well as frank apoptosis, in cancer cells, which include c-Myc, E2F-1 and HPV. Apoptosis and its connection to cell cycle-related proteins is of interest therapeutically, as these types therapies could ultimately lead to the cancer cell annihilation *via* apoptosis. Recently, a shift has occurred, changing the focus of chemotherapy from exploration of agents that cause cell growth arrest to those that favor apoptosis.

5. Biomedical applications of microarrays in clinical trials

5.1 Microarray applications to gene expression: identifying signaling pathways

Changes in homeostasis can be followed through various experimental strategies that monitor gene expression profiling, for example, by employing high-throughput microarray technology. This section discusses briefly the successful use of microarray technology in RNA expression studies aimed at identifying signaling pathways that are regulated by key genes implicated in carcinogenesis/ tumorigenesis. A primary objective of tumor-profiling experiments is to identify transcriptional changes that may be the cause of the transition from the normal to the tumor phenotype. Such changes may, however, occur also as a consequence of various neoplastic transformation(s). More importantly, this approach may allow the identification of molecular fingerprints that can be utilized for the classification of different tumor types, and are therefore valuable diagnostic molecular tools in cancer
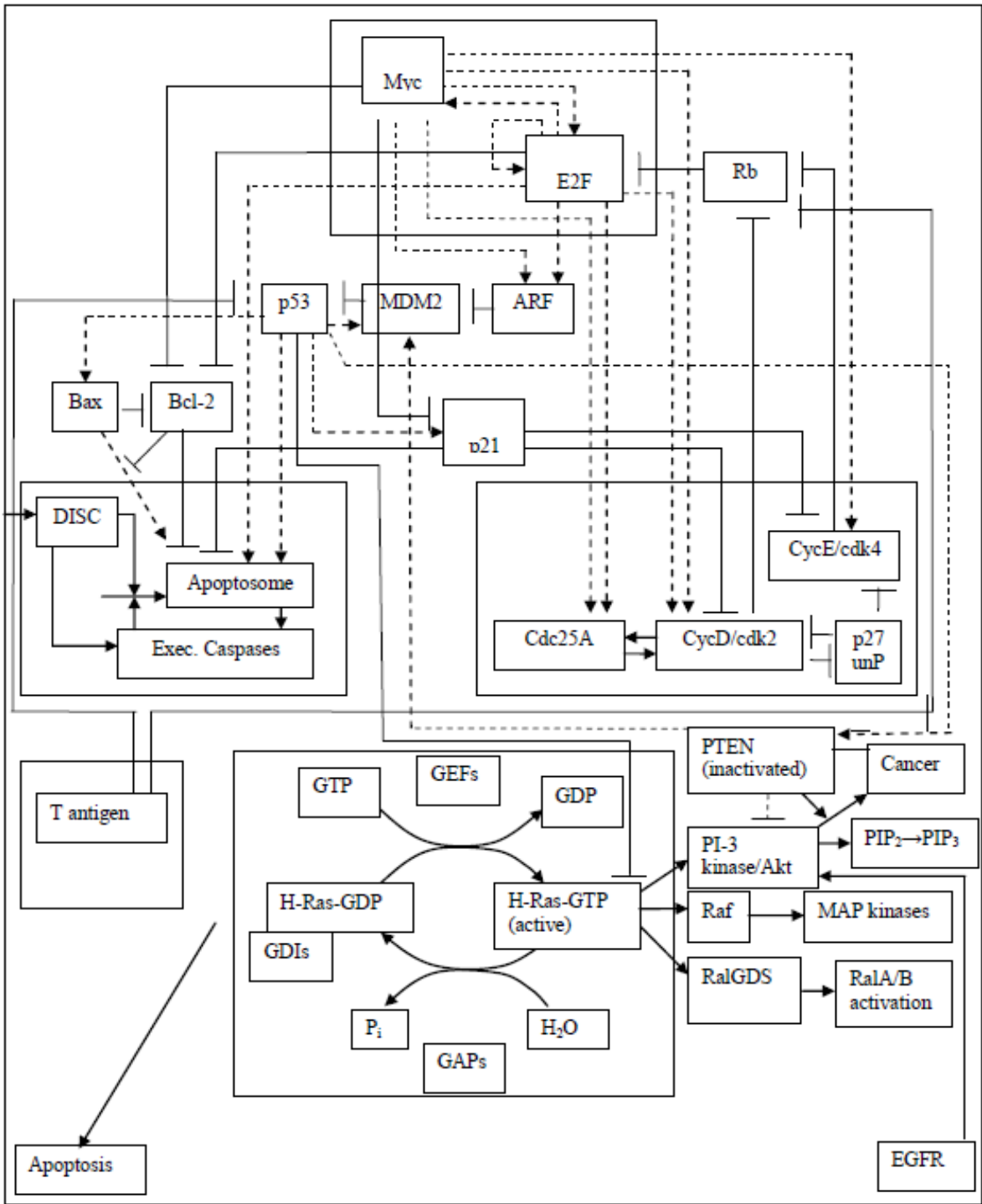
Fig. 2. Pro-Apoptotic Cancer Cycling Model: an update based on the previous model of Aguda et al. (2003).

patients. For example, Alizadeh et al. (2000) have successfully used such an approach to identify molecularly distinct subclasses of diffuse large B-cell lymphoma that could not be distinguished by conventional diagnostic tools. In another study, a molecular fingerprint comprising approximately 50 genes has been isolated from a total of over 6,000, and this fingerprint can reliably differentiate between acute myeloid leukemia and acute lymphoblastic leukemia Golub et al (1999).

5.1.1 Identification of specific transcriptional targets in cancer

The approach requires, however, multiple independent experiments with several large groups of samples in order to enable one to reliably and reproducibly separate the biologically relevant changes from false ones that may occur as a result of the genetic heterogeneity between individual samples from the same tumor, for example. The two examples quoted above were able to reproducibly identify tumor type-specific molecular determinants through multiple experiments with various tissue samples.

A different experimental approach to the one presented above is, however, needed for identifying specific targets such as defined genes that are implicated in cancer progression; this involves monitoring changes in transcriptional profile that occur as a result of modulation of the expression level of the defined gene, or genes, selected for such studies. The altered expression profile can be viewed as a 'blueprint' by which the defined gene controls its cellular function. The transcriptional profiles are thus employed to define *downstream signaling pathways* that have been previously validated through other techniques such as differential display Tanaka et al (2000) and serial analysis of gene expression Yu et al. (1999). This approach combined with microarray technology allows the simultaneous identification of all potential targets. Its only drawback is the reliance upon the prior knowledge of the selected genome for such investigations. The caveat is, however, that the investigator who employs this approach needs also to devise additional experiments in order to confirm that genes identified with the microarray are indeed *physiologically relevant* targets.

5.1.2 Identification of downstream transcriptional targets of the BRCA1 tumor-suppressor gene

The breast and ovarian cancer susceptibility gene BRCA1 is probably the most studied gene in the breast cancer field because of its clinical significance and multiple functions. BRCA1 was shown to be mutated in the germ line of women with a genetic predisposition to either breast or ovarian cancer Mikki et al (1994). Most mutations identified reported have resulted in the premature truncation of the BRCA1 protein. BRCA1 is known to encode a 1863 amino acid phosphoprotein that is predominantly localized to the nucleus, presumably with a unique function. Protein sequence analysis identified a C-terminal BRCT motif, which was then postulated to play a role in cell cycle checkpoint control in response to DNA damage Koonin EV, Altschul and Bork (1996). Consistent with this postulated role, BRCA1 becomes hyperphosphorylated in response to various agents that damage DNA such as γ/X--ray-irradiation, an effect that was reported to be partially mediated by chk2 kinases (Lee et al. 2000). Furthermore, BRCA1 has been shown to be implicated in at least three functional pathways:

- Mediating the cellular response to DNA damage,
- Acting as a cell cycle checkpoint protein, and
- Functioning in the regulation of transcription.

However, the physiological significance of such BRCA1 actions as well as their relationships with the function of BRCA1 as a tumor-suppressor gene still remain to be defined. Further details are presented next.

The BRCA1-BARD1 ubiquitin ligase

As shown above the BRCA1 gene encodes a 1863-amino-acid protein (Miki et al 1994) which consists of a RING-finger domain in its terminal N-region, a region that includes a nuclear

localization signal and a domain that binds to many cellular proteins, and tandem BRCT domains in its C-terminal region. BRCA1 is associated with a diverse range of biological processes, such as DNA repair, cell cycle control, transcriptional regulation, apoptosis and centrosome duplication. Thus, a specific role has already been postulated for BRCA1 in transcriptional regulation. The C-terminal domain of BRCA1 was reported to contain a potent transactivation domain when this was fused to a heterologous DNA binding motif (Monteiro, August and Hanafusa, 1996). The oligonucleotide array-based expression profiling described above in Section 2.2 was employed by Haber (2000) in collaboration with Affymetrix Co. to identify the downstream transcriptional targets of the BRCA1 tumor-suppressor gene in order to define its function (Harkin et al 1999). A known biochemical function of BRCA1 is its E3 ubiquitin ligase activity. The following reported observations provide only indirect, additional clues to the tumor-suppressor gene function of BRCA1. Germ line mutations of BRCA1 were reported for half of breast-ovarian cancer pedigrees and for approximately 10% of women with early onset of breast cancer, uncorrelated with their family history (Fitzgerald et al 1996). It was also shown in other studies that somatic inactivation of BRCA1 is rare in sporadic breast cancers (Futreal P, Liu Q, Shattuck-Eidens D et al., 1994) and mutations were reported for approximately 10% of sporadic ovarian cancers, therefore suggesting potentially distinct genetic mechanisms for sporadic, breast and ovarian cancers (Berchuk et al 1998). The reduced BRCA1 protein expression reported for the majority of sporadic breast cancers indicates that *epigenetic mechanisms* such as those described in **Section 6** was suggested to play a significant role in regulating the BRCA1 expression (Wilson et al 1999). Furthermore, a defect was reported in the transcription-coupled repair of oxidative-induced DNA damage in mouse embryo fibroblasts with attenuated BRCA1 function (Gowen et al 1998); this observation would suggest that BRCA1 plays a more general role in mediating the cellular response to DNA damage. Thus, BRCA1 has also been reported to be involved in cell cycle checkpoint control, by becoming hyperphosphorylated during late G_1 and S cell phases, and then changing to transiently dephosphorylated early after the M phase (Ruffner and Verma, 1997). Moreover, the BRCA1 overexpression has been reported to induce a G_1/S arrest in human colon cancer cells (Somasundaram et al, 1997). By comparison with the cancer regulation model in **Figure 2**, it seems very significant for oncogenesis that BRCA1 is *physically associated* with the transcriptional regulators *p*53 (Ouichi et al 1998), CtIP (Yu et al 1998), c-Myc (Wang et al 1998), as well as the histone deacetylases HDAC1 and HDAC2 (Yarden and Brody 1999). The physical association of BRCA1 with c-Myc acquires special significance as c-Myc seems to be involved in controlling telomerase activity, whereas p53 is involved in DNA-repair, cell-cycling and apoptosis. Therefore, in the simplified model presented in Figure 3, one should add the BRCA1 links to both p53 and c-Myc in order to facilitate an understanding of the BRCA1 possible roles in oncogenesis.

5.1.3 Selecting gene expression systems

There are several related problems in studying gene function by expression profiling. For example, it has been often reported to be difficult to generate cell lines that overexpress genes such as BRCA1, or p53, because their forced overexpression can lead either to growth suppression or apoptosis (as shown for example in Figure 3, and at the end of the previous section). However, in the case of BRCA1, it was reported that the *tet-off* inducible expression system (Gossen and Bujard 1992) can be utilized to generate cell lines with highly regulated inducible expression of BRCA1 (Harkin et al, 1999). This inducible

expression system introduces into the cells a chimeric transactivator; the latter consists in the *tet* repressor fused to the VP16 transactivation domain. This chimeric transactivator is inactive in the presence of tetracycline, whereas in the absence of tetracycline it can bind to promoters that contain the *tet* operator sequence; the latter sequence is then utilized to drive the expression of BRCA1. This expression system has a major advantage in that it allows the change in just one parameter involved in the induction of BRCA1. The BRCA1 induction in one population is the only difference between the genetic backgrounds of the two populations that are being compared by oligonucleotides arrays. A number of BRCA1 transcriptional targets can thus be identified with Affymetrix oligonucleotides arrays, and among these, the stress and DNA damage-inducible gene *GADD45* was the gene that exhibited the greatest degree of differential signal intensity (Harkin et al, 1999). The specific target genes thus identified were also verified by Northern blot or quantitative reverse transcriptase-PCR analysis in order to confirm induction in response to the stimulus, that is, the induction of BRCA1 (Harkin et al, 1999). Total RNA was extracted from cells in which the exogenous BRCA1 was either switched off (+ *tet*) or switched on (– *tet*). Fluorescent images were generated using the Affymetrix human cancer G110 array containing approximately 1,700 genes that were previously reported to be implicated in cancer; such fluorescent images were then scanned and analyzed. Two lanes were present in such images that corresponded to individual arrays hybridized with biotinylated cRNA probes and were generated from cells in which exogenous BRCA1 was either induced (+ *tet*) or repressed (- *tet*). Each gene on the array was represented by 16 probe pairs, one being wild-type and one containing a mismatch at the central nucleotide. In such fluorescent images, two genes, GADD45 and ATF3 were identified (and confirmed by Northern blot analysis) as being the *transcriptional targets of the BRCA1 tumor-suppressor gene*. Furthermore, in this BRCA1 study, the induction of GADD45 by BRCA1 was reported to be correlated with the BRCA1-mediated activation of the c-jun *N*-terminal kinase/stress-activated protein kinase JNK/SAPK pathway. Significantly, the activation of JNK/SAPK was then shown to be required for the BRCA1-mediated apoptotic cell death in this cell line system. This finding suggests an interesting model for the BRCA1-mediated apoptosis, as presented in some detail by Harkin et al (1999). Most significantly, the experimental approach reported by Harkin et al (1999) was indeed able to defin*e physiologically relevant* target genes. In another recent report, Yu et al (2001) utilized a modified version of the *tet-off* inducible expression system to define the downstream transcriptional targets of the *p*53 tumor--suppressor gene (Yu et al 1999). A total of 34 genes were identified that exhibited at least a 10-fold upregulation in response to the inducible expression of *p*53. Somewhat surprisingly, there was a marked heterogeneity of the response when it was evaluated in different cell lines derived from the same tissue of origin. Among the 33 genes studied only nine were found to be induced in a panel of five unrelated colorectal cell lines, and 17 were induced in a subset; eight were not induced at all in any of the five cell lines examined. This can be interpreted as being due to a high degree of cell type specificity. Furthermore, *p*53 was not absolutely required for induction -- for the majority of the genes identified-- in response to either adriamycin or 5-FU. Therefore, these agents do not seem to act exclusively through *p*53, suggesting that there is inherent redundancy in the majority of signaling pathways. Such inherent redundancy in signaling pathways of cancer, and untransformed, cells might be important in understanding the results of clinical trials in cancer treatment with signal transduction modulators that will be discussed in the next subsection **(5.2).**

5.2 Clinical trials with signal transduction inhibitors -- novel anticancer drugs active in chemo-resistant tumors

Recently, there is an increasing number of reports suggesting that human cancers frequently involve pathogenic mechanisms which give rise to numerous alterations in signal transduction pathways. Therefore, novel therapeutic agents that target specific signal transduction molecules or signaling pathways altered in cancer are currently undergoing clinical trials often with remarkable results in cancer treatments of patients in which chemo- and/or radio- therapy resistant tumors have become apparent. For example, several new classes of such anti-cancer drugs are:

- tyrosine/threonine kinase inhibitors, including: STI-571
  ('Gleevec', or Imatinib Mesylate), ZD-1839 ('Iressa'), OSI-774, and flavopiridol, which are ATP-site antagonists and have recently completed phase I and phase II trials (see for example, Liu et al, 1999);
- several other kinase antagonists that are currently undergoing clinical evaluations, including UCN-01 and PD184352;
- other strategies for downmodulating kinase-driven signaling include 17-allyl- amino-17 demethoxygeldanamycin and rapamycin derivatives. Phospholipase-directed signaling may also be modulated by alkylphospholipids.
- Farnesyltransferase inhibitors, originally developed as inhibitors of *ras*-driven signals, may attain activity by affecting other/or additional targets (see for example, Zujewski, Horak, Bol, et al., 2000; End, Smets, Todd, et al., 2001).
- monoclonal antibodies Herceptin and C225.

Signal transduction is an efficient method for fine-tuning the development and modeling of cancer treatments (Ideker at al., 2001, 2002). There is also a detailed NCI report on clinical trial and signal transduction modulators as novel anticancer (Sausville, Elsayed, Monga and Kim, 2003).

5.3 Interactome-transcriptome analysis and differential gene expression in cancer

It has been claimed that high-throughput yeast-two-hybrid (HT-Y2H) methods will allow a systematic approach to functional genomics, by placing individual genes in the global context of cellular functions (Mendelsohn and Brent, 1999). One finds that high-throughput screening methods such as HT-Y2H have indeed allowed the mapping of the first interactomes for three eukaryotes (Giot et al 2003; Li et al 2004; Uetz et al 2000). Because of the human interactome's much larger size and its very high-degree of complexity there will be quite high costs and labor involved in obtaining the data necessary, for example, for an HT-Y2H mapping of a complete human cell interactome. Furthermore, the complete data analysis together with the assembly of the complete interactome network is likely to require both conceptual and computational advances, in addition to a significant amount of time and collective effort(s) by one or several research teams. In view of the high, potential importance of the human interactome for cancer therapy, and also for improved diagnosis and 'rational' clinical trials, such an effort should now be a top priority. Such an effort should also be coordinated with an improved mapping of the complete yeast interactome as a model, or test, system. Meantime, there have been since 2005 a few reports of 'surrogate', or partial, human cancer cell interactomes in the form of predicted maps of human protein interaction networks based on partial data and comparative analysis. Such studies emphasize even further the need and urgency for the complete mapping of several human

cancer cell interactomes. Following the seminal studies of DeRisi et al (1996) that utilized cDNA microarray to analize gene expression patterns in human cancer, there have been relatively few attempts at deriving hypothetical gene expression patterns in human cancer. The first claim of such an attempt was recently made by Wachi, Yoneda and Wu (2005) for genes that were differentially expressed in squamous cell lung cancer tissues from five patients who had undergone surgical removal of the tumor(s). cRNA samples were prepared and hybridized to arrays obtained from Affymetrix® (Hg-U133A™.). These authors were able to carry out paired *t*-test analyses for each *individual* patient in order to distinguish the genes in which expression levels in their squamous lung cancer cells differed from the paired normal lung tissue (control samples) obtained from the same five individuals. The authors' prediction methodology will be briefly discussed in the next subsection as some of the details are relevant for the evaluation of these results which were the first to be reported for the (hypothetical) interactome—transcriptome analysis of human cancer cell data for a group of five patients with the same diagnosed form of (lung) cancer, and with the same treatment (tumor removal by surgery). The hypothetical human protein interaction maps are a relatively new endeavor (Brown and Jurisica, 2005; Lehner and Fraser, 2004) perhaps because they are likely to have many false positives, as well as miss a significant fraction of the relevant/real protein-protein interactions. Currently, microarray analysis still suffers inherently from relatively high noise levels and the accompanying information loss (buried in noise); although this inherent noise problem is partially eliminated through multiple replicate analyses, the number of replicates is often limited by the availability and the material cost. Another significant problem of such microarray projects is the huge amount of data that needs to be processed in order to obtain useable information (Claverie, 1999).

5.3.1 Analysis of human protein-protein interactions (HPPI) and integration of array data into a predicted protein-protein interaction network (PPIN)

Wachi, Yoneda and Wu (2005; WYU05) employed for their human cell data analysis a web-presented database (OPHID, April 25, 2005) of predicted interactions between human proteins (Brown and Jurisica, 2005) based on data for human and other four organisms which included the intensely-studied yeast and fruit fly. (OPHID is freely available to academic users at http://ophid.utoronto.ca). This protein interaction database listed 16,034 known human protein interactions obtained from various public protein interaction databases, as well as 23,889 additional, predicted interactions which are evaluated using protein domains, gene co-expression and Gene Ontology terms. The results can be visualized in OPHID using a customized, graph visualization program. The data comprises literature-derived human PPI from BIND, HPRD and MINT, "with predictions made from *Saccharomyces cerevisiae*, *Caenorhabditis elegans*, *Drosophila melanogaster* and *Mus musculus*". The genes in the WYU05 array were matched to those in OPHID using gene symbols and protein sequences. In this manner, 2137 genes in the WYU05 microarray experiments were 'matched to the protein network from OPHID'. These predictions should, however, be thought of only as 'hypotheses' until they are experimentally validated. On the other hand, there is increasing evidence that at least certain PPIs may be conserved through evolution (Pagel et al 2004; Wuchty et al 2003). Recently, Sharan et al (2005) claimed that about 50% of the protein-protein interactions predicted by using *interologs* between microorganisms are also experimentally validated. The interologs approach might play therefore a role in the

partial validation of the HT-Y2H protein network mapping without, however, necessarily achieving the claimed, global validation of the predicted (hypothetical) interactome. Differentially expressed genes (DEGs) from squamous cell carcinomas (SCCs) were then identified as discussed above and their connectivity in the network graph was examined to determine their 'topological' properties, such as the edge distribution for DEGs in comparison with the surrounding graph subnetwork.

5.3.2 Differentially expressed genes –DEG- results for SCC of human lung

The genes that are upregulated in SCC were found to exhibit a positive correlation (Pearson's r-coefficient of 0.82) with the number of edges associated with them (Fig. 1a of Wachi, Yoneda and Wu 2005), which was interpreted as indicating that DEGs that are upregulated in SCC are also highly connected. However, the downregulated genes were reported also to have a positive correlation (r = 0.75) to connectivity, albeit slightly lower (Fig. 1b of Wachi, Yoneda and Wu, 2005). On the other hand, microarray probesets that matched the genes in the protein network (n =-2,137) had a negligible correlation coefficient (r =0.06) to link number, proving that the genes on the test microarrays did not contribute to bias in the number of links for DEGs in SCC.

A k-core analysis of DEGs in SCC of the human lung was also carried out *(loc. cit.)* which were reported to measure "*how close are the DEGs to the topological 'center' of the human PPI network*". Based on the k-core analysis, it was concluded that: "*the upregulated genes are more centrally located in the protein network than the down-regulated genes*". If duplicated and validated, such studies would be important as the 'topological centrality' of the genes in the interactome was previously reported to be associated with the *essential* functions of the genes in the yeast (Jeong et al 2001). Such essential genes, are lethal when mutated, and also tend to have high connectivity. Moreover, other genes that are not essential in this sense, but provide a vital function in toxin metabolism were reported to have a high number of edges associated with the nodes, and to be less well connected than the essential genes in yeast (Said et al 2004). Furthermore, a *k*-core analysis has also been performed on the yeast essential genes and they were reported to be global hubs, whereas the non-essential genes were not hubs (Wuchty and Almaas, 2005). It was also claimed that these essential, global hubs are conserved throughout different species; however, one notes that, thus far, there is insufficient data and evidence to prove this claim, or hypothesis. Nevertheless, one may consider as a 'working hypothesis' that "*there should be a core set of genes that needs to be maintained throughout the course of somatic evolution in the tumor microenvironment*" (Wachi, Yoneda and Wu, 2005). This hypothesis is thus consistent with the *somatic evolution model* of cancer. Such conserved genes might be the 'essential genes' in cancer cells, and they may also have somewhat analogous to the global hub, essential genes reported in yeast (Wuchty and Almaas, 2005). DEGs would thus be essential for the survival and proliferation of cancer cells in SSC of the human lung, and the upregulated genes would be centrally located in the protein network as well as have higher connectivity, perhaps suggesting their possible essential role(s) in human (SSC) lung cancer. As this is the first report of a predicted/ hypothetical human cancer interactome network one should definitely consider 'replicating' the reported studies and also evaluating such potentially important findings in the context of a complete human cancer interactome (differential) analysis. This possibility that DEGs might be essential for the survival and proliferation of cancer cells in SSC of the human lung has much too important consequences to be ignored; therefore, it must be thoroughly

investigated and also tested with sufficiently extensive, translational genomics and transcriptional databases that do not seem to be currently available (Han et al. 2006). **Further supporting analyses for this conjecture made by** Wachi, Yoneda and Wu (2005) are considered in the next section.

5.4 Cancer proteins and the global topology of the human interactome network

A recent and extensive study of both cancer and non-cancer proteins (Jonsson and Bates, 2006) was integrated into a validated protein-protein interaction (PPI) network, or interactome, of human proteins. In their report, the connectivity properties were investigated for all proteins previously shown to be modified as a result of mutations leading to cancer (Furteal, et al 2004). A global protein-protein interaction network was then constructed by a homology--based method which is claimed to accurately predict protein-protein interactions. It was then suggested that human proteins that are involved in cancer, or 'cancer proteins', exhibit a network topology which is substantially different from that of other proteins which are considered not to be involved in cancer. Notably, increased connectivity was pointed out for cancer proteins involved in the following subnetworks: cell growth and apotosis-related, signal transduction (MAPK, TGF-beta, insulin, T-cell and B-cell receptor, adipocytokine, cytokine-cytokine interaction), cell motility/cytoskeleton, cell communication, adherence junction, focal adhesion, leukocyte migration, antigen processing and folding/sorting/degradation. Furthermore, it was proposed that such observations '*indicate an underlying evolutionary pressure to which cancer genes, as genes of central importance, are subjected.*' Linking these claims with previous proposals by Wuchty and Almaas (2005) that globally central proteins form an *evolutionary backbone* of the proteome and are *essential* to the organism, (and also with the conjecture made by Wachi, Yoneda and Wu, 2005, discussed here in Section 5.3.), Jonsson and Bates (2006) suggested that cancer proteins may generally be older than the non-cancer ones in evolutionary age. Furthermore, they also suggested that the somatically mutated cancer proteins may be of somewhat younger evolutionary average age in comparison with those from the germline, as a consequence of the evolutionary selection pressure postulated to affect germline mutated proteins. Note also that the previous study of (SCC) human lung cancer by Wachi, Yoneda and Wu (2005) also reported increased interaction connectivity in differentially expressed proteins in human lung cancer tissues.

6. Epigenomics in mammalian cells and multi-cellular organisms

6.1 Epigenetic controls

Upon completion of the US Human Genome Mapping Project and related studies, it became increasingly evident that a sequence of 30,000 or so 'active' genes that encode and direct the biosynthesis of specific proteins could not possibly exhaust the control mechanisms present in either normal or abnormal cells (such as, for example, cancer cells). This is even more obvious in the case of developing embryos or regenerating organs. Subsequently, more than 120,000 genes were suggested to be active in the human genome (*Nature, 2004*). Furthermore, specific control mechanisms of cellular phenotypes and processes were recently proposed that involve *epigenetic* controls, such as the specific acetylation **<=>** de-acetylation reactions of DNA-bound histones (for an overview article on epigenomics see, for example, *Scientific American* 2003, December issue). Such controls intervene from outside the genome but ultimately they also affect gene expression. Therefore, gene profiling

techniques would need to be combined with epigenomic tools and analysis in order to gain an improved understanding of functional genomics and interactomics. Epigenomic tools and novel techniques begin to address the complex and varied needs of epigenetic studies, as well as their applications to controlling cell division and growth. Such tools are, therefore, potentially very important in medical areas such as cancer research and therapy, as well as for improving 'domestic' animal phenotypes *without* involving genomic modifications of the organism. This raises the interesting question if 'epigenomically controlled-growth organisms' (ECGOs) -- to be produced in the future-- would be still argued against by the same group of people who currently objects to GMOs, even though genetic modifications would be neither present nor traceable in such ECGO organisms?'

6.2 Novel tools in epigenomics: rapid and ultra-sensitive analyses of nucleic acid – protein interactions

Several novel techniques could also be applied for the highly-selective detection of epigenomic changes in mammalian cells related to diseases such as individual types of cancer (Jones and Laird, 1999; Plass, 2002) and Alzheimer disease. Such novel tools are likely to be utilized in a wide range of applications in biotechnology research related to Post-Genomics and Epigenomics. Tumor suppressor genes are transcriptionally silenced by *promoter hypermethylation* that also appears to lead to alterations in chromatin structure- a possible mechanism for such repression of the suppressor genes. In contrast to the genetic mutation or deletion mechanism of tumor suppressor gene inactivation, epigenetic inactivation of tumor suppressor genes would occur *via* methylation of specific DNA regions that could be prevented by DNA methyl-transferase or histone deacetylase inhibitors. Aberrant CpG--island methylation has non-random/tumor-type-specific patterns (Costello et al 2000). Such patterns can be identified by employing methylation--specific PCR (MS-PCR; Herman et al 1996), and can also be employed either for tumor class prediction by microarray-based DNA methylation analysis (Adorjan et al 2002) or for high-throughput microarray-based detection and analysis of methylated CpG islands (Yan et al 2002). Hypermethylation profiling is important for both accurate diagnosis and the development of optimal strategies in cancer therapy. Gene promoter hypermethylation has been reported in both tumors and serum of patients diagnosed with several types of cancer: head and neck cancers (Sanchez-Caspedes et al. 2000), nasopharyngeal carcinoma (Wong et al. 2002), non-small cell lung cancer (Belinsky et al 1998; An et al 2002), gastric carcinoma (Lee et al 2002), liver, prostate, bladder and colorectal cancers (Wong et al 1999; Jeronimo et al 2002). Substantial efforts are being made recently for the development of new methods and tools that are capable of sensitive and quantitative DNA methylation analysis, as well as early and accurate diagnosis of cancer. Among such tools are: Fluorescent methylation--specific polymerase chain reaction assay (FMS-PCR; Goessl et al 2000), SNIRF (Mahmood and Weissleder, 2003), indocyanine green-labeling (IGL) for human breast carcinomas (Ntziachristos et al 2000), ConLight-MSP (Rand et al 2002), COBRA (Xlong and Laird, 2002), Methylation-Sensitive *Single Nucleotide* Primer Extension (Ms-SnuPE; Gonzalgo and Jones, 1997), DNA microarray sensitive detection by Metal-Enhanced Fluorescence (MASD/MEF; Lakowicz, 2001; Malicka et al 2003 a, b)), and NIR Fluorescence Micro-Spectroscopy (NIRFMS), single cancer cell detection (Baianu et al 2004a). Specific molecular markers of cancer (Sidransky, 2002) hold the promise to identify those molecular signatures that are *unique* to specific types of cancer, and are essential for the *early accurate diagnosis* and treatment of

cancer. Such novel molecular tools and methodologies could be employed to rapidly and accurately identify molecular signatures of cancer and aging-related diseases in mammalian cells in culture in order to determine how specific epigenomic mechanisms involved in the control of cell division and apoptosis operate throughout the cell cycle. Among the specific epigenomic control mechanisms that one could investigate with such new tools are: CpG-island methylation, p15 (INK4b) and p16 (INK4a) hyper-methylation (in synchronous hepatic carcinoma cells), GSTP1 methylation in non-neoplastic/ synchronous cells, as well as histone-deacetylation and its effects on histone- nucleic acid interactions in stable synchronous cell populations in culture. Both cancer and aging were reported to involve DNA methylation of specific genome regions (van Helden & van Helden, 1989; Ahuja et al 1998). Gene expression profiling and epigenomic testing could be carried out with both ultra-sensitive, novel human and mouse microarrays. Powerful spectroscopic and microspectrosopic techniques can be then employed for the analysis and further improvement of such tools for the investigation of nucleic acid--protein interactions.

- High-field 2D NMR of protein--protein and protein--nucleic acid interactions
- NIR Chemical Imaging of protein clusters in cells and single cancer cells in tissue; NIR-FMS; SNIRF
- MEF and FCS/FCCS/ FRET detection of single molecules amplified-ELISA; NASBA
- Ms-SnuPE; FMS-PCR; Lux ™ Fluorogenic Primers*/ RT-PCR*,
- MyArray ™ DNA- Human*, GeneFilters ᴿ Human Regular Arrays**.
- Specific Knock-out or silencing shRNAi's (SuperArrayTM).

**The testing of these new tools can be carried out for example with stable and synchronous mammalian (human HeLa and mouse) cells in culture.

Table 1. Techniques under Development and Related Applications that are commercially supported *,**.

7. Conclusions and discussion

Novel translational oncogenomics research is rapidly expanding with a view to the application of new technologies, findings and computational models in both pharmaceutical and clinical areas. Sample analyses in recent clinical studies have shown that gene expression data can be employed to distinguish between tumor types as well as to predict outcomes. Important, potential applications of such results are *individualized* human cancer therapy (Pharmacogenomics) and 'personalized medicine'. There is clearly a need for individualized cancer therapy strategies based on high-throughput microarray information recorded for isolated tumor cell lines from stage I through stage III cancer patients. Studies of Differential Gene Expression in human cancer cell lines are clearly required for developing new strategies for efficient cancer therapies for patients whose tumors have developed resistance to existing therapies. Such gene profiling expression, proteomic, interactomic and tissue array data is essential for improving the survival rate of stage III cancer patients undergoing clinical trials with novel signaling pathway inhibitors/ blocker medicines, such as those discussed in some detail in **Section 5**. Several technologies aimed

at future applications in oncogenesis are currently under development both in the direction of improved detection sensitivity and increased time resolution of cellular events, with the limits of single molecule detection and picosecond time resolution already being reached (Sections 2.5, 2.6 and 6.2). The urgency for funding and carrying out the complete mapping of a human cancer interactome with the help of such novel, high-efficiency / low-cost and ultra-sensitive techniques is pointed out for the first time in the context of recent findings by translational oncogenomics and human cancer interactome predictions.

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9. References

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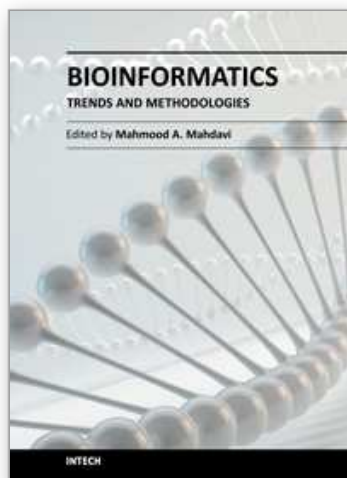
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