1. Introduction

Rapid advances in the genome sequencing of human and dozens of other species resulted in accumulation of extensive DNA sequence information (Gregory et al., 2002; McPherson et al., 2001). While it represented an unparalleled achievement in biology, the DNA sequences by themselves are not particularly functionally informative. In order to understand how the genome sequence helps to shape a functioning organism, more works have to be done beyond sequencing. To characterize the precise function of each gene in the genome is one of the most important tasks in the so-called post-genomic era. The traditional methods that are performed on individual genes separately can hardly meet the needs of such large-scale studies. Hence, there is a growing demand for high-throughput technological solutions in the field of functional genomics.

In order to investigate the gene function on a global scale, a whole range of novel experimental strategies have been developed in the last decade. Among them, microarray technology is one of the most widely applied methodological approaches and has revolutionized molecular biology. The technology uses hundreds to millions of probes fixed on a solid support to interrogate the targets consisting of multiple RNA or DNA molecules within individual samples. Emerged as a new technique for large-scale DNA mapping (Poustka et al., 1986) and sequencing (Cantor et al., 1992), microarray technology has been successfully applied to measure the transcription levels of thousands of genes in parallel (Lockhart et al., 1996; Schena et al., 1995). Since then, the concept of miniaturization by high-density arraying has been expanded into many areas, ranging from SNP genotyping to characterization of DNA copy number changes. In addition to DNA arrays, protein arrays, carbohydrate arrays, tissue arrays and small-molecule arrays were also developed. Likewise, protein arrays were being used for probing protein abundance, protein modifications, and protein-protein, protein-DNA or protein-small molecule interactions (Huang et al., 2004; Stoll et al., 2005; Walter et al., 2000). The combination of computational tools and the large data sets gained from these microarray-based mRNA and/or protein profiling studies provides the opportunity to understand not only the function of each gene in isolation but the complexity of functional networks and control systems.

Most of the data produced by DNA and protein microarrays, however, are still being generated in vitro, although in vivo data has always been preferred since they can elucidate the function of genes under their native cellular environment.

Currently, in vivo analysis of gene function is usually accomplished on single gene scale using cell-based assays. It is usually done by delivering the DNA constructs or RNA molecules into cells that can lead to the overproduction or inhibition of a gene product. Then, the consequent effects on cellular physiology are measured and the function of the gene product is inferred from these gain- and loss-of-function assays. The platforms for this cell-based functional study are typically constructed on petri dish or multi-well (6-, 12-, 24-, or 96-well) plates. Although in some cases it has ever been up-scaled to 384-well plate to achieve higher throughput, there are still some limitations of the micro-well plate platform in characterizing the huge number of targets revealed by other genomic, proteomic or bioinformatic surveys (see following sections).

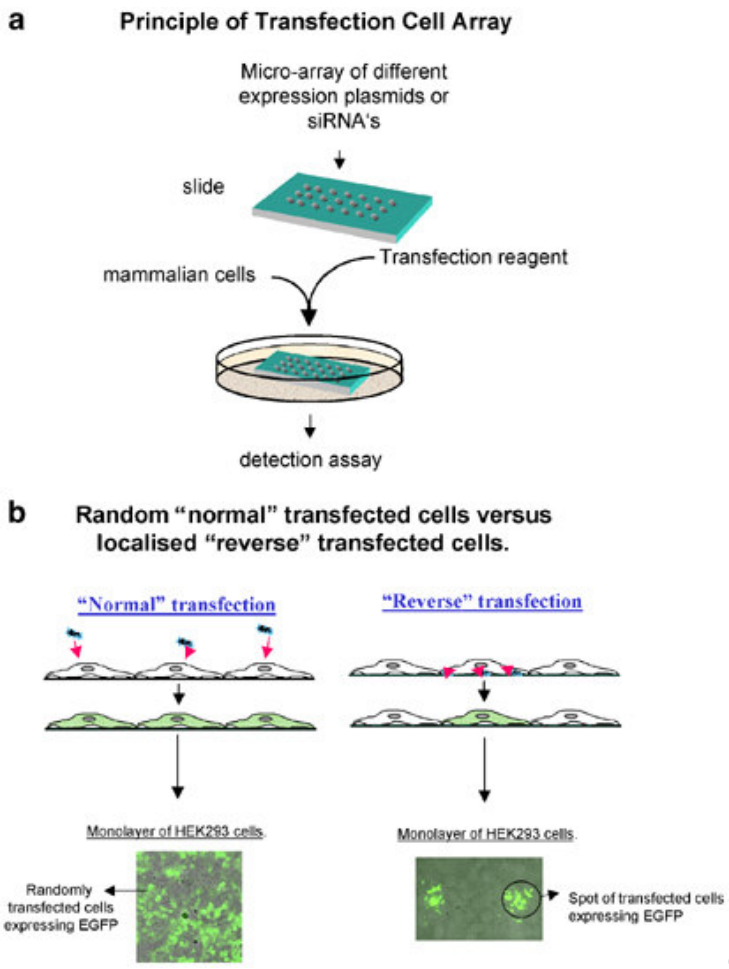
1.1. Transfected Cell Array—a novel technology for high-throughput functional analyses in living cells

1.1.1. Concept of transfected cell array

The success of DNA and protein microarrays motivated researchers to miniaturize the cell-based functional study in a high-density arraying format. Developed by Ziauddin and Sabatini, Transfected Cell Array (TCA) represents a robust alternative for high-throughput analysis of gene function in mammalian cells (Ziauddin and Sabatini, 2001). The principle of the TCA technique is based on the transfection of a set of DNA or RNA molecules that are immobilized on a solid surface in a microarray format into mammalian cells. Only the cells growing on top of the spotted nucleotides become transfected, creating spots of localized transfection within a lawn of non-transfected cells. Using appropriate cell-based assays, the phenotypic effects of the transfection of up to thousands of different molecules can be monitored in parallel. TCA combines DNA or RNA microarray platform with cellular biology techniques

such as cell transfection and detection assays, to achieve high-throughput functional analyses in mammalian cells.

Briefly, the process of a TCA can be divided into three distinct steps: (i) DNA or RNA microarray preparation, (ii) cell reverse transfection and (iii) functional detection assay (Fig.1.1 a. (Vanhecke and Janitz, 2004)).



(Vanhecke and Janitz, 2004)

Fig. 1.1 Principle of transfected cell array technique

TCA technology combines the microarray technique with living cells (a). Reverse transfection allows for multiple, spatially separated transfections of nucleic acids in a single experiment (b).

Microarray preparation In the preparation of microarrays for TCA, expression vectors containing the opening reading frame of protein-coding genes can be printed on commercial glass slides, as described in its original protocol (Ziauddin and Sabatini, 2001). Recently, DNA plasmids containing the sequence coding for short hairpin RNAs (shRNAs) or simply the chemically synthesized small interfering RNAs (siRNAs) have also been successfully arrayed for high-throughput transfection

and gene knock-down in mammalian cells (Kumar et al., 2003; Mousses et al., 2003). For DNA or RNA spotting on cell arrays, the same robotic arrayers for traditional DNA microarrays can be used. Design of cell arrays does not differ from the classical DNA or protein microarray, and the same robotic arrayer software can be used to arrange the spots throughout the slide. Preparation of DNA samples for cell arrays differs, however, from the sample preparation for traditional DNA or protein microarrays, where spotted probes are used for hybridization. In cell arrays, the expression vectors and/or RNAs cannot be immobilized permanently on the solid surface since they have to be subsequently transfected into the cells growing on top of the array. Therefore, DNA/RNA samples are prepared in gelatin solution to equilibrate the adherence of nucleotides on the glass slide before transfection and the release of molecules into the cells during transfection.

Reverse transfection In the second step of TCA experiments, adherent cells are added on top of arrayed slide and form a cell monolayer that covers the spotted nucleic acids. This step is dubbed by Ziauddin and Sabatini as "reverse transfection" since cells are transfected by the nucleic acids that are immobilized underneath rather than by the nucleic acids that are added from the top in normal transfection. Along with the release of DNA or RNA from the solid surface, only the cells growing on top of the DNA or RNA spots take the nucleotide molecules up and become transfected. Consequently, in contrast to normal transfection, where transfection events occur throughout the entire cell layer, reverse transfection takes place in spatially separated group of cells and allows for the investigation of multiple targets in single experiment (Fig.1.1 b). The arrayed DNA or RNA spots must be incubated with suitable transfection reagents before the addition of cells on top of the slides. Similar to normal transfection, the ratio of nucleic acids to transfection reagent and the incubation time are also critical for the efficient transfection of cell arrays, and has to be optimized for each cell line. Moreover, in the transfection of cell arrays, more attention should be paid to the amount of cells used. The cells must be sufficient to grow as a monolayer coving every nucleic acid spot throughout the slide. On the other hand, the number of cells should not be too high in order to avoid the cell death before the functional detection.

Functional detection Depending on the purpose of experiments, a variety of assays can be carried out on either living or fixed cells to monitor the transfection effects. Some extreme cell morphological changes such as cell death can be simply monitored

through light microscope. Most of phenotypic effects, however, have to be detected using specific facilities or cell-based bioassays. In principle, the functional assays that are generally carried out on adherent cells can also be applied to TCA. This composes, for example, a number of fluorometric, colorimetric, and luminescent assays, which monitor the apoptosis, cell proliferation and signaling, and individual protein features. Practically, the most common method used for TCA is based on the detection of fluorescence emitted from the fluophors (e.g. Cy3, Cy5 or Alexa Dyes) that are coupled to cellular components by immunoreactions or non-immunologic labeling. Alternatively, autofluorescent reporter such as green fluorescent proteins (GFP) and/or its derivatives, which allows for direct signal detection, can be coupled to target protein using recombinant cloning strategy. For signal acquisition, fluorescent microscope and fluorescence-based scanner are often used in most of cases. Standard microarray-based software can be also used for image analyses including identification and quantification of the signal recorded on cell arrays. Recent advances in automated-microscopy and accompanying image process software allow for high-throughput slide scanning and data analyses of cell arrays, making the genome-wide screening of gain- or loss-of-function phenotypes feasible (Baghdoyan et al., 2004; Liebel et al., 2003).

1.1.2. Aadvantages of cell array technique

Transfected cell array (TCA) offers a robust platform for high-throughput functional studies of genes and proteins. One of the biggest advantages of TCA is the opportunity to perform large-scale study of gene function in the context of living cells. Proteins expressed in living mammalian cells undergo a series of complex processing such as post-translational modification and intracellular translocation. Functional study of human proteins in human cells guarantees the *in vivo* processing networks intact, and may reveal features that cannot be shown in prokaryotes, yeast and other model organisms. Owing to the advances in automated microscopy, transfected cell arrays can be used to monitor live cells in culture, allowing the discovery of transient or time-dependant phenotypes, such as programmed cell death and the intracellular movement of a GFP-tagged protein.

TCA have a number of advantages over the conventional microwell plate-based expression format. One leading advantage of cell arrays is the increased local single-

to-background ratio, which facilitates the identification of the sample signal. Originated from highly organized arrays of DNA constructs or RNA molecules, the sample signals on cell arrays are concentrated in the well-defined small area on top of nucleotide spots surrounded by non-transfected cells (Fig.1.1 b). This allows for the identification of weak signal out of the background, so that the false-negative results are reduced. Another prominent feature of cell array technique is cost-effectiveness. Using the conventional robotic arrayer, it is feasible to achieve densities of up to 10,000 spots per standard microscope glass slide. At these densities, the consumption of DNA/RNA materials as well as transfection and functional detection reagents can be substantially reduced. This makes TCA, at the moment, the most cost-effective tool for genome-wide gene functional analyses (Janitz et al., 2006).

Several groups have created cell arrays with spotted plasmid vectors or linear PCR products designed to express proteins of interest (Conrad et al., 2004; Redmond et al., 2004). In the meantime, a number of groups combined RNAi technology with TCA technique to perform large-scale gene loss-of-function studies (Erfle et al., 2004; Kumar et al., 2003; Mousses et al., 2003; Silva et al., 2004; Yoshikawa et al., 2004). In parallel of the efforts for cell arrays establishment, there is a demand of biological read-outs that can be used for measurement of the phenotypic effects as a result of protein overexpression or RNAi silencing. Among them, protein subcellular localization charaterization and cell apoptosis detection could provide hints to gene functions through their influence on complex cellular processed.

1.2. Protein Subcellular localization in functional genomics

1.2.1. Importance of protein subcellular localization study

The subcellular localization of a protein can provide important information about its function within the cell. As eukaryotic cells, in particular mammalian cells, are characterized by a high degree of compartmentalization, most protein activities can be assigned to particular cellular compartments. For example, DNA-binding transcription factors can only alter transcriptional activity when they are localized to the nucleus, and transmembrane (TM) receptors can only bind their soluble ligands

when they are at least partially exposed to the extracellular environment. Categorization of proteins by subcellular localization is therefore one of the essential goals for functional annotation of the human genome.

The knowledge on protein location within their cellular environments is also critical for understanding the regulatory mechanism by which it is controlled. The accurate functions of protein interaction networks greatly rely on the proper localization of each protein component. A conventional method to identify protein-protein interaction at single cell level is to trace the mutual localization of proteins under physiological environment (Relic et al., 1998; Surapureddi et al., 2000). This approach is very often used to confirm the protein interactions obtained from large-scale screening system such as yeast two-hybrid (Wong and Naumovski, 1997). Another common strategy to study the regulation or interaction networks is to see if the localization of proteins alters by disrupting the networks intentionally. Molecular approach such as partial cloning or site mutation can be used for the introduction of changes into protein sequence in order to destroy its interaction with functional partners, which then reveals the localization mechanism of the given protein (Kawana et al., 2003; Queralt and Igual, 2003). On the other hand, the regulation networks can be disrupted from the "partner-side" by using specific inhibitors to the partner. For example, Zuckerbraun et al. used a pharmacological inhibitor of RhoA (ras homolog gene family, member A) and observed altered localization of extracellular signal-regulated kinases (ERKs), thus determining RhoA as a regulator of ERK through its influence on ERK subcellular localization (Zuckerbraun et al., 2003).

Aberrant translocation of proteins, in many cases, correlates with pathological changes in the cell physiology and account for the clinical manifestation of several genetic diseases. One example is "primary hyperoxaluria," a rare disease, which results in kidney stones already at an early age. This disease is due to the aberrant localization of an enzyme called alanine:glyoxylate aminotransferase. Normally, a targeting signal in the enzyme directs it to the peroxisome, while in diseases, this signal is altered and the protein mislocalizes to the mitochondria where it is unable to perform its normal function (Danpure et al., 1993). A growing list of the diseases caused by improper localization of proteins makes the protein translocation a promising target for the development of therapeutic agents. In recent reports, two small chemical compounds were found to specifically inhibit the translocation of vascular-cell adhesion molecule 1 (VCAM1), a protein that is associated with various

chronic inflammatory conditions, into the endoplasmic reticulum (ER) (Besemer et al., 2005; Garrison et al., 2005). This opens the opportunity to manipulate the translocation of secretory and membrane proteins in a signal sequence-specific manner.

The localization of proteins affects the usefulness of, and the strategies for, using a protein as a diagnostic marker or a target for therapy. Plasma membrane proteins and secreted signaling proteins are candidate targets for monoclonal antibody-based therapies (Brekke and Sandlie, 2003). There are already more than ten Food and Drug Administration (FDA)-approved monoclonal antibody therapeutics and dozens more in clinical development. Some of these, including Trastuzumab (Herceptin), Rituximab (Rituxan), and Cetuximab (Erbitux), target membrane proteins on the surface of malignant cells and are well-established in the treatment of cancer (Diehn et al., 2006). On the other hand, intracellular proteins that are released into the extracellular space during cell injury or death provide the basis for sensitive assays to diagnose specific organ injury (e.g. creatine kinase for the diagnosis of acute myocardial infarction) (Puleo et al., 1994). Thus, large-scale identification of protein localizations that are specific to organs, tissues, or disease has potential value for further development of these therapeutic and diagnostic approaches.

1.2.2. Conventional approaches for localization study and their limitations

A variety of experimental and computational approaches have been developed for identifying proteins subcellular localization.

1.2.2.1. Computational approach

Computational biologists have made large efforts in developing programs to predict protein subcellular localization. Till now, a batch of such software has come out in this field, applying various biological concepts and computational methods. Presently, four leading methods are commonly used: 1) the overall protein amino acid composition. For example, SubLoc predicted the protein localization based on the fact that the proteins with different subcellular localization usually had different amino acid composition (Hua and Sun, 2001); 2) known targeting sequences. One of

the most important principles of protein sorting mechanism is the existence of targeting signal in the amino acid sequence, leading the proteins to different organelles or outside the cell. Hence, several computational approaches are focused on predicting the presence of certain targeting motifs, e.g. signal peptides (SPs), mitochondrial targeting peptide (mTP), nuclear localization signals (NLS), and transmembrane alpha-helices in protein sequences (Bannai et al., 2002; Claros and Vincens, 1996; Emanuelsson, 2002); 3) sequence homology and/or motifs. For example, Proteome Analyst was based on the keywords from the protein database SWISS-PROT and the annotation of homologous proteins (Lu et al., 2004) ; 4) combination of different information obtained from the first 3 categories. For example, PSORTII based its prediction on the overall amino acid composition, N-terminal targeting sequence information and motifs (Horton and Nakai, 1997).

Being able to perform in an automated and high throughput fashion, computational methods appeared appealing for assignment of protein subcellular location. However, no matter what algorithm it used, the computational prediction was always based on the biological knowledge, which is far from complete. The enormous complexity of the protein sorting process, alternative means of transportation pathways, and lack of complete data for every organelle, still limited the application of computational methods.

1.2.2.2. Experimental approach

Commonly employed experimental approaches for protein subcellular classification include the immunohistochemical labeling of endogenous protein, and the construction of fusion proteins with autofluorescent protein tags (e.g. GFP). By either of these approaches, protein localization data have traditionally been accumulated at single protein base. Until recently, following the completion of the genome sequencing of various organisms, large efforts were made to bring the protein localization studies to genome-wide scale. The aim is to understand not only the function of each gene in isolation but also the complexity of the functional networks governing cellular physiology. The first large-scale study was carried out in yeast. Ding et al. expressed 250 proteins in fission yeast and studied their intracellular distribution (Ding et al., 2000). The localization study in yeast cells was proceeded by two other projects with 2,744 (Kumar et al., 2002) and 4,165 yeast proteins (Huh et

al., 2003). The later number has reached 75% of the budding yeast proteome. In mammalian cells, the first large-scale localization study was performed in microwell-plate format by Simpson et al. and comprised 107 human genes (Simpson et al., 2000). In their study, GFP was fused to the cDNA sequence and then transfected into monkey Vero cells for expression. The classification of each protein to particular cellular compartment was based on the empirical determination of subcellular structures through their morphology. Recently, an automated reagent dispensing system has been established to facilitate the transfection and immunostaining in 96-well plates (Liebel et al., 2003). A microwell-plate based approach, however, is characterized by high reagent consumption and the requirement for sophisticated automation equipments. This is especially disadvantageous for multiplied co-localization experiments, which requires a more economic and high-throughput approach to reduce the reagent consumption to absolute minimum.

1.2.3. Organelles colocalization—a requirement in genome-wide protein localization study

Identification of intracellular compartments of a mammalian cell plays a crucial role in the subcellular localization study. In previous large-scale studies, the localization identification of a given protein was only based on its morphology. For example, GFP and its derivatives were used as a fusion tag to visualize a protein distribution pattern, and this pattern is the only resource for examiners to classify the protein to a certain intracellular compartment (Simpson et al., 2000). In this case, localization determination can be quite subjective, and relies mostly on the acquaintance of investigators with subcellular morphologies. For several reasons, the chance of a false classification can be relatively high. First, the morphologies of cellular compartments are associated with cell physiological status, e.g. the stage of cell cycling. Second, extracellular influence such as change of components of culture media, and/or expression of exogenous proteins may also result in the alteration of cellular structure. Third, the morphology of cell organelles varies from cell type to cell type. Finally, the test protein itself may localize at multiple sites or present an atypical distribution pattern that does not resemble any organelle morphologies. A labeling strategy for cellular organelles should be therefore carried out in each experiment to indicate the localization of a given protein. Very often, a batch of organelle-specific

antibodies and/or dyes is used to label each cell compartment with a fluorescence whose spectrum is different from the one used to stain recombinant proteins. This procedure is termed as organelle counterstaining. As a result, the colocalization of a protein with specific cell organelle can be objectively reflected from the merged color due to overlap.

Colocalization experiments, however, are difficult to carry out for a large number of genes using conventional transfection platform such as microwell-plate form. In large-scale analyses, the counterstaining of each protein with all organelle markers is required to determine its unique or multiple localizations. This leads to a multiplication in the number of test samples and a dramatic increase in the cost of experiments.

Based on the high-density of immobilized DNA spots, transfected cell arrays allow the simultaneous counterstaining of all recombinant proteins and cell clusters throughout the slide, making it currently the most efficient technique for high-throughput colocalization screening.

1.3. Cell death and apoptosis

1.3.1. Types of cell death

There are two primary pathways by which cells die: accidental cell death and programmed cell death. The accidental cell death is called necrosis, which occurs when cells received irreversible injury from structural or chemical insult. Examples of such insults include ischemia (lack of oxygen), extreme temperature, and physical trauma. The hallmark of necrosis is that cells die because they are “damaged” and cannot recover.

In contrast, cells that die by programmed cell death commit suicide as a result of activation of an inherited intracellular program. This may occur as a response to developmental or environmental cues, or in response to physiological damage detected by the cell’s internal surveillance networks. Very often, the cells appear completely healthy prior to committing suicide. The ability to undergo programmed cell death is a built-in latent capacity in virtually all cells of multicellular organisms.

This cell death is extremely important in embryonic development, maintenance of tissue homeostasis, and many pathological conditions

For programmed cell death, the most described pathway is apoptosis.

Apoptosis is an orderly process that leads cell death through several morphological phases (Duvall and Wyllie, 1986; Kerr et al., 1972). Coming from the ancient Greek, apoptosis refers to shedding of the petals from flowers or leaves from trees, which describes vividly the particular morphological changes appearing during this type of programmed cell death. Apoptotic death comprises two phases. During the latent phase, the first phase of apoptosis, the cell looks morphologically normal but the death program has been activated. This is followed by the executive phase, which is characterized by a series of dramatic structural and biochemical changes that culminate in the fragmentation of the cell into membrane-enclosed apoptotic bodies. Activities that cause cell to undergo apoptosis are said to be pro-apoptotic, whereas activities that protect cells from apoptosis are said to be anti-apoptotic.

1.3.2. Necrosis versus Apoptosis

Necrosis is a disorderly mode of cell death (Duvall and Wyllie, 1986). Typically, loss of plasma membrane integrity is an early event in necrotic death. This allows water to enter the cells, causing it to swell so that the plasma and organelle membrane burst. As a result, the cell undergoes a process of autodigestion and dissolution, leading to the leak of cytoplasmic contents into the intercellular space. This leakage, in turn, causes local inflammation as phagocytic cells flock to the site, ingest the debris, and become activated.

In contrast to necrosis, rather than cell swelling, apoptosis involves cell shrinkage, where there is a marked condensation of both the nucleus and the cytoplasm. Besides size change, apoptosis is characterized also by a reproducible pattern of structure alteration of both the nucleus and cytoplasm. In order of appearance, apoptosis involves the changes including (1) loss of microvilli and intercellular junctions; (2) shrinkage of the cytoplasm; (3) dramatic changes in cytoplasm motility with activation of a violent program of blebbing; (4) loss of plasma membrane asymmetry, with the distribution of phosphatidylserine being randomized so that it appears also in the outer membrane leaflet; (5) changes in the organization of the cell nucleus, typically involving the hypercondensation of the chromatin and its collapse against

the nuclear periphery; and (6) the "explosive" fragmentation of the cell into membrane-enclosed apoptotic bodies containing remnants of the cell nucleus, mitochondria, and other organelles. Usually, the progress through these morphological changes is quite rapid, occurring with hours.

One of the most important features of apoptosis is that the plasma membranes on the dying cell and the individual apoptotic bodies remain intact throughout the entire process. There is no spillage of cellular contents as cells condense and fragment into apoptotic bodies. In the last stage, apoptotic bodies are rapidly phagocytosed and degraded in the lysosomes of surrounding cells. Apoptosis can thus be considered as the disassembly of the cell into "bite-sited" vesicles with bound membrane. Since the cellular contents are rarely released into the environment and surface markers on apoptotic bodies can suppress the activation of macrophages, apoptotic death does not lead to an inflammatory response.

1.3.3. Importance of apoptosis in human diseases

Apoptosis is observed in all multicellular organisms including both animals and plants. It is essential in many physiological processes, including maturation and effector mechanisms of the immune system (Bidere et al., 2005; Cohen et al., 1992), embryonic development of tissue and organs (Clarke, 1990), development of the nervous system (Batistatou and Greene, 1993; Yuan and Yankner, 2000) and hormone-dependent tissue remodeling (Strange et al., 1992). Inappropriate regulation of apoptosis may play an important role in many pathological conditions like ischemia, stroke, AIDS, autoimmunity, hepatotoxicity and cancer (Bidere et al., 2005; Geng, 2003; Gill et al., 2002; Reeve et al., 2005). In oncology, extensive interest in apoptosis comes from the observation, that this mode of cell death is triggered by a variety of anti-tumor drugs, radiation and hyperthermia, and that the intrinsic propensity of tumor cells to respond by apoptosis is modulated by expression of several oncogenes and may be a prognostic marker for cancer treatment (Hickman, 1992; Lyons and Clarke, 1997). Recently it has been reported that apoptosis is also a key factor in neurodegenerative diseases, such as Huntington's disease and Alzheimer's disease (see review in (Yuan and Yankner, 2000).

Despite the importance of apoptosis in biological researchs, our understanding of the regulation of programmed cell death in health and disease is far from complete. In

functional genomics, large-scale identification of novel cell death regulators is of particular importance in order to understand the complex regulation of cell death as well as to reveal new therapeutic strategies.

1.3.4. Apoptosis analysis on transfected cell arrays

1.3.4.1. Conventional apoptosis detection methods

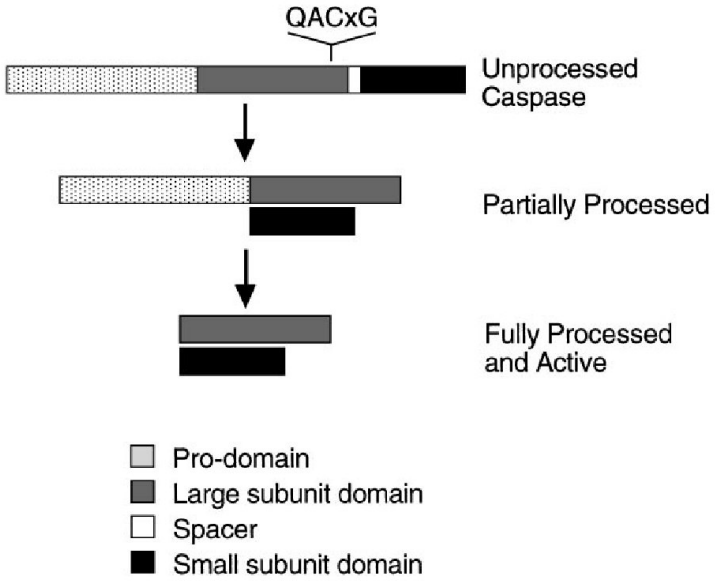
Gross changes in cell morphology and chromatin condensation, which occur during apoptosis, can be detected based on altered light scatter from dying cells. Biochemical assays, however, are required to assess apoptosis at molecular level. Several biological hallmarks of apoptosis have been utilized to develop the detection assays characterized with respective technical advantages and drawbacks.

Caspases activation

Caspases (cysteine-aspartic acid specific proteases) are present as dormant enzymes in healthy cells but become activated in response to different inducers of apoptosis (Martin and Green, 1995). The process of their activation is considered to be the key event of apoptosis and its most specific marker (for reviews, see (Budihardjo et al., 1999; Earnshaw et al., 1999; Shi, 2002)).

The detection of caspase activation therefore provides the most definitive evidence of apoptosis. The detection can be done directly by specific reaction with activated enzyme (e.g., using antibody or FLICA inhibitors) or indirectly by the presence of cleavage product of caspase, e.g. PARP p85, the cleaved unit of poly(ADP-ribose) polymerase (PARP)).

Immunocytochemical detection of activated caspases Caspases are activated by transcatalytic cleavage of their zymogen procaspase molecules into large and small subunits (Fig. 1.2, (Rathmell and Thompson, 1999)). The subunits then assemble to form the heterotetramer consisting of two small and two large subunits, constituting the active caspase (Budihardjo et al., 1999; Earnshaw et al., 1999). Antibodies that are specific to activated caspases, e.g. caspase-3, -7, -8, and -9 are now commercially available. It is possible, therefore, to detect caspase activation by immunocytochemical means.



(Rathmell and Thompson, 1999)

Fig. 1.2 Caspase domains and activation process

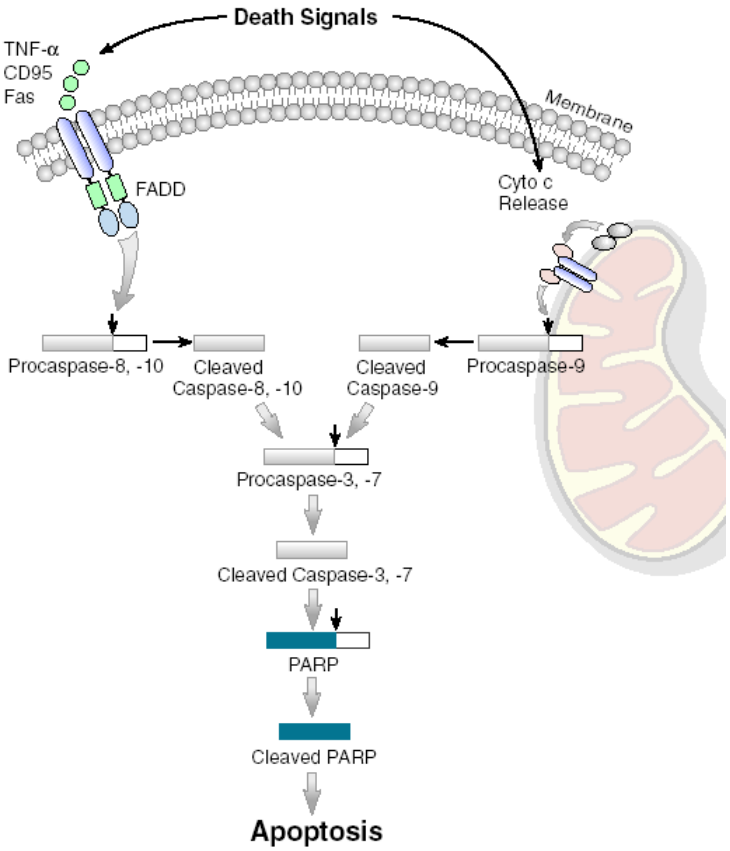
Caspase contain three primary domains that are processed in a two-step mechanism to produce an activated enzyme.

FLICA Fluorochrome-labeled inhibitors of caspases (FLICAs) are a series of reagents designed as affinity labels for the active enzyme center of caspases. Exposure of live cells to FLICAs results in uptake of these reagents by apoptotic cells only (Smolewski et al., 2001). The most potent caspase inhibitor consists of 3 or 4 amino acids, including the activated caspase recognizing aspartate residue, which is linked to fluoro-methyl-ketone (FMK).

Composed with different peptides, FLICAs possess varied specificities to different caspases, for example, Acetyl-Asp-Glu-Val-Asp-FMK (DEVD-FMK) targets predominantly on caspase-3 cleavage, whereas Val-Ala-Asp-FMK (VAD-FMK) reacts nearly with all mammalian caspases. The FMK adduct renders the peptide permeable to live cells, thereby eliminating the need for cell permeabilization. Fluorochrome such as fluoroisothiocyanate (FITC) or sulforhodamine can be conjugated to FLICAs, which facilitates detection of activated caspases in apoptotic cells by fluorescent microscopy or scanner in addition to flow cytometry.

Determination of poly (ADP-ribose) polymerase (PARP) PARP is a nuclear enzyme that is involved in DNA repair and activated in response to DNA damage (Satoh and Lindahl, 1992). PARP is important for cells to maintain their viability. During apoptosis, PARP is cleaved by caspases (primarily by caspase-3) (Kaufmann et al.,

1993; Lazebnik et al., 1994; Tewari et al., 1995) Nicholson DW et al. 1995 Nature) (Fig. 1.3 (Cell Signaling Technology, Inc.)). Cleavage of PARP promotes cell disassembly and serves as a hallmark of the apoptotic type of cell death (Oliver et al., 1998). In human PARP, the cleavage occurs between Asp214 and Gly215, which separates PARP's amino-terminal DNA binding domain (24 kDa) from its carboxy-terminal catalytic domain (85-kDa) (Tewari et al., 1995); Nicholson DW et al. 1995 Nature). The development of antibodies that recognize the cleaved 85-kDa PARP fragment (PARP p85) can be used as immunocytochemical markers of apoptotic cells.



(Cell Signaling Technology, Inc.)

Fig. 1.3 PARP signaling pathway

During apoptosis, PARP is cleaved by caspases (primarily by caspase-3) to form its catalytic 85-kDa fragment (PARP p85).

Annexin V

Phospholipids of the plasma membrane are asymmetrically distributed between the inner and outer leaflets of the membrane. Normally, phosphatidylcholine and sphingomyelin are exposed to the external leaflet of the lipid bilayer, while

phosphatidylserine is located on the inner surface. During apoptosis, this asymmetry is disrupted and phosphatidylserine becomes exposed on the outside surface of the plasma membrane (Fadok et al., 1992; Koopman et al., 1994). Because the anticoagulant protein Annexin V binds with high affinity to phosphatidylserine, fluorochrome-conjugated Annexin V can serve as a marker of apoptotic cells, in particular for their detection by flow cytometry (van Engeland et al., 1998).

DNA fragmentation

Extensive DNA fragmentation is also regarded as a specific marker of apoptosis. Cleavage of genomic DNA during apoptosis results in double-stranded, low molecular weight DNA fragments (mono- and oligo-nucleosomes) as well as single strand breaks ("nicks") in high molecular weight DNA. Based on this phenomenon, a variety of detection methods are developed. Two general approaches have been used: DNA electrophoresis and in situ DNA end-labeling.

Gel electrophoresis Gel electrophoresis of the DNA fragments cleaved intranucleosomally results in a so-called "DNA ladder". In spite of its convenience, this method cannot provide information on apoptosis in individual cells nor relate cellular apoptosis to histological localization or cell differentiation.

TUNEL DNA strand breaks that are generated during apoptosis can be identified through labeling the free 3′-OH termini with modified nucleotides in an enzymatic reaction. Terminal deoxynucleotidyltransferase (TdT) has been commonly used for the incorporation of nucleotides (preferentially dUTP) to the breaks in a template-independent manner. Using labeled dUTP, DNA strand breaks can thereby be labeled with a fluorochrome either directly (e.g. FITC-dUTP) or indirectly (e.g. BrdUTP followed by FITC-conjugated anti-BrdU antibody). This tailing reaction using TdT is described as TUNEL (TdT–mediated dUTP Nick End Labeling). Compared with another *in situ* end-labeling method where free 3'-OH groups are labeled by nick translation (Darzynkiewicz et al., 1992) , the TUNEL method is considered to be more sensitive and faster (Gorczyca et al., 1993). The number of DNA strand breaks in apoptotic cells is so large that the intensity of their labeling in the TUNEL reaction ensures the positive identification and discriminates them from cells that have undergone primary necrosis (Gorczyca et al., 1993; Gorczyca et al., 1993).

Fractional (sub-G1) DNA content using PI/DAPI The fragmented, low-molecular-weight DNA can be extracted from the cells following their fixation in precipitating fixatives such as ethanol. Generally, the extraction occurs during the process of cell staining in aqueous solutions after transfer from the fixative. As a result, apoptotic cells often end up with deficient DNA content, and when stained with a DNA-specific fluorochrome, e.g. propidium iodide (PI) or DAPI, they can be recognized by cytometry as cells having less DNA than G1 cells. On the DNA content frequency histograms, they form a characteristic sub-G1 peak (Hotz et al., 1994; Nicoletti et al., 1991).

1.3.4.2. Application of cell arrays for apoptosis study

Most apoptosis analyses are currently carried out using flow cytometry. Together with different detection assays flow cytometry can be used to: (1) identify and quantify dead or dying cells, (2) reveal the type of cell death (apoptosis or necrosis), and (3) study mechanisms involved in cell death. However, using flow cytometry, the large-scale screening remains a difficult task.

Combined with suitable detection assays, TCA provides an alternative to flow cytometry for apoptosis analysis especially due to its high throughput manner. Fluorescence-based imaging systems based on microscopy and laser scanner have recently been developed for automated analysis on TCA. Fluorescent assays such as immunocytochemical detection of caspases activation and TUNEL appear to be promising for the usage on cell arrays. However, a few considerations have to be taken when establishing apoptosis detection on TCA. First, cell lysis is not possible to be performed on cell arrays. Second, cell arrays are normally subjected to fixation for follow-up detection and long-term storage. As a result, the conventional assays such as those detecting “DNA ladder” and DNA content in (sub-G1) fractional are not applicable for cell arrays. It is possible to carry out the detection reactions such as FLICAs and Annexin V in living cells on TCA; however, the question if the apoptotic signals can be retained after cell fixation has to be addressed.

1.4. Objectives

Transfected cell array (TCA) offers a robust platform for high-throughput functional analyses of genes and proteins in the context of living cells. Along with the maturing of TCA technique, currently, there is a demand for the development of biological read-outs that can be effectively used on the cell arrays. The aim of this study is to establish cell array-based subcellular localization and cell death detection approaches for high-throughput functional annotation of human chromosome 21 (Chr21) proteins.

Functional analysis of Chr21 proteins is of high medical relevance. This refers, in particular, to trisomy of human Chr21, which results in Down's syndrome, a complex developmental and neurodegenerative disease. The phenotype of Down's syndrome includes various organ dysmorphies, stereotypic craniofacial anomalies and brain malformations (Roizen and Patterson, 2003). Molecular analysis of this syndrome, however, poses a particular challenge because the aneuploid region of Chr21 contains many genes of unclear function. Genomic sequencing and expression annotation of human Chr21 (Cawley et al., 2004; Gitton et al., 2002; Hattori et al., 2000; Kampa et al., 2004; Reymond et al., 2002), as well as studies of the transcriptome of Down's syndrome mouse models (Amano et al., 2004; Dauphinot et al., 2005; Kahlem et al., 2004; Lyle et al., 2004; Saran et al., 2003), provided a comprehensive resource for the systematic functional characterization of Chr21 genes. However, functional characterization at the protein level has mainly been performed using protein prediction algorithms. Therefore, the subcellular distribution of Chr21 proteins and their overexpression effects on cell growth and cell death would provide an important insight into their cellular functions.

To achieve unambiguous subcellular localization information, in this study, a large set of organelle counterstains was introduced to cell arrays to reach high-throughput colocalization characterization. In the meantime, it was also aimed to facilitate phenotyping the cellular morphological changes as a consequence of overexpression of Chr21 genes.

To construct efficient cell death detection on cell arrays, a variety of conventional apoptosis assays were examined on cell arrays. Another goal of using different apoptosis assays in parallel was to elucidate the mechanism of cell death induction.

The candidate Chr21 genes that could result in cellular morphological changes were subjected to cell array-based apoptosis detection.

In the last part of this dissertation, the cell death detection platform was also applied to identify the siRNA-induced apoptosis on cell arrays. This strategy has the significance in revealing the genes functioning as apoptosis suppressors.