5. Conclusion

5.1. Cell array-based localization and apoptosis studies reveal novel functional features of Chr21 proteins

In this study, two functional platforms based on cell array technique were established for high-throughput protein co-localization study and cell apoptosis detection. The experimental approaches were applied to human chromosome 21 proteins, with the aim to reveal their novel functional features that could possibly contribute to further understanding of molecular pathologies of the diseases related to this chromosome, such as Down's syndrome.

The results provided comprehensive information on the subcellular distribution of Chr21 proteins and the physiological effects following overexpression of these genes. The utility of small His_6 and Myc epitope tags has been aimed to reduce the possible localization artifacts derived from large fusion tags such as GFP. The localization of 34 Chr21 proteins have been described for the first time in this study, and the data may serve as a reference to study the aberrant protein localization as a result of trisomy 21.

The particular morphological alterations as a result of overexpression of two Chr21 proteins (claudin-14 and –8) were further evaluated using cell array-based cell death assays. The utility of multiple death assays confirmed the cell death-inducing effect of the claudins, and suggested a non-classic apoptosis mechanism. Together with other functional annotations, such as gene expression profiling and *in situ* hybridization, our cell phenotype-based information should contribute to creating the system biology of chromosome 21, as well as to further understanding of the molecular pathology of Down's syndrome.

5.2. Cell array facilitates high-throughput colocalization study

In this study, we established colocalization approach using transfected human cell arrays for a large set of cellular compartments including the nucleus, ER, Golgi apparatus, mitochondrion, lysosome, peroxisome, and the microtubule, intermediate

and actin filament. The application of this “organelle-colocalized celll arrays” facilitated the precise determination of the localizations for numerous recombinant proteins in a single experiment. It also provided a chance to record all possible location sites for every studied protein, which, in some cases, reflected the dynamic protein sorting and translocation behaviors inside the cells, and thus provided more indications about its function.

Furthermore, organelles-colocalization allowed detailed investigation of the cell morphological alterations as a result of the expression of exogenous proteins. Changes in the cellular morphologies (including the organelles phenotypes) between transfeced and non-transfected cells could be easily distinguished. The abnormal physiological phenotypes, together with the protein localization features, might provide important clues to understand the cellular function of proteins as well as their regulation mechanisms.

The usage of organelle-counterstains also opened an opportunity for automated protein localization studies, which may facilitate genome-wide analyses. Microscope-based automatic image acquisition systems were commercially available from a number of companies, and have been established in several research centers (Conrad et al., 2004; Liebel et al., 2003; 2004; Wiemann et al., 2003). Some of the systems are compatible to microscopy glass slides format, thus allowing for cell array-based applications. In the meantime, the advances in software development allowed the signal quantification at single-cell level. How to identify a number of cell organelles, however, still presents a bottleneck in automatic classification of protein localizations. Conrad et al. implemented a machine-learning method to train an automatic classification of subcellular patterns, using several GFP-tagged proteins with known localizations (Conrad et al., 2004). An averaged accuracy of 82% was achieved for the classification of several distinct organelles. However, low accuracies (less than 50%) were obtained for the ER, microtubules and mitochondria. These organelles, even in manual study, are difficult to be distinguished from each other due to their visual similarities. Moreover, the similarities among certain organelles, the biological variations of a same compartment in different cell types, as well as the variations among individual cells derived from the expression of different exogenous proteins, pose the leading challenges for automatic phenotype analysis. These problems can be partially solved by using the organelles-counterstaining approach that was established in this study. With suitable software, the signal can be automatically quantified for the

labeled cell organelles, the expressed recombinant proteins, and their overlapped areas. The overlapping level of different organelles can then be calculated for a given protein, and the best-overlapped case indicates its most possible localization site. In principle, the process can be fully automated for high-throughput localization classification, independent from the compartment phenotypes, cellular environments and the property of expressed proteins.

For drug development, the "colocalization cell arrays" can be extended to the functional screening of therapeutic agents that specifically act on some particular proteins. Protein mislocalizations, in some cases, can directly cause the clinical manifestation (Mueller et al., 2004). On the other hand, for the proteins causing particular diseases, their native post-translational sorting and translocation pathways can also be the targets for drug development. For example, secreted proteins and transcription factors with disease-specific expression patterns are transported out of cells and into the nucleus, respectively, where they provoke pathological phenotypes. In those cases, using "colocalization cell arrays", it is possible to perform a functional screening of chemical libraries on large sets of proteins at a same time. The compounds that can interrupt with the subcellular localization of the disease-specific proteins will be of great therapeutic potentials.

5.3. Cell array facilitates large-scale identification of cell death regulators

In the second part of this study, transfected cell array technique was combined with apoptosis detection assays to achieve high-throughput screening of apoptosis regulators. A series of apoptosis assays that are usually used with flow cytometry were, for the first time, applied on cell arrays. This led to the discovery of the cell death resulting from the overexpression of several human chromosome 21 proteins and from the endogenous protein knockdown mediated by siRNAs.

5.3.1. Multiple death assay enhance the detection sensitivity

When using cell arrays, the time of detection is especially critical for apoptosis detection. In the case of single detection assay, the optimal time of detection is difficult to be determined for several reasons. Firstly, the cells growing on cell arrays

differentially respond to the exogenous nucleic acids. In the cell cluster covering even the same DNA or siRNA spot, the transfection kinetics and efficiency was found to differ from cell to cell, leading to nonsynchronous apoptosis. Secondly, the huge variation in the time of apoptosis onset can result from different exogenous inducers targeting at different pivots in apoptosis pathways. Finally, to avoid the high spontaneous-apoptosis background due to high cell density from long incubation time and to diminish the loss of dead cells from the cell monolayer, apoptosis detection on cell array is preferentially performed shortly after the cell transfection. In this study, the apoptosis assays were normally performed in less than 48 and 60 hours after the transfection of Hek293T and HeLa cell array, respectively. At the time of detection, however, the apoptotic signal might not reach the climax.

To improve the detection sensitivity, the “multiple detection” was constructed on cell arrays for the first time. Several apoptosis assays were combined to collect the death signals from different apoptotic stages, and thus enhance the signal intensity. This is of particular interest in large-scale screening of novel apoptosis inducers where false negative results appear to be the major drawback when using single detection assay.

5.3.2. Multiple death assay identify non-apoptotic programmed cell death

In recent years, the terms apoptosis and programmed cell death have been mistakenly viewed as synonymous. A number of well-documented examples exist of cells that undergo programmed cell death without exhibiting the dramatic structural changes that are the basis of the term apoptosis (Kitanaka et al., 2002; Pollard and Earnshaw, 2004). By using multiple apoptosis assays that utilize different detection principles, we could actually reveal the programmed cell death undergoing apoptotic and non-apoptotic pathways.

Caspases activation is the essential event of apoptotic cell death. In this study, the antibodies against cleaved caspase-3, a central caspase player, and that against cleaved PARP protein, were used to reflect the physiological status of caspases. FLICAs, the activated caspase inhibitors, were also tested on cell array and proved to be functional with limited applications.

TUNEL reaction and Annexin V binding assay were introduced in this study in order to identify both apoptotic- and non-apoptotic programmed cell death. TUNEL reaction is commonly applied as a standard technique to detect apoptosis. However, positive detection of TUNEL has also been shown by autophagic cells (type II non-apoptotic programmed death), necrosis, and by the cells with elevated activity of DNA repair (Kanoh et al., 1999; von Bultzingslowen et al., 2001). The ability of cells to bind Annexin V is another general marker of cell apoptosis. However, cells undergoing necrosis and non-apoptotic form of programmed cell death, as well as macrophages and the cells engulfing apoptotic bodies may also be positive to the Annexin V assay (Castro-Obregon et al., 2002; Marguet et al., 1999). Therefore, TUNEL reaction and Annexin V binding should be considered as the assays rather to identify the cells with severe DNA damage and the loss of distribution asymmetry of phosphatidylserine across the membrane, which may also occur in the cell death with non-apoptotic origination, than to detect classic apoptosis only.

5.3.3. Multiple death assay open a chance for the development of therapeutic agents

The realization that many successful chemotherpeutic agents act by inducing cancer cells to undergo apoptosis has led to large efforts in discovering new drugs that will elicit this response. Better understanding of the events during the latent phase of apoptosis may inspire the development of agents that will trigger an apoptotic response in tumor types that are resistant to current chemotherapy. On the other hand, identification of apoptosis suppressors will be protective against the diseases due to severe apoptosis, such as the cell death following the stroke and infarct and the apoptosis in some neurodegenerative diseases.

Compared with apoptosis, the molecular mechanisms of non-apoptotic programmed cell death are much less known. However, many substances including small exogenous chemicals and endogenous proteins have been found to trigger cell death undergoing non-apoptotic pathway (Castro-Obregon et al., 2002; Kitanaka et al., 2002; Mochizuki et al., 2002; Tan et al., 2005; Trulsson et al., 2004). This opens a chance to use the combined cell death assays on cell arrays to hunt, in a high-throughput way, for novel cell death inducers or inhibitors with therapeutic potentials.

5.4. Advantages and limitation of transfected cell arrays

The results described in this study have demonstrated usefulness of transfected cell arrays (TCA) for large-scale functional analyses through gene overexpression and/or knockdown. With the immobilization of nucleotides in a highly organized 'microarray' format, TCA achieves a significant miniaturization of transfection and signal detection, and hence allows for high-throughput functional screening.
Like all microarray-based techiniques (e.g. DNA or protein microarrays), TCA is a "positional" array with defined location of spotted material on solid surface. On cell arrays, known cDNAs are printed separately with difined coordinates, therefore it is unnecessary to isolate the cDNA and identify it when a phenotype of interest is discovered. This circumvents the substantial work usually required for fluorescence-activated cell sorting or successive rounds of "sib" selection (in sib selection, clones conferring the phenotype of interest are isolated from clone libraries by iteratively subdividing the clone population) (Bailey et al., 2002).

TCA has also certain advantages over protein microarrays, in which pure proteins are immobilized on a surface. Although of potentially broad use, highly representational protein microarrays are difficult to make. This is because large numbers of individually purified proteins are needed, and some proteins cannot be expressed in prokaryotic cells, which are commonly used for large-scale protein production. Thus, TCA can be a substitution for protein microarrays for some applications, such as identification of small molecule targets or antibodies screening.

TCA has shown several general advantages over conventional cell-based strategies. Through immobilization and miniaturization, a high signal-to-background ratio and cost-effectiveness due to less consumption of materials were achieved (see also in section 1.1.2). Moreover, TCA is more amenable to high-content observation of single cell phenotype because it is implemented on microscope slides, which is accessible to a broad range of detection methods, from microscope, confocal system to laser scanner. In contrast, the widely used microtiter-plate readers typically detect signals, such as fluorometric or colorimetric intensity, that is averaged over all cells in a well (Bailey et al., 2002).

Several intrinsic limitations, however, exist when using transfected cell arrays. Vanhecke and Janitz have listed several advantages and limitations of cell arrays

compared to microwell-plate platform (Table 5.1 (Vanhecke and Janitz, 2005)). Application of cell arrays was limited to adherent cells, which can form a cell monolayer on array surface. Even for adherent cells, the cell lines that can be used for TCA were limited to those with a high transfectable ability, such as Hek, HeLa or COS cells. Thus, the hard-to-be-transfected cells such as most primary cells were barely applicable to cell arrays. However, these technical limitations can possibly be conquered with the recent development of new chemical and biological strategies. Kato et al. designed a biocompatible anchor for cell membrane components. The slides fabricated with such anchors were amenable to the attachment of non-adherent human erythroleukemic K562 cells (Kato et al., 2004). Very recently, Sabatini's group described lentivirus-transfected cell arrays, a novel approach for overexpression and loss-of-function screens in a broad range of mammalian cells including primary and nondividing cells (Bailey et al., 2006). These efforts are important in expanding the application of cell arrays on cell types that were thought to be intractable in the past.

Table 5.1 Comparison of microwell plate- and microarry-based transfection and functional plateform

96-microwell plate	Cell array
Advantages	
Possibility of flow-cytometric analysis	Low cell & reagent consumption (reagents, e.g. siRNAs, necessary for single-well transfection are sufficient for hounds of reverse transfections)
Possibility of transfection by electroporation (primary cells)	Higher throughput transfection, expression and detection (up to 10 000 samples) in a single experiment
Suitable for non-adhesive cells	Suitable for large-scale protein colocalization study
Cost-effective for smaller sample sets	Possibility of long-term storage of printed microarrays
Well-established functional read-out for microwell plate format	Suitable for analysis on a single cell level (e.g. subcellular protein distribution)
Disadvantages	
Not suitable for large-scale protein colocalization study	Limited to adhesive cells
Higher consumption of cells and reagents	Not suitable for screening of protein targets with a long turn-over (over 72 h)
High throughput experiments require expensive automated dispensing system	Not possible for electroporation transfection. Transfection limited to lipid-based approaches
Detection of the collective signal from many cells	Low number of targeted cells per sample hampers advanced statistical analysis

One of the most important aspects for successful application of cell arrays is the development of the suitable biological read-outs that can be used to infer gene/protein functions in a high throughput manner. The transient or stable expression of reporter constructs containing the coding sequence of GFP or luciferase allow the monitoring of signaling pathway activation and can be used for identification of novel pathway elements. For example, in a study aiming to monitor mitogen-activated protein (MAP) kinase signaling, a serum response elements (SRE) reporter was fused to GFP and co-transfected with multiple candidate genes on cell array. By this way, the inhibition of upstream MAP kinase signaling proteins could be identified by certain inhibitor (Webb et al., 2003). Instead of reporter construct, many phosphorylation-specific antibodies can be alternatively used as immunofluorescent read-outs on cell arrays for signaling transduction analyses. Nevertheless, there is still an urgent need for the development of functional read-outs on cell arrays to analyze the activity of overexpressed or knock-downed proteins. This motivated us to establish the organelles-colocalization and apoptosis detection assays on cell arrays as described in this dissertation.

TCA may also evolve to accommodate assays on living cells in real time. Currently, functional assessment of transfeced cell arrays is normally carried out on fixed cells. Automated systems for real-time and/or time-lapse imaging sets are also in development. These limitations, however, will be overcome with technical supports, making possible the real-time analysis of transient or kinetic phenotypes, such as protein translocation and cellular morphological changes during division or apoptosis. Very recently, an automated time-lapse imaging facility has been constructed on TCA to facilitate phenotyping of the chromosomes during cell devision (Neumann et al., 2006). Such fully antomated system has demonstrated powerful in their pilot RNAi-screening and will effectively assist more real-time analyses at genome-wide scale.

The main difficulty in creating pan-genomic arrays is the work required in making the high-content library of cDNA expression constructs and siRNAs. Currently, projects to make collections of full-length cDNAs for all human genes are underway (e.g. the German cDNA Consortium) and genome-wide siRNA library is in the meantime commercially available, shedding light on pan-genomic cell array uses. Very recently, a huge collection containing 104,000 lentiviral shRNA vectors has been constructed to target each of 22,000 human and mouse genes (Moffat et al., 2006). An efficient

gene silencing has been achieved using a subset of the library to identify candidate regulators of mitotic progression in human colon cancer cells. Such resource would allow researchers to perform comprehensive and reliable loss-of-function screens at genome-wide scale in a broad range of cell types.

With the advances in functional genomics including genome sequencing and genome-wide cDNA cloning and sh/siRNA construction, we believe that cell array-based gene overexpression and knockdown approach will facilitate functional characterization of vast sequence information produced from genome sequencing projects. The cell array-based protein colocalization and cell death platforms described here could also contribute greatly to the identification of novel genes that affect a wide range of cellular processes including protein sorting and localization, cell growth, apoptosis, and non-apoptotic cell death.