Systemic and Cell-Type Specific Profiling of Molecular Changes in Parkinson's Disease

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**SYNOPSIS**

Parkinson's disease (PD) is a complicated neurodegenerative disorder although it is oftentimes defined by clinical motor symptoms originated from age dependent and progressive loss of dopaminergic neurons in the midbrain. The pathogenesis of PD involves dopaminergic and nondopaminergic neurons in many brain regions and the molecular mechanisms underlying the death of different cell types still remain to be elucidated.

There are indications that PD causing disease processes occur in a global scale ranging from DNA to RNA, and proteins. Several PD-associated genes have been reported to play diverse roles in controlling cellular functions in different levels, such as chromatin structure, transcription, processing of mRNA, translational modulation, and posttranslational modification of proteins. The advent of quantitative high throughput screening (HTS) tools makes it possible to monitor systemic changes in DNA, RNA and proteins in PD models. Combined with dopamine neuron isolation or derivation of dopamine neurons from PD patient specific induced pluripotent stem cells (PD iPSCs), HTS technologies will provide opportunities to draw PD causing sequences of molecular events in pathologically relevant PD samples.

Here I discuss previous studies that identified molecular functions in which PD genes are involved, especially those signaling pathways that can be efficiently studied using HTS methodologies. Brief descriptions of quantitative and systemic tools looking at DNA, RNA and proteins will be followed. Finally, I will emphasize the use and potential benefits of PD iPSCs-derived dopaminergic neurons to screen signaling pathways that are initiated by PD linked gene mutations and thus causative for dopaminergic neurodegeneration in PD.

**Key Words:** Parkinson's disease; cell type specific; high-throughput; systemic; quantitative profiles; dopaminergic neuron; neurodegeneration; induced pluripotent stem cell

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INTRODUCTION

Parkinson’s disease (PD) is the most common neurodegenerative movement disorder1. Progressive loss of dopamine neurons in the midbrain which normally form a nigrostriatal circuitry accounts for canonical motor symptoms in PD, such as bradykinesia, tremor, rigidity, and postural instability. Although age-dependent and robust dopaminergic demise is the most prominent characteristic of PD and the patients are diagnosed by this feature, there are numerous non-motor symptoms associated with the degeneration of non-dopaminergic neurons in other brain areas3,2. Non-motor symptoms include depression, sleep disorder, autonomic disorder, gastrointestinal problems that are controlled by catecholaminergic and serotonergic neurons. Thus, pathogenic signaling in PD may involve more complicated molecular components depending on diverse cell types and brain regions which are disease-affected, although there might be common pathways that contribute to cell death to some extents for all regions. Although motor symptoms in PD patients in the early disease stages are satisfactorily controlled in most PD patients, there are few treatment options for non-motor symptoms. Moreover, the progressive nature of PD pathogenesis and long incubation time with average onset age of 60 years hamper the identification of important initial molecular changes which set in motion characteristic multi-neuronal degeneration in PD, which is critical in developing treatment options to reverse or halt the disease progression.

About 10% of PD cases are caused by mutations in PD-linked genes (e.g. autosomal dominant PD genes: α-synuclein, LRRK2; autosomal recessive PD genes: parkin, PINK1, DJ-1). In other word, mutations and alterations of cellular signaling by loss or gain of function of these PD proteins can initiate PD disease progression and eventually recapitulate dopaminergic neurodegeneration which in most cases are indistinguishable from sporadic PD cases. It is believed that understanding PD-linked genes and associated signaling pathways will eventually lead us to better understanding of more general and complex neurodegeneration in PD3. Indeed hypothesis driven and focused studies have yielded valuable downstream and upstream molecular mechanisms by which the mutations in PD genes contribute to PD-related phenotypes4,5,7. But, along with several types of neurons involved in PD, a number of PD genes and linked chromosome loci make it difficult to identify distinct molecular changes or network of signaling pathways that contribute to dopaminergic or non-dopaminergic cell types in PD. Fur-thermore, there are cells that are not affected by PD gene mutations even at the same incubation periods. Even those neurons affected in PD exhibit different time course of degeneration7,12. Although focused studies on single proteins or macromolecules with which PD proteins associate may provide potential therapeutic points to intervene, more systemic approach should be employed to obtain a big picture of altered molecular network for different PD genes and for different cell types to expand our knowledge in PD neurodegeneration.

In this review, I will provide logics in applying systemic -omics (Chromatin immunoprecipitation coupled with deep sequencing or microarray; RNA deep sequencing, microarray, BAC array technology for translational RNA profiling; quantitative proteomics such as SILAC) technologies for PD genes (e.g. α-synuclein, LRRK2, c-Abl, PINK1, DJ-1, parkin, and substrates or modifiers for PD genes) which will be followed by brief introduction to the technologies. The potential scheme to study cell type specific profiling of systemic molecular alterations by relevant PD genes will be further discussed with emphasis on technologies which will offer an opportunity to dissect distinct signaling pathways in cell populations. Key points of each section are presented as an illustration for an overview of the manuscript (Figure 1).

DNA BINDING AND MESSENGER RNA PROFILES AND ALTERATIONS OF CHROMATIN STRUCTURES IN PD

Parkin is an E3 ubiquitin ligase which is composed of ubiquitin like domain in the N-terminus and two RING domain and In between Ring (IBR) domain5. Mutations in parkin accounts for more than 50% cases of early onset recessive PD3. Many of mutations have been found in E3 ligase domain and shown to impair its ubiquitin ligase functions which can result in the accumulation of its substrate proteins6,7,10,11. However, there are several mutations in non-catalytic regions and some of mutations don’t affect its ligase function, implicating non-canonical role of parkin in PD pathogenesis1. Interestingly, parkin can bind to the promoter region of p53 and regulate its transcription12. Given that p53 is critical element in mediating cell cycle arrest and cell death, DNA binding and gene regulation of parkin can be critical to maintain dopamine or non-dopamine cells in normal physiological condition or pathological condition following PD-related stress such as oxidative, dopamine, and nitrosative stresses which are known to regulate parkin’s function13,15. The ability of parkin to bind p53 promoter prompts the hypothesis that parkin regulation of other promoters may be altered by PD-linked parkin mutation or parkin modifications which have been shown to affect parkin function.

The potential regulation of promoters is not limited to parkin’s noncanonical function of DNA binding and transcriptional repression. Parkin’s role in PD pathogenesis is more widely studied in terms of K48 polyubiquitination and proteasomal degradation of potential toxic substrate proteins5. Among dozens of parkin substrates which accumulate following dysfunctional
parkin, AIMP2 and FBP1 can bind to the promoter region of the deubiquitination enzyme USP29, which is involved in p53 regulation\(^6\). FBP1 directly binds to the promoter and AIMP2 collaborates as a cofactor in this transcriptional activation. Since transcription factors rather regulate multiple genes in a given context, AIMP2/FBP1 regulated promoters are likely to be globally distributed in the chromosomes and this regulation can be altered by accumulation of AIMP2/FBP1 downstream of parkin inactivation. Interestingly, parkin regulation of DNA binding and regulation has been also reported for mitochondrial genome\(^17\).

One of the prominent pathological hallmark of PD is the presence of Lewy body, the intracellular protein inclusion\(^18\). Lewy body comprises many proteins but mainly \(\alpha\)-synuclein. Although physiological roles of \(\alpha\)-synuclein are thought to be in synaptic neurotransmission, the gain of function mutations in \(\alpha\)-synuclein such as A53T, E46K, A30P or elevation of protein levels due to multiplication of this gene tend to form aggregates, toxic oligomer to protofibril and fibril forms of aggregates\(^19,20\). Until the elegant study by Olszcha et. al, the mechanisms by which these aggregate formation causes cell death were largely unknown\(^21\). There was also some hypothesis that Lewy body is indeed a byproduct of cellular protective function in order to sequester toxic proteins to keep them from interacting with other proteins\(^22,23\). By quantitative mass spec analysis of interactome of artificially produced \(\beta\)-sheet aggregate which mimics structural features of many aggregate prone disease proteins including \(\alpha\)-synuclein, huntingtin, amyloid-\(\beta\), they found that many physiologically important proteins are sequestered by aggresome through nonspecific interaction largely mediated by physicochemical properties of those interacting proteins\(^21\). The sequestration of proteins results in malfunction of many cellular processes in which these proteins are involved. Transcription regulation is one of the major processes that are potentially affected by aggresome formation\(^24\). In the disease progression, Lewy body pathology spreads from brain stem to the cortical regions, accompanying neurodegeneration of different neuronal types. Given that many cell types are affected by protein aggregation, it would be insightful to examine how differently transcriptome is altered in different neurons by \(\alpha\)-synuclein protein aggregation.

**Figure 1.** Schematic illustration of potential application of proteomics and transcriptomics in PD model- or iPSC-derived dopamine neurons. Sinaing pathways in PD dopamine neurons are briefly depicted with placement of PD associated genes in singlaing pathways where they have been shown to be involved. They include protein translocation into different organelles such as nucleus, mitochondria, membrane, Lewy body protein inclusion, posttranslational modifications such as phosphorylation, ubiquitination, nitrosylation, and others, and transcriptional, and translational regulations. Potential points of application of quantitative analytical tools such as SILAC, microarray and deep RNAseq were indicated next to the levels of potential alterations by PD associated genes.
Besides PD genes, MPTP intoxication has been widely used to produce selective dopamine neurodegeneration and PD modeling in mice and monkeys. Selectivity for dopamine neuron is derived from MPTP metabolite, MPP+, uptake into cells through the dopamine transporter located in the axon terminal of dopamine neuron. Once MPP+ is transported into the cell, it binds to mitochondrial electron transport chain complex I and inhibits oxidative phosphorylation, leading to reactive oxygen species (ROS) generation. One of the main cell death pathways elicited by MPP+ induced mitochondrial dysfunction and ROS generation is nitric oxide synthase activation and NO radical formation. Ultimately DNA damage-induced poly (ADP-ribose) polymerase activation kills dopaminergic neurons. Important insight from MPTP studies in PD research is that mitochondrial complex I inhibition can be sufficient to result in the cell death of dopamine neurons. In this respect, several PD genes, especially parkin and PINK1, have been extensively studied to show association with mitochondrial maintenance. Radical-induced DNA damage response possibly relates mitochondrial dysfunction with transcription factor or chromatin structure changes in a systemic manner. Oxidative stress which is one of the characteristic features of PD has been reported to impact gene transcription by the regulation of histone deacetylase (HDAC). It is highly probable that chromatin remodeling is widespread in the disease progression which may ultimately contribute to PD pathogenesis.

**CHROMATIN IP**

Chromosome is a complex structure with DNA intertwined around histone complex, and forming an ordered, highly compact structure. Histone modifications (e.g., acetylation by histone acetyl transferase or deacetylation by histone deacetylase) can result in the opening or closing of promoter regions where transcription factors will have access to regulate gene expression. The DNA element or sequence can be studied by using chromatin immunoprecipitation technology followed by oligo chip microarray or direct sequencing. Briefly, to study global promoter profiles targeted by specific DNA binding factors (i.e., parkin, AIMP2, FBP1, modified histone), cells are fixed to form crosslink between DNA and transcription factors or histone. Optimized sonication produces around 200 bp fragments among which immunoprecipitation using antibody specific to the protein pull down the sequences that are bound to the protein complex. The decrosslinked promoter DNA can be hybridized to promoter sequence array or directly sequenced after brief amplification steps. The analysis and mapping of promoter sequences that are associated with the transcription factor will give an insight into a direct and global regulation of genes in PD related models depending upon which transcription factors are investigated. The model systems and PD related proteins where CHIP analysis is directly applicable will be covered in more detail in the section. Mouse models coupled with Laser capture microdissection.

**MICROARRAY AND RNA DEEP SEQUENCING**

If CHIPseq or CHIP on chip is the screening for directly targeted promoter regions by a transcription factor, transcript microarray or RNA deep sequencing can provide data about how whole messenger RNA expression is altered in disease condition. In other words, we can get profiles of not only directly regulated but also indirectly regulated genes following PD-related molecular changes including PD gene mutations, and PD associated stresses. Microarray is done by hybridizing Cy2- and Cy3-fluorescent labeled messenger RNAs from two different samples, respectively. Oligos which should be complementary for predicted gene mRNA are imprinted on a microarray slide. Once differentially fluorescence labeled mRNAs from two samples bind to microarray, the relative intensities on each spot corresponding to certain genes can be easily determined because more mRNA will reflect stronger hybridized signal. Relative easiness and low price make this technology as an attractive option for highthroughput screening of global gene expression profiling. The potential ability of PD genes and oxidative stress to directly bind to DNA and also regulate chromatin condensation will definitely affect gene transcription which potentially contributes to set in motion PD pathogenesis. Virtually every genetic alterations can cause changes in global transcriptional profiles to some extent. To differentiate direct pathogenic changes from compensatory responses will be the next challenges. And this is why we need good model systems or genetic PD models in the beginning to initiate this transcriptomic analysis. PD model systems and dopaminergic specific application of transcription profiling will be further covered in the sections. Bacterial artificial chromosome (BAC) array ribosomal profiling and Stem cell derived dopamine and non-dopamine neurons.

**PROTEIN ABUNDANCE, SUBCELLULAR DISTRIBUTION, AND POSTTRANSLATIONAL MODIFICATION PROFILES IN PD**

While gene regulation in transcription levels provides substantial diversity in physiological cellular function, messenger RNAs of differentially expressed genes only partially represent the whole protein expression and network which indeed play functional roles in executing important biological functions. For example, although there are certain levels of correlation in mRNA and protein levels, increasing evidence indicates layers of regulations in translation efficiency either by microRNA inhibition.
of translation or by more sophisticated unknown mechanism involving the process of polysome formation in certain mRNA species. Moreover, there are tremendous amount of post-translational modifications which are known to be important in the regulation of activities of proteins or for their translocation and interaction with downstream effectors. This is also true for PD linked genes and their interactors including modulators and substrates. More thorough understanding of molecular changes in PD environments would be only possible by parsing out the systemic analysis of differentially regulated proteome either in their expression levels or in the levels of modification and translocation into different compartments of organelles.

Parkin is an multi-faced E3 ubiquitin ligase which mediates diverse lysine linkages (K-63 or K48) of ubiquitin conjugation either of mono, multi or poly ubiquitin chains to many different substrates residing in different compartments. Different modes of ubiquitin modification mediate diverse cellular processes including protein degradation (K-48 linkage poly ubiquitin), stress response, cell cycle regulation, protein trafficking, endocytosis (mono- or multi-ubiquitination), signaling (K63 poly ubiquitin) and transcriptional regulation. As such, parkin is likely to be involved in many important cellular functions of which disruption by PD linked mutation induced dysfunctional ligase activity of parkin can lead to PD pathogenesis. The systemic and thorough understanding of parkin substrate network and processes involved can be only achieved by quantitative and global assessment of proteomics, not only in their ubiquitin modification but also subcellular localizations and interactions. K-48 polyubiquitination and proteasomal degradation of parkin substrates are the most well characterized parkin function in relation to PD associated pathological changes, which include intracellular protein inclusion Lewy bodies or Lewy neurites and accumulation of toxic protein substrates like AIMP2 or PARIS. Although there are dozens of other parkin substrates reported so far, comparative analysis and association studies of all of these parkin substrates remain to be elucidated in proper dopamine cell models of PD animals or familial PD patient derived iPSC cells. Moreover, there are tremendous amount of post-translational modifications which are known to be important in the regulation of activities of proteins or for their translocation and interaction with downstream effectors. This is also true for PD linked genes and their interactors including modulators and substrates. More thorough understanding of molecular changes in PD environments would be only possible by parsing out the systemic analysis of differentially regulated proteome either in their expression levels or in the levels of modification and translocation into different compartments of organelles.

There are two PD genes encoding putative kinase, LRRK2 and mitochondrial PINK1. Tremendous attention has been given to this two PD associated kinases since, once proven, aberrant kinase activities related to disease are potential therapeutic targets which can be modulated by small molecule screening. Several dominant mutations found in LRRK2 have been shown to enhance its kinase activity, while recessive mutations in PINK1 impair its catalytic activity. Correlation between suppression of G2019S-LRRK2 kinase activity and dopaminergic cell survival has been documented in a virally induced LRRK2 PD animal models. Thus, changes in phosphorylation signaling cascades supposedly initiated by aberrantly increased LRRK2 kinase activity appear to be responsible for dopaminergic degeneration and cessation of this pathological signaling by blocking LRRK2 kinase activity either by using chemical inhibitors or LRRK2 kinase dead mutants version is sufficient to prevent neurodegeneration even with increased protein levels of LRRK2. Like it is the case with all the kinase signaling, signaling amplification and diversification through many downstream effectors would be one of key features of LRRK2 signaling cascades. Therefore, systemic profiling of differentially regulated phosphoproteomics in not only LRRK2 related PD mouse models or patient derived iPSC cells but also in sporadic PD conditions might provide the basis of significant signaling pathways which contribute to pathological changes in this mouse models, including fragmentation of dopamine neurites, and of course neurodegeneration. It should be noted that LRRK2 has been shown to be involved in many different biological processes such as protein aggregation, mitochondrial function, and protein translation. Therefore, organelle specific dissection of global proteomics can provide filtered information on how pathological LRRK2 can regulate different compartments of cells.

In contrast to LRRK2, maintenance of PINK1 kinase function is thought to be protective for dopamine cell survival. Initial report on PINK1 activity upon oxidative stress indicates gradual increase of PINK1 activity and corresponding elevation of its substrate TRAP1 phosphorylation that are related in protective response against oxidative challenge for mitochondria. Furthermore, several PD linked mutations in PINK1 not only impair its kinase functions but also impair its ability to recruit parkin to the mitochondria when mitochondrial potential is dissipated due to damage. Interestingly, PINK1 is a mitochondrial kinase of which expression levels on mitochondrial outer membrane change in a short time scale depending on the status of mitochondrial function. CCCP treatment dissipates mitochondrial potential and inhibits rapid import, proteolysis, and proteasomal degradation of PINK1 in a normal condition. This robust increase of PINK1 stability and protein levels on the surface of damaged mitochondria along with its kinase activity have been suggested as an initiating event in recruiting the E3 ligase parkin to the mitochondria and subsequent autophagy.

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degradation of these organelles. Both kinase activities and protein levels and potentially its micro localization in mitochondria appear to be critical in its biological functions and dysregulation of PINK1 by familial mutations or by PD environment stress can be imperative in the mitochondrial abnormalities and neurodegeneration in PD.

Afore mentioned PD enzymes themselves are affected in their catalytic activities either by PD linked familial mutations or posttranslational modifications imposed by PD associated aging process like oxidative, dopamine, and nitrosative stresses. And this potential regulation of E3 ligase function of parkin or kinase activities of LRRK2 or PINK1 provides the basis to extend these PD genes implication in more general sporadic PD cases in which there are no known genetic mutations accounting for PD development.

QUANTITATIVE MASS SPECTROMETRY ANALYSIS

Mass spectrometry is not inherently for quantitative analysis but for identification of proteins. So traditionally, for relative abundance of proteins, the proteins of differential expression were indirectly identified by comparing two dimensional gel electrophoresis and coomassie staining of whole proteome. The spots which show difference in intensities are analyzed by mass spectrometry to identify potential proteins accounting for the changes. It is essential to validate the result by western blots to make certain the authentic proteins of changed expression. And although mass spectrometry is a very sensitive tool to identify proteins, the staining methods such as coomassie or silver staining limit the repertoire of candidate proteins which can be analyzed by mass spec to those of abundant expression.

On the other hand, stable isotope labeling by amino acids in cell culture (SILAC) is a tool that enables quantitative mass spectrometry analysis of whole proteome. It is also possible to isotope label whole proteome in animals like drosophila or mice by feeding them either with natural or the 13C6- or 15N-substituted lysine or arginine over two to four generations without affecting development, growth, fertility and behavior. Typically, two groups between which the proteome is going to be compared are grown in light or heavy media containing either natural amino acids of 12C6-lysine or heavy isotope-substituted version of 13C6-lysine, respectively. When whole proteins of cells grown in heavy isotope media are saturated with 13C6-lysine, total proteins from each sample among which treatment was given to only one group can be mixed and run together for mass spectrometry analysis. Treatments can be drug, PD related stressors, or acute induction or knockdown of PD genes. Since all the proteins or peptides digested by Lys-C or N endoprotease, peptides from cells in heavy isotope will be separated from peptides from the other cells grown in regular media by a mass of 6 Da. The comparison of peak intensities and identification of peptide via tandem mass spectrometry will indicate relative abundance of target proteins. SILAC is also comparable with other enrichment steps to study posttranslationally modified proteomes, including phosphopeptides, ubiquitinated peptides, or nitrosylated peptides. Purification and enrichment of modified proteins or peptides and subsequent tandem mass spec analysis of SILAC labeled samples will identify proteins of which modification is increased or decreased compared to control sample.

In addition to protein abundance or modification revealed by SILAC aided quantitative proteomics, physiological or pathological subcellular translocation of certain proteins can be investigated when SILAC mass spectrometry is used in conjunction to subcellular fractionation enrichment steps. For example, PD linked E3 ubiquitin ligase has been shown to translocate to mitochondria upon dissipation of mitochondrial potential or to the nucleus following damage stress. More global network of PD protein dynamic distribution in subcellular compartment and association with distinct protein complex will broaden our understanding on molecular mechanisms of PD-related gene function.

The application of SILAC mediated quantitative proteomics is not limited in studying relative abundance of proteins. Pulsed growth for certain time duration in regular media after saturated labeling in heavy media followed by quantitative analysis can allow to study the relative stability of each protein species just like it is routinely done in a small scale to study protein degradation kinetics by radioisotope labeling in cells. In PD animal models or postmortem tissues from PD patients, there are abnormal accumulation of several protein species, notably α-synuclein oligomer or fibrils and parkin lysine-48 polyubiquitin substrates such as AIMP2, FBK-1, and PARIS. Given the observation of proteasome inhibition in PD, the hindered clearance of proteins can be global. Still quantitative comparison of stability of global proteomes in disease models like parkin acute deletion mice or human dopamine cells derived from iPSCs harboring parkin mutations is likely to yield a class of proteins of which degradation is altered and as such serves as a potential candidate substrate of parkin contributing to PD pathogenesis.

MODEL SYSTEMS APPLICABLE FOR CELL TYPE SPECIFIC GENE EXPRESSION PROFILING

There has been extensive transcriptome and proteome analysis in postmortem PD patient midbrain to identify commonly altered mRNAs, proteins and signaling pathways compared to age matched control. Although important insights have been obtained from these previous studies, it retains limitations. In
the end stage of disease, there are few dopamine neurons survived in the midbrain. Therefore, mRNAs or proteins purified from these regions are not likely to represent mainly dopamine cell population. Moreover, even though laser capture microdissection can be employed to concentrate only TH expressing dopamine cells, the cells are in the end stage of neurodegeneration which will mask the initial gene expression changes that actually elicit all the cell death causing alterations. These are some of the reasons that genetic or toxin induced PD mouse models or patient derived induced pluripotent stem cell derived dopamine neurons are likely to provide pictures of transcriptome and proteome that more closely reflect early PD initiation.

MOUSE MODELS COUPLED WITH LASER CAPTURE MICRODISSECTION

For the profiling of promoter regions regulated by parkin or transcriptome altered by parkin deletion or parkin mutation, conditional parkin knockout mice can be a choice of starting material. Although adult onset deletion of parkin has not yet proven to lead to dopaminergic neurodegeneration in mice, transcriptome analysis by microarray or RNAseq coupled with parkin binding promoter analysis by CHIPseq will provide parkin associated early changes in gene expression. Rather than midbrain regional dissection, LCM collection of dopamine neurons is more preferred methodology in order to prevent dilution of small but physiologically significant alterations of important genes in dopamine neurons. Highly specific TH antibody labeled dopamine neurons can be efficiently dissected by LCM\(^{67,68}\). Although microarray may require more RNA amount than RNAseq, highly pure samples will reduce noise which can be caused by dilution with other neurons and glial cell types in a traditional brain dissection technique, eventually leading to more sensitive detection of mildly changed genes. Coupled with LCM, other aforementioned PD associated proteins which have implications of acting as transcription factors (i.e. AIMP2, FBPI) can be studied to identify not only targeting promoter regions but also transcriptional changes. Lentivirus or adeno-associated viral delivery of AIMP2 or FBPI to the midbrain can efficiently transduce various cell population in the mibrain including dopaminergic neurons. LCM purified dopamine cells can then be further analyzed for CHIPseq and RNAseq. It should be noted that antibody quality is quite important for successful purification of promoters bound to a target protein. If CHIPseq quality antibody for the protein of interest is not available, tagging the protein with a tag for which CHIPseq quality antibody is available like GFP can be conjugated and delivered to the brain via a stereotaxic viral injection.

Although LCM isolation is quite laborious and the amount of samples are limited for application to RNA analysis in which oftentimes signal amplification is feasible, scaling up dopamine neuron isolation and the use of more sensitive mass spectrometer theoretically make it possible to study quantitative proteomic analysis in dopamine neurons from PD relevant animal models.

BACTERIAL ARTIFICIAL CHROMOSOME (BAC) ARRAY RIBOSOMAL PROFILING

BAC array translational profiling is a recently developed technology which enables isolation of mRNAs that are actively engaged in translation in a certain genetically defined cell population\(^{69,70}\). By using specific BAC drivers which are well characterized in terms of its promoter activity throughout CNS (www.gensat.org), EGFP-L10a fusion protein is cloned downstream of BAC promoters that showed activities in genetically defined cell population. For example, dopamine transporter (DAT) BAC-eGFP-L10a is available to label dopaminergic neuronal population in the substantia nigra. By regional dissection of ventral midbrain followed by affinity purification of ribosomes consisting of eGFP-L10a for dopamine neurons, isolation of polysomal RNAs from dopamine neurons is possible. The availability and highly specific affinity of monoclonal eGFP antibodies which are renewable source enable large scale isolation of polysomal RNAs from dopamine neurons of several experimental groups. Moreover, since genetic crossings are possible with this BAC-eGFP-L10a lines, there is no limit in studying and profiling translational mRNA changes in PD related animal models and dopaminergic selective toxin models such as MPTP intoxication.

The benefit of BAC array comparative analysis is that it is possible to identify previously unknown cell-specific transcripts by the power of the efficient enrichment of mRNAs from a given cell population which expresses eGFP-L10a ribosomal protein in desired CNS cell types by the BAC promoters used\(^{69,70}\). RNA isolation in those eGFP-L10a expressing target cell population reduces potential dilution of RNA profiles which often occurs in traditional regional dissection. This is especially true in highly heterogeneous tissues like brains. Compared to laser capture microdissection, the workload is relatively small and large amount of mRNA purification is feasible for microarray or deepsequencing analysis. Another advantage of BAC array technology is that one can analyze mRNA population which is located in microdomains of certain cell types. For example, active mRNA distribution and translation are ongoing in axon terminal of neurons. Oftentimes, cell body and axon or dendrite are located in anatomically distinct regions of brain. Thus, by dissecting corresponding brain regions containing desired structures such as axon fibers, it is possible to get a translation
profile of genes that are actively transported and expressed in those microdomains.

**STEM CELL DERIVED Dopamine AND NON-DOPAMINE NeURONS**

Along with the use of PD related genetic or toxin induced animal models coupled with isolation of specific cell types, emergence of iPSC technology and derivation of these pluripotent cells into desired neurons has led to unprecedented research opportunities on human diseases and cell therapy via individualized disease modeling of dopamine and non-dopamine neuronal populations in a dish.

Pluripotency can be achieved by reprogramming through somatic cell nuclear transfer into enucleated zygotes or early blastomeres or by induction of genes (i.e., Yamanaka factors [Oct3/4, Sox2, Klf4, c-Myc]) that are highly expressed in embryonic stem cells and are capable of reverting end-differentiated chromatin to the one in stem cells. Embryonic stem cells are naturally occurring truly pluripotent cells but the limited supply of oocytes and ethical restriction on the use of embryo make induced pluripotent stem cells more attractive in research, although iPSC retain intrinsic technical problems such as epigenetic profiling/modification landscape, the use of c-myc, an oncogene, random insertion when retrovirally induced, all of which can potentially interfere with derivation of authentic dopaminergic neuron and characterization. There have been a number of efforts still going on to improve these issues in iPSC production. And it is quite promising that iPSC derived human disease models will be more available to many researchers with low costs and little specialties in producing these cells in conjunction with technology driven robust advancement of iPSC methodology. For instance, the use of Sendai virus, an RNA virus not integrating into host chromosome, not only increased reprogramming efficiency but also eliminate the issue of integration and residual activity of reprogramming factors in many of traditional methods. Sendai virus derived transgenes are diluted and removed during cell division and the selection for virus free iPSCs resulted in fully functional iPSC generation with high efficiency of maximum 1%, sur-passing those achieved by retrovirus transduction of 0.02%. Subsequent refinement of specification and derivation into midbrain dopaminergic neurons by treating small molecule activators of sonic hedgehog and wnt signaling improved survival, function, and reduced overgrowth problems, which can be essential to study disease phenotypes in vitro with long term cultures.

Induced pluripotent stem cells (iPS cells or iPSCs), like embryonic stem cells, are capable of turning into any cell types of body and can be artificially produced via reverse differentiation from a fully differentiated adult somatic cells, like fibroblast, the feature which is the key in modeling human neurodegenerative disorder in which often cases specific neurons are more vulnerable against genetic predisposition or age dependent molecular changes. But iPSC’s pluripotency to become any types of neurons at the same time imposes problems of heterogeneous neural cell types derived from typical differentiation protocol. Combined with innovative differentiation protocols such as lentiviral delivery of LMX1A to facilitate A9 type midbrain dopamine neuron derivation and cell type specific BAC fluorescent reporters, and purification steps based on cell surface marker and FACS, it became possible to obtain ES or iPSC derived neuronal population highly enriched for dopaminergic neurons.

Availability of PD patient derived iPSC and its derivation to human dopamine neurons provided the opportunity to work with in vitro human PD models in a dish with unlimited supply of dopamine cells to investigate molecular changes that are specific and important for PD pathogenesis, which will eventually contribute to the selective neurodegeneration in that individuals who harbor the genetic mutations along with certain genetic composition. Monogenic mutations found in rare familial PD can be possible basis in developing in vitro PD models via iPSC technologies, including PARK1, PARK4/SNCA, PARK2/parkin, PARK5/ubiquitin COOH-terminal hydrolase L1 (UCHL1), PARK6/PTEN-induced kinase 1 (PINK1), PARK/DJ-1, and PARK8/Leucine-rich repeat kinase 2 (LRRK2).

As a collaborative efforts, so far, NINDS funded consortium has supported the generation of iPSCs lines for early onset familial forms of PD, including SNCA triplication, LRRK2 R1441C, PINK1 deletion, and unaffected family control. 40 fibroblast lines and 10-20 iPSC lines are expected to be available from the Coriell NINDS repository with multi-investigators’ efforts. In addition, Michael J. Fox foundation for Parkinson’s research also supports in the generation of patient-specific inducible pluripotent stem cells (parkin mutations) to enhance the study of PD by providing iPSC resources.

iPSCs have been developed and the derived dopamine neurons were characterized for α-synuclein triplication, LRRK2 GS mutation, PINK1 deletion, and parkin mutation. For autosomal dominant PD alleles, synuclein and LRRK2, human dopamine neurons exhibit elevated levels of α-synuclein with increased signs of oxidative stress with some induction of oxidative stress-response genes. Similar way for the main autosomal recessive genes, PINK1 and parkin which have been shown to be critical for mitochondrial maintenance and function, human dopamine cells with loss of these proteins (i.e., Q465X or V170G for PINK1 and exon3 deletion for parkin) result in PD related phenotypes which have been previously reported in cell or animal models, which include defects in parkin recruitment to mitochondria in PINK1 mutations and alterations in dopa-
mine metabolism and neurotransmission in parkin mutation\textsuperscript{39}. Those pathologically PD relevant changes and especially transcriptional changes determined for several genes in LRRK2 and parkin support the notion that these human dopamine neurons derived from PD patient specific iPSC can be subjected for transcriptome analysis to identify systemic alterations of gene expression networks.

Most insights into biological and pathological significance of certain signaling pathways have been derived from comparative analysis of experimental groups versus control group where there is usually single variation which will be responsible for phenotypic consequences. And this is why inbred strains of mice have served as optimal genetic animal models to understand the effect of genetic alterations because the genetic background or constituents of every single allele other than knockout or transgenic sites should be identical. This will reduce noise and increase signal to noise ratio, eventually strengthening the scientific and genetic power of results obtained from this comparative analysis. Studies on human postmortem tissues, on contrary, have a number of variations in genetic background other than single monogenic mutation which is thought to be responsible for PD progression because every human individual has a distinct set of genetic composition. Comparison has been made at best between PD patient and unaffected family members but oftentimes age-matched healthy individual at the time of tissue acquisition have been used to compare with PD patients. Although several reports on familial PD patient derived iPSC and human dopamine cells were compared to normal subjects of highly heterogeneous genetic background, though still providing some insights into PD relevant signaling changes, the use of zinc finger proteins to generate isogenic lines revolutionized the way to do comparative analysis of PD derived dopamine cells versus corrected version of wild type dopamine cells in the same genetic background. The feasibility of this zinc finger protein mediated genome editing of specific locus in human embryonic stem cells or iPSCs has been demonstrated for Parkinson’s-causing mutation A53T\textsuperscript{38}. A53T α-synuclein was successfully repaired to wild type with a precise double strand break in α-synuclein and homologous recombination aided by a specifically engineered zinc-finger nuclease. Interestingly, they also replaced wild type α-synuclein into A53T allele in embryonic stem cells using donor template containing the Parkinson’s-causing mutation A53T to introduce this single-base-pair change.

The zinc finger nuclease assisted editing of PD genes not only facilitates the comparative analysis in an appropriate isogenic condition but also enables introduction of PD-causing alleles in otherwise normal individual cells. Introduction of multiple alterations on many PD associated alleles will be instrumental to study genetic interaction studies in human dopamine cells, as commonly exploited in mouse models by combining different PD gene models such as bigenic mice of α-synuclein and LRRK2 mutants overexpression or triple knockouts of three recessive PD genes, namely parkin, PINK1, and DJ-1.

Collectively, development of zinc finger mediated editing of PD linked mutant allele to wild type genomic sequence establishes an unarguable and appropriate control to be compared to cells harboring mutant allele. Molecular and signaling changes between these two groups will provide authentic targets which are truly regulated by the monogenic mutation and thus important for disease progression. Additional control can be ectopic expression of wild type allele if the mode of inheritance is loss of function mutations.

CONCLUDING REMARKS

Patient specific IPS cells together with corrected version of control are ideal system to apply systemic analysis tools since the phenotypes induced by patient specific alleles are more likely global and subtle molecular changes, or pre-degenerative changes, that predispose these cells for neurodegeneration when associated with age-dependent processes. Identification of novel signaling or validation of previously identified pathways in animal models or cell lines will lead to therapeutic chemical screening or genetic modifier screening in a patient specific manner. Moreover, potential of PD iPS derivation into many different types of neurons, some of them are fairly spared in the disease process of dopaminergic neurodegeneration, can be a valuable starting point to tackle the fundamental question of PD pathogenesis which is why dopamine neurons in the substantia nigra are more vulnerable than other cells, even those neighboring dopamine cells in ventral tagmental area. Examination and discrimination of altered signaling pathways specific for dopamine neurons can be actively investigated by increased sensitivity and systemic quantitative power of SILAC, microarray, and BAC array technologies. Combination of appropriate model systems, isolation of dopamine and nondopamine neurons, and systemic analysis technologies would be a reasonable approach to understand differential vulnerability of neurons against disease relevant genetic alterations.

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