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Review

Discovery of new epigenomics-based biomarkers and the early diagnosis of neurodegenerative diseases

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ABSTRACT

Treatment options for many neurodegenerative diseases are limited due to the lack of early diagnostic procedures that allow timely delivery of therapeutic agents to affected neurons prior to cell death. While notable advances have been made in neurodegenerative disease biomarkers, whether or not the biomarkers discovered to date are useful for early diagnosis remains an open question. Additionally, the reliability of these biomarkers has been disappointing, due in part to the large dissimilarities between the tissues traditionally used to source biomarkers and primarily diseased neurons. In this article, we review the potential viability of atypical epigenetic and/or consequent transcriptional alterations (ETAs) as biomarkers of early-stage neurodegenerative disease, and present our perspectives on the discovery and practical use of such biomarkers in patient-derived neural samples using single-cell level analyses, thereby greatly enhancing the reliability of biomarker application.

1. Introduction

Neurodegenerative diseases, characterized by the physical decay of disease-associated target neurons and the eventual loss of surrounding non-specific neural cells, affect tens of millions of people worldwide every year. Limiting the damage caused by neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), Lou Gehrig's disease/amyotrophic lateral sclerosis (ALS), and Huntington's disease (HD), remains one of the most important challenges faced by humanity. Considerable research effort has been spent on the discovery of neurodegenerative disease biomarkers (measurable signals indicative of disease, infection, or injury) so that disease onset can be diagnosed before neural damage becomes too severe for reversal of disease by therapeutic agents. It remains elusive, however, whether traditional biomarkers identified so far can be applied for early detection of neurodegenerative disorders (Trojanowski and Hampel, 2011). Furthermore, the difficulty of sampling primarily disease-associated neurons has relegated researchers to source samples from peripheral patient-derived bio-material, which may not reliably represent neurodegenerative disease conditions (Khan and Alkon, 2015).

In this article, we highlight epigenetic and/or consequent transcriptional alterations (ETAs) for their promising potential as early diagnostic biomarkers in neurodegenerative disease. Next, we introduce an integrative approach to discover these biomarkers by combining patient-derived neural organoids with single-cell level analyses of epigenomic and/or consequent transcriptional profiles. Finally, we present a practical method for the use of these biomarkers in undiagnosed human subject-derived iPSC neurons. Although the application of the introduced strategy may be limited to genetically caused neurodegenerative diseases for now, the prospect of early diagnosis of even a limited subset of previously undiagnosable diseases is highly compelling.

2. Clinical importance of early-stage diagnosis and treatment of neurodegenerative diseases

The effectiveness of treatment in neurodegenerative diseases often depends on the timing of drug administration. For example, the dietary supplements folic acid and vitamin B have been shown to ameliorate cognitive deficits in patients with mild AD (Chen et al., 2016), while conferring little benefit to patients suffering from severe AD (Aisen et al., 2008). Given the importance of timely therapeutic intervention, early diagnosis of neurodegenerative diseases may greatly enhance the effectiveness of administrated drugs. Early signs of neurodegenerative

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disease, such as nascent disease-associated neural features, are therefore examined with great attention.

Early neural features of neurodegenerative diseases are diverse (Kwon et al., 2017). For example, in early-stage AD, PD, ALS, and HD, before significant neural death occurs, neurons exhibit dendrite defects, impaired axonal transport, and mitochondrial perturbations, among other features. Disease-associated dendrite defects may be caused by structural support alterations in the dendritic cytoskeleton (Kweon et al., 2017), or by a blocked local supply of plasma membrane components by Golgi outposts (Chung et al., 2017; Lin et al., 2015). Impaired axonal transport has been observed in fruit fly (Gunawardena and Goldstein, 2001), mouse (Kamal et al., 2000), and human patient (Stokin et al., 2005) models of AD, fruit fly (Alami et al., 2014) and mouse (Bilsland et al., 2010) models of ALS, and squid (Szebenyi et al., 2003), fruit fly (Gunawardena et al., 2003), and mammalian (Trushina et al., 2004) models of HD. This impaired transport is thought to amplify neural damage by disrupting the delivery of a number of vital organelles (Kweon et al., 2017). Mitochondrial disturbances, such as problems with mitochondrial respiration, trafficking, inter-organelle communication, and quality control, are thought to be associated with most, if not all, neurodegenerative disorders (Schon and Przedborski, 2011).

Collectively, the extensive diversity of abnormal neural features seen in neurodegenerative disorders suggests system-wide atypical changes in cellular activity. More importantly, by virtue of their early manifestation, these system-wide atypical changes may be exploited as early neurodegenerative disease signals. In the following section, we review how system-wide changes relate to ETAs, and in what way these ETAs can be used as early-stage biomarkers for neurodegenerative disorders.

3. ETAs in neurodegenerative diseases

One potential mechanism responsible for these system-wide changes is epigenetic alteration. Epigenetic alteration, i.e. changes in gene expression that occur without any changes in the underlying nucleotide sequence (Hwang et al., 2017), is now recognized to be closely associated with neurodegenerative diseases. In fact, drugs that restore neurodegenerative disease-associated epigenetic alterations have been shown to ameliorate disease toxicity in neurodegenerative disease models of AD (Ricobaraza et al., 2012; Govindarajan et al., 2011), PD (St Laurent et al., 2013), and HD (Hockly et al., 2003). For example, Entinostat (MS-275), a histone deacetylase (HDAC) inhibitor, ameliorates memory deficits, behavioral impairment, neuroinflammation, and aberrant amyloid deposition in the cerebral cortex and/or hippocampus in the APP/PS1 mouse model of AD (Zhang and Schluesener, 2013).

As of recent, researchers have invested considerable efforts into the investigation of ETAs as biomarkers for neurodegenerative diseases. Peripheral samples, such as those based in biofluid, have been instrumental in the non-invasive detection of ETA-based biomarkers (Runne et al., 2007; van Rheenen et al., 2018; Santiago et al., 2018; Schmitt et al., 2015). For example, Ai et al. was able to demonstrate blood leukocytes from PD patients significant hypomethylation patterns in SNCA intron-1 (Ai et al., 2014), which are associated with the increase of SNCA expression and pathogenesis of familial and sporadic PD (Ross et al., 2008; Singleton et al., 2003). However, three important limitations listed below must be addressed before ETA-based biomarkers can be generally applied.

First, while evidence suggests that ETAs occur early in the course of neurodegenerative diseases (Calligaris et al., 2015), the characteristics of those epigenetic alterations are currently poorly understood. ETA-based biomarkers have the potential not only to reveal disease presence or vulnerability to it, but also to measure the outcome of therapy and thus improved clinical trials (Boessen et al., 2014; Simon and Maitournam, 2004). However, to achieve this potential for improved diagnosis, prognosis, and personalized medicine treatment, a deeper understanding of the characteristics of ETA-based biomarkers is needed. For instance, reliable early-stage neurodegenerative disease biomarkers require an understanding of the state of biomarker along the axis of disease development. As such, it remains to be elucidated whether increased epigenetic aberration reflects disease severity [as proposed by J. L. Jakubowski et al. (Jakubowski and Labrie, 2017)] or whether certain epigenetic divergence patterns peak at an early-stage and then diminish over the course of disease progression.

Second, ETA-based biomarkers (and biomarkers in general) must be discovered and applied in model systems that faithfully mimic human epigenetic and/or consequent transcriptional profiles. Epigenetic and/or consequent transcriptional profiles are highly species-specific (Kanton et al., 2019; Pollen et al., 2019; La Manno et al., 2016; Khrameeva et al., 2019; Zhu et al., 2018; Xu et al., 2018a; Xu et al. 2018b; Zeng et al. 2012), thus, biomarkers discovered in non-human species may not be practical in humans. It is important to note that ETA-based biomarkers discovered in non-human models will inevitably require a validation process, which both decreases accuracy and slows the biomarker identification process. Although epigenetic biomarkers were traditionally discovered in non-human models, recent technological advances allow us to identify species-specific biomarkers in samples of human origin. We will elaborate on these advances in the following section.

Third, the human-derived biomaterial used to discover biomarkers must accurately reflect the cellular aberrations that occur in early-stage neurodegenerative diseases. Because the early stages of neurodegenerative disease primarily involve disease-specific neuronal subtypes, the use of peripherally-derived biomaterial greatly limits our ability to detect not only ETA-based biomarkers, but also biomarkers in general. Furthermore, a few compelling reports question whether the epigenetic profiles of peripheral tissues reliably reflect those of neurons (Varley et al., 2013; Coetzee et al., 2016). Hence, for the early diagnosis of disease, detecting cellular aberrations in primarily disease-associated neurons is essential, which calls for an alternative method of sampling patient biomaterials.

In summary, the discovery and application of ETA-based biomarkers necessitates a deeper understanding of ETAs, which in turn requires the use of human-derived samples that retain the cellular aberrations that occur in the early stages of neurodegenerative disease.

4. Perspectives on the discovery of ETA-based biomarkers and their application for early diagnosis of neurodegenerative diseases

4.1. The discovery of ETA-based biomarkers using patient-derived neural organoids

As discussed above, there are important constraints that must be applied in order to discover useful biomarkers for early-stage neurodegenerative disease: first, the biomarker needs to exploit the early ETAs associated with neurodegenerative disease; second, the biomarker must be discovered using cellular samples of human origin that are primarily relevant to the disease of interest. Given these constraints, we propose that ETA-based biomarkers should be discovered using patient-derived neural organoids (Fig. 1).

An organoid is a self-organizing 3D structure, grown from stem cells, that realistically mimics the micro-anatomy of the organ in question. When using neural organoids to discover ETA-based biomarkers, it is necessary to qualify the biomarkers of interest. Neurodegenerative diseases are caused by a myriad of factors, many of which are environmental or extrinsic in nature. Although such external factors cause neurodegenerative diseases and may result in ETAs in patients, the aberrations caused by these factors may not be represented in patient-derived neural organoids. This is because the cells used to generate organoids go through essentially a “reprogramming process” during the induction of pluripotency and will not regain, in the organoid, the epigenetic signatures induced by environmental or other

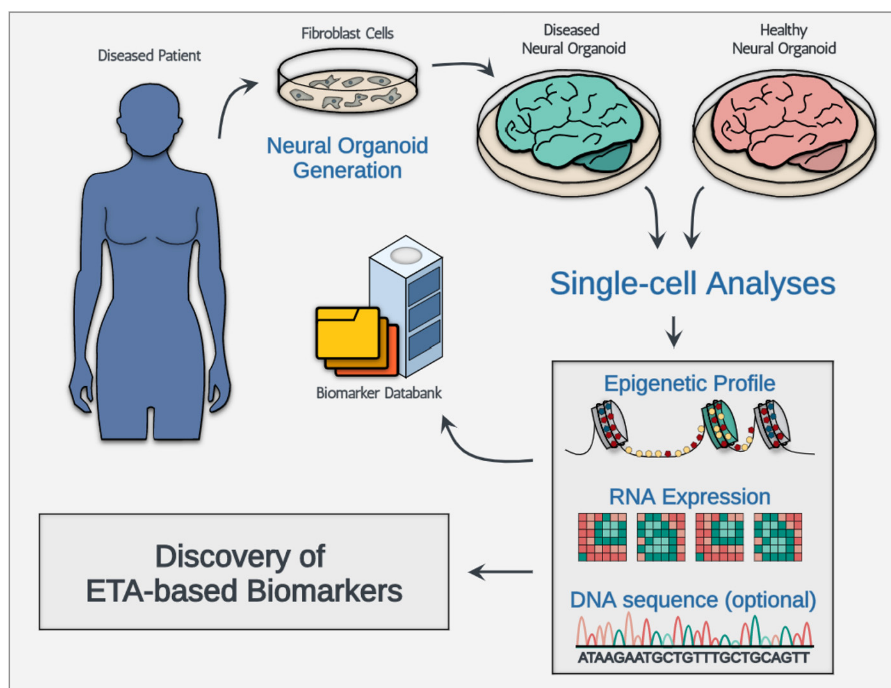

Fig. 1. A schematic illustration showing the conceptual procedures for the discovery of epigenetic and/or consequent transcriptional alteration (ETA) - based biomarkers through patient-derived neural organoids.

extrinsic causes. Accordingly, we must exclude ETAs that originate due to environmental factors as biomarkers. Similarly, we must exclude ETAs that arise due to complex cell-to-cell interactions, as these ETAs are fundamentally inoperative as biomarkers. In other words, of the myriad ETAs associated with the neurodegenerative disease, our principal focus must be on those modifications that originate due to disease-associated genetic mutations. As a matter of fact, theoretically, only one ETA-based biomarker is needed for the accurate early detection of neurodegenerative disease: one that is shared by disease patients and conserved in neural organoids. The feasibility of this approach becomes more apparent when considering previous reports that identified, in patient-derived samples, abnormal ETAs associated with genetic factors of neurodegenerative diseases, as summarized in Tables 1a and 1b.

Another important issue worth exploring is whether iPSC-driven neurons or neural organoids do, in fact, retain their epigenetic profiles through the reprogramming and differentiation processes. While the extent to which iPSC-derived neurons mirror *in vivo* neurons has not yet been fully elucidated, accumulating evidence suggests that the epigenetic profiles of iPSC-derived neurons and neural organoids may mimic those of their *in vivo* counterparts (de Boni et al., 2018; Luo et al., 2016) and that epigenetic profiles are actually preserved. One example is a timely article by Luo et al, which compared epigenomic features in cerebral organoids and in human fetal brains, using genome-wide, base resolution DNA methylome and transcriptome sequencing (Luo et al., 2016). The study revealed that *in vitro* and *in vivo* brain development shares epigenomic and transcriptional signatures and that cerebral organoids recapitulate the epigenomic landscape of human fetal brain. Researchers have also actively incorporated 3D *in vitro* modeling and neural organoids to understand the fundamental mechanisms underpinning the epigenetic aberrations in particular, and the pathogenesis in general, of neurodegenerative diseases (Table 2a). To discover ETA-based biomarkers and better investigate organoid biology, it will be important to continue to explore and narrow down the classes of ETAs that are vulnerable to the process of inducing pluripotency.

Once patient-derived neural organoids have been generated, it will be highly advantageous to incorporate an unbiased screen that identifies both ETAs and the appropriate cellular subtypes that best reflect early neurodegenerative disease conditions. We conjecture that there exist primarily disease-associated neural subtypes possessing early ETAs, as can be hinted at in a study by Fernandez-Santiago et al. (2015). Neural organoids display spontaneous neural development that contains various types of brain cells (neural progenitor cells, excitatory neurons, inhibitory neurons, astrocytes, oligodendrocytes, etc.) that, as mentioned earlier, largely retain *in vivo* epigenetic and/or transcriptional profiles. Thus, neural organoids effectively mimic *in vivo* developmental processes and neural structures *in vitro*. However, the production of heterogeneous cells is a double-edged sword: although it can be used to screen for optimal cellular subtypes that are indicative of neurodegenerative disease-associated ETAs, it also makes bulk-level analyses challenging. In heterogeneous bulk-level samples, the epigenetic and transcriptional profiles of relevant cells may be masked by the sheer number of surrounding extraneous cells, especially when the proportion of cells from the cellular subgroup of interest is small. To understand the epigenetic and/or transcriptional profiles of patient-derived neural organoids associated with neurodegenerative diseases, single-cell level analyses is, therefore, strongly advisable. The integrative method that joins organoid technology and single-cell level analyses allows for investigations into various specific cell-subtypes, many of which are extremely difficult to explore *in vitro*. Human neurons that reside in specific cortical layers, for example, are difficult to investigate using single cell-type cultures as these neurons rely on the spontaneous development of progenitor cells. It is these cells, and countless others, that can be investigated to identify optimal cellular subtypes that are indicative of neurodegenerative disease-associated ETAs.

In fact, single-cell level analyses have already attracted considerable attention as methods that allow improved characterization of neurodegenerative diseases. As previously mentioned, bulk-level tissue sampling provides limited resolution for the examination of cell-type-specific abnormalities. With single-cell transcriptome analysis, however, each cell is characterized by its transcriptome and grouped by its cell type and state. Exemplifying the capability of single-cell transcriptome analysis, Mathys et al. sampled post-mortem brain tissues from the prefrontal cortexes of 48 individuals with AD, and analyzed the

Table 1a
Selected reports demonstrating the association between genetic aberrations and epigenetic profile perturbation in patient-derived neurodegenerative disease samples (Frontotemporal dementia: FTD, Dopaminergic neuron: DAn, c9ALS: ALS with G_4C_2 repeat expansion mutation in C9orf72 gene).

	Sample Origin	Genetic Mutation	Modification	Epigenetic Mark	Observation/Implications	Reference
PD	Fibroblast of fPD and sPD patients	G2019S LRRK2 mutation	DNA Methylation	1,261 differentially methylated CpG sites in L2PD and 2,512 in sPD	Epigenetic changes manifest only in iPSC-derived DAn from fPD and sPD patients	(Fernandez-Santiago et al. 2015)
ALS/FTD	Human frontal corticies and cerebella tissues	c9ALS/FTD patients with G_4C_2 repeat expansion mutation	Histone Modification	Trimethylation of lysine residues within histones H3 and H4	Toxic effect characterization of histones H3 lysine residue trimethylation	(Belzil et al., 2013)
	Human cerebellum	c9ALS/FTD patients with G_4C_2 repeat expansion mutation	DNA Methylation	Hypermethylation of the CpG sites within the C9orf72 promoter region	Report of cerebellar hypermethylation in c9ALS/FTD	(Belzil et al., 2014)
HD	Brain of HD patients	PolyQ expanded mutant huntingtin	DNA Methylation	5hmC levels in the 5′UTR region of ADORA2A	Reduction of A2AR levels by polyQ expanded mutant huntingtin though abnormal epigenetic regulations	(Villar-Menendez et al., 2013)

Table 1b
Selected reports demonstrating the association between genetic aberrations and transcriptional perturbation in patient-derived neurodegenerative disease samples.

	Sample origin	Genetic mutation	Transcriptional mark	Observation/Implications	Reference
AD	iPSC-derived neurons from fibroblast of patients with late-onset AD	CRISPR/Cas9-mediated editing of APOE genes	Neural differentiation genes such as DCX, ASCL1, and AD-associated genes such as MAPT, among others	Similar cellular disturbances including mRNA regulations between sAD and APOE4 gene-edited iPSC lines	(Meyer et al., 2019)
PD	iPSC-derived neurons from fibroblast of PD patients	GBA-N370S mutation	Genes involved in neuronal development, neuronal differentiation, and synaptic activity, among others	Identification of transcriptional repressor histone deacetylase 4 as an upstream regulator of disease progression	(Lang et al., 2019)
HD	iPSC-derived neurons from fibroblast of Juvenile-onset HD patients	Expanded polyQ repeats in huntingtin	Genes associated with cellular morphology, cell movement, and neurological diseases, among others	Abnormal transcriptomics, morphology, and function seen in iPSC-derived cortical neurons harboring huntingtin mutations	(Mehta et al., 2018)
	Genome edited hESC lines carrying varying length of polyQ repeats	Expanded polyQ repeats in huntingtin	In neurons, genes associated with cell cycle progression, among others	PolyQ repeat length-related abnormalities include mitochondrial respiration, oxidative stress, and enhanced susceptibility to DNA damage	(Ooi et al., 2019)
ALS	iPSC-derived neurons from fibroblasts of C9orf72 ALS patients	G_4C_2 repeat expansion in C9orf72	Genes involved in membrane excitability, including DPP6, among others	Altered gene expression by abnormal G_4C_2 repeat expansion leading to the diminished capacity for motor neurons to fire continuously	(Sareen et al., 2013)

Table 2a
Selected reports utilizing 3D iPSC models and organoid technologies for the characterization of neurodegenerative diseases (Spinal Muscular Atrophy: SMA).

	Sample origin	*In vitro* model	Observation/Implication	Reference
AD	Aβ oligomer treated hiPSC	3D iPSC model	Characterization of 3D *in vitro* AD model with disease mirroring qualities	(Zhang et al. 2014)
	fAD patients	3D hNSC model	High levels of phosphorylated tau aggregates in 3D *in vitro* model with fAD mutations	(Choi et al. 2014)
	fAD patients with APP or PSEN1 mutation	Brain organoid model	Amyloid and tau pathology significantly reduced by β- and γ-secretase inhibitors	(Raja et al. 2016)
	sAD patients	3D iPSC neuro-spheroid model	Differential effects of candidate drugs, such as BACE1, to 2D and 3D *in vitro* AD models	(Lee et al. 2016)
	Mutant APP expressing hNPCs	3D triculture (neurons, astrocytes, and microglia) model	Neuron and astrocyte loss resulted from microglial recruitment in *in vitro* AD models	(Park et al. 2018)
FTD	FTD patient with Tau P301 L mutation	Cerebral organoid model	Neuroinflammation, synaptic loss, Aβ accumulation, and tau hyperphosphorylation induced by abnormal Cdk5 activation in p25 accumulated *in vitro* FTD model	(Seo et al. 2017)
PD	iPSC-derived dopaminergic neurons	3D microfluidic cell model	Characterization of 3D microfluidic PD model for industrial-scale personalized drug discovery	(Moreno et al. 2015)
	Human neuroepithelial stem cells	Mid-brain specific organoid models	Characterization and development of mid-brain specific organoid for *in vitro* PD model	(Monzel et al. 2017)
	fPD patient with LRRK2 gene mutation	3D human neuroectodermal spheres intestinal organoid models	Known PD defects recapitulated in 3D intestinal organoids	(Son et al. 2017)
	G2019S mutated LRRK2 containing iPSC	Mid-brain organoid model	Characterization of the functional importance of TXNIP in LRRK2-associated PD	(Kim et al. 2019)
	PD patients with LRRK2 G2019 K mutation	Mid-brain organoid model	Neurodevelopmental defects related to increased FOXA2 in PD midbrain organoids	(Smits et al. 2019)
SMA	SMA Patient	Spinal organoid model	Motor neuron degeneration prevented by CDK4/6 in SMA spinal organoids	(Hor et al. 2018)

transcriptomes of 80,660 single cortical nuclei (Mathys et al. 2019). The authors identified six major pathological subpopulations that were transcriptionally distinct, and, surprisingly, found major cell-type-specific transcriptional changes that appeared in the early stages of pathological progression. Late-onset AD patients exhibited upregulated genes that were common across cell-types and were generally involved in the global stress response. Although the authors speculate that the transcriptional alterations stemmed from changes in cell state, the biological underpinnings of those changes remain unclear. Investigations involving the disease-associated epigenetic alterations may provide additional missing details that may explain the early manifestation of transcriptional aberrations. In sum, not only does the study by Mathys et al. demonstrate the utility of single-cell transcriptomics in AD research (by revealing the complexity of altered cell-type-to-cell-type interactions in AD), but it also hints at the importance of sampling primarily disease-associated neurons from undiagnosed human subjects early in disease development. We will further explore the latter issue in the following section.

Single-cell transcriptome analysis can identify distinct gene expression patterns and specify the cell-type and state of a given cell in tissue samples. Epigenetic marks such as DNA methylation (Mulqueen et al., 2018; Smallwood et al., 2014), histone modification (Rotem et al., 2015), chromatin accessibility (Buenostro et al., 2015; Jin et al., 2015; Cusanovich et al., 2015), and chromatin conformation (Nagano et al., 2013; Flyamer et al., 2017; Kind et al., 2015) have also been profiled at the single-cell resolution to improve the characterization of cell-types or states associated with neurodegenerative diseases. For example, co-profiling transcriptomes and epigenomic alterations at the single-cell level allowed Lake et al. to successfully map the AD-associated gene BIN1 to microglia (Lake et al., 2018).

The technical limitations of utilizing neural organoids for the discovery of ETA-based biomarker are worth discussing as well. Two potential concerns are in the organoid technology itself: high organoid-to-organoid variability and lack of key neural structures (such as vesicular structures) may prohibit reliable *in vitro* modeling of *in vivo* neural compartments. While these concerns are indisputably valid, extensive research in recent years has yielded major improvements in organoid technology. The high organoid-to-organoid variability, and, consequently, low organoid reproducibility, stems from extremely complex experimental conditions which may differ between labs and experimenters. These experimental conditions are of prime interest in the generation of faithful *in vitro* neural organoids that display disease-associated epigenetic and/or consequent transcriptional profiles. In addition, the disproportionate distribution of oxygen and nutrients due to the lack of proper organoid microvasculatures may cause ectopic influences that extraneously alter the epigenetic and/or consequent transcriptional profile of cells. With recent improvements in organoid-generating protocols, however, many of these obstacles can be addressed. For example, protocols for producing 3D brain organoids and spheroids with high reproducibility were published by Velasco et al., showing that 95% of the organoids generated virtually indistinguishable compendium of cell-types in 166,242 cells isolated from 21 individual organoids (Velasco et al., 2019). This study, as well as many others, is expected to make the protocols for generating neural organoids uniform, and thus are expected to yield uniform organoids. In terms of organoid-associated vesicular structures, Cakir et al recently engineered a human cortical organoid with functioning vasculature-like structures that possess several characteristics of the *in vivo* blood-brain barrier (Cakir et al., 2019). Although there is still room for improvement, neural organoid technology is advancing at an accelerated pace. Recognizing the potential of organoid technology and anticipating future improvements, we reason that organoid technology will be highly effective in the identification of ETA-based biomarkers for the early diagnosis of neurodegenerative diseases.

Table 2b
Selected reports utilizing iPSC models for the characterization of neurodegenerative diseases.

	Sample origin	*In vitro* model	Observation/Implication	Reference
AD	fAD and sAD patients	iPSC-derived neurons	Higher levels of pathological AD markers in iPSC-derived neurons	(Israel et al., 2012)
	fAD and sAD patients	iPSC-derived neurons	Stress responses alleviated by docosahexaenoic acid treatment in AD iPSC-derived neural cells	(Kondo et al., 2013)
	fAD patient	iPSC-derived forebrain neurons	Increased level of both APPsβ and Aβ by elevated β-secretase cleavage of APP	(Muratore et al., 2014)
	Isogenic iPSC lines with APOE4 mutation	iPSC-derived brain neurons, microglia, and astrocyte cells	Increased synapse number and elevated Aβ42 secretion shown in APOE4 mutated neurons	(Lin et al., 2018)
	Early-onset type fAD with PSEN1 exon 9 deletion	iPSC-derived astrocytes	Manifestation of AD hallmarks, including increased β-amyloid production, altered cytokine release, and dysregulated Ca2+ homeostasis, in iPSC-derived astrocytes	(Oksanen et al., 2017)
PD	PD patient with homozygous p.G2019S mutation in the LRRK2 gene	iPSC-derived DAn	Manifestation of increased expression of key oxidative stress-response genes and α-synuclein protein in iPSC-derived PD neurons	(Ha et al., 2011)
	PD patients with Parkin mutations	iPSC-derived midbrain DAn	Elevated oxidative stress though abnormal transcription of monoamine oxidases A and B seen in iPSC-derived midbrain DAn	(Jiang et al., 2012)
	Isogenic iPSC lines with SNCA-A53 T mutation	iPSC-derived DAn	A characterization of a transcriptional pathway related to basal and mitochondrial toxin-induced nitrosative stress	(Ryan et al., 2013)
	Gaucher's disease and PD patients with GBA1 mutations	iPSC-derived midbrain DAn	Reduction in glucocerebrosidase activity and increase in glucosylceramide and α-synuclein levels in iPSC-derived neurons	(Schondorf et al. 2014)
	Young-onset PD patient with no known PD mutations	iPSC-derived midbrain DAn	Accumulation of soluble α-synuclein protein and phosphorylated protein kinase Cα and reduction of lysosomal membrane proteins seen in iPSC-derived DA neurons	(Laperle et al., 2020)
ALS	ALS patients with SOD1 mutations	iPSC-derived motor neurons	Neurofilament aggregation followed by neurite degeneration manifested in spinal motor neurons when glia were absent	(Chen et al., 2014)
	ALS patient with TDP-43 or C9orf72 repeat expansion mutations	iPSC-derived motor neurons	Initial hyperexcitability followed by progressive loss of action potential output displayed in patient iPSC-derived motor neurons	(Devlin et al., 2015)
	Sporadic ALS patients	iPSC-derived motor neurons	Characterization of sporadic ALS subclassifications by *in vitro* characteristics	(Fujimori et al., 2018)
	ALS patient with C9orf72 repeat expansion mutations	iPSC-derived astrocytes	Secretion downregulation of several antioxidant proteins in C9-mutated astrocytes	(Birger et al., 2019)
FTD	FTD and presymtomatic patients with long C9orf72 hexanucleotide repeat expansion	iPSC-derived neurons	Manifestation of instability during reprogramming and neuronal differentiation of iPSCs in G_4C_2 expanded cells	(Almeida et al., 2013)
HD	HD patients with polyglutamine expansion in huntingtin gene	iPSC-derived neural stem cells and striatal neurons	Reduction of toxicity in HD patient-derived iPSC lines when polyQ repeat length decreases	(An et al. 2012)
	HD patients with polyglutamine expansion in huntingtin gene	iPSC-derived neural stem cells and striatal neurons	Altered gene and protein expression patterns in HD patient-derived iPSC	(Consortium, 2012)
	HD patients with low polyQ repeat expansions in huntingtin gene	iPSC-derived GABA MS-like neurons	Disease pathology such as huntingtin protein aggregation, abnormal lysosomes/autophagosomes, nuclear indentations, and enhanced neuronal death during cell aging is seen in iPSC-derived neurons	(Nekrasov et al., 2016)
	Juvenile-onset HD patients with polyQ huntingtin mutations	iPSC-derived deep and upper cortical neurons	Altered neuronal development, neurite morphology, and electrophysiology is seen in HD iPSC-Derived cortical neurons	(Mehta et al., 2018)

4.2. The practical application of found ETA-based biomarker for the early diagnosis of neurodegenerative diseases using human iPSC-derived samples

Once neurodegenerative disease-associated ETA-based biomarkers have been characterized in organoids containing heterogenous cell types, the diagnosis of neurodegenerative diseases can be achieved through iPSC-based sampling of specific disease-associated neurons. In other words, while the discovery of ETA-based biomarkers benefits from the heterogeneous nature of organoids, for the targeted detection of already characterized ETA-based biomarkers, iPSC technology is highly preferred as it allows for the production large number of a single cell type (e.g. dopaminergic neurons for the diagnosis of PD). The advent of iPSC technology enables sampling of primarily disease-associated cells that retain genetic risk variants and the epigenetic and/or consequent transcriptional profiles of patients, enabling the discovery of valuable signals indicative of disease onset. Of note, *in vitro* modeling of human neurodegenerative diseases has frequently been carried out using genetically engineered iPSCs that have been differentiated into disease-associated cell-types (Table 2b). These studies indicate the feasibility of using iPSCs for the non-invasive sampling of primarily disease-associated neurons in neurodegenerative diseases.

We propose the use of primarily disease-associated neurons, obtained from differentiated iPSC, for the detection of ETA-based biomarkers in undiagnosed patients (Fig. 2). This requires the development of efficient, standardized culture conditions that allow the differentiation of stem cells into specific disease-associated cell-types (Li et al., 2019). A process that maximizes cellular uniformity in the *in vitro* differentiation process will be necessary, as iPSC-derived neural culture cells are notoriously (and undesirably) heterogeneous. If the efficiency of differentiation is low, the target cell purity may also be low, and it may therefore be difficult to observe the phenotype of interest. In such case, here too, would be advisable to incorporate single-cell level analyses to detect our ETA-based biomarkers.

The feasibility of our proposal has been best hinted at in an article by Fernández-Santiago et al. which pointedly demonstrated that iPSC-derived neurons from PD patients exhibit differentially methylated CpG sites. Notably, such disturbances were observed when familial PD patient-derived fibroblasts were differentiated into dopaminergic neurons but, importantly, not in non-dopaminergic neurons (Fernandez-Santiago et al., 2015). Surprisingly, sporadic PD patient-derived dopaminergic neurons, too, had comparable DNA methylation aberrations, suggesting not only that familial and sporadic PD may partly converge on similar pathogenetic mechanisms, but also that our proposed strategy may be effective in some cases of sporadic neurodegenerative disease. This hypothesis will, of course, require scrutiny before it is applicable to clinical use.

Another study worth mentioning is by Lang et al. In this study, the effect of GBA mutation was examined in both bulk and single-cell resolution via cell-type-specific collection of iPSC-derived dopaminergic neurons using fluorescence activated cell sorting (FACS). Using a set of genes that captured an axis of variation between control cells and cells from patients with PD carrying GBA mutation, an epigenetic modifier, histone deacetylase 4, was identified as an upstream regulator of disease progression. This epigenetic modifier was subsequently identified as a new therapeutic target of certain cases of PD (Lang et al., 2019).

5. Conclusion

Our proposed strategy requires ETA-based biomarkers that originate from disease-associated genetic aberrations. It may be presumed, therefore, that our proposed model is primarily applicable to inherited forms of neurodegenerative diseases, which make up only 5-10% of neurodegenerative disease cases. Although our proposed model is ideal for the study of inherited forms of neurodegenerative diseases, the potential utility of our proposed strategy extends far beyond these cases. In fact, the strategy we propose may be applicable to some cases of sporadic neurodegenerative diseases without clear genetic contributions (as mentioned above in our discussion of the article by Fernández-Santiago et al.) and sporadic neurodegenerative diseases that are associated with sporadic genetic mutations acquired during early development. For example, the abnormal G_4C_2 repeat expansion in the C9orf72 is observed in one third of familial cases and, in some locations, up to 10% of sporadic cases of ALS (Al-Chalabi and Hardiman, 2013; Hodges, 2012). It follows, therefore, that if G_4C_2 repeat expansions can induce ETAs, then even sporadic cases of G_4C_2 repeat expanded ALS can be diagnosed using our proposed strategy.

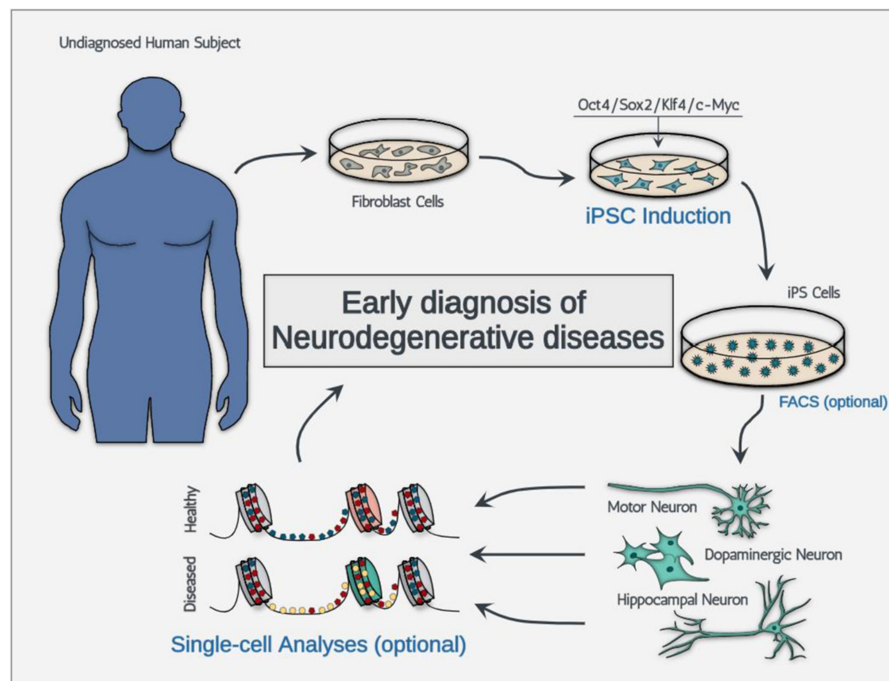

Fig. 2. A schematic illustration showing the conceptual diagnostic workflow consisting of iPSC - derived neurons for early diagnosis of neurodegenerative diseases.

Although we believe that our proposed strategy should be used in conjunction with genomics-based diagnosis of neurodegenerative diseases, there are some important advantages to the ETA-based biomarker strategy. First, hidden genetic risk factors may lead to ETAs, which can be exploited as early biomarkers for neurodegenerative diseases. Like familial inherited disease cases, some cases of sporadic neurodegenerative diseases originate from sporadic genetic mutations acquired during early developmental stages (as in the G_4C_2 repeat expansion ALS example above). It is therefore conceivable that a proportion of sporadic neurodegenerative diseases, which represent 90-95% of neurodegenerative disease cases, are associated with unknown or otherwise hidden genetic causes or risk factors [this was recently hinted at in a recent article by Laperle et al. which identified a molecular signature in iPSC-derived models of sporadic PD, suggesting that there might be acting unknown genetic contributor(s) in previously categorized sporadic PD (Laperle et al., 2020)]. Assuming that these hidden genetic aberrations induce disease-associated ETAs, ETA-based biomarkers may be used to diagnose more than the 5-10% of neurodegenerative disease cases that are known to arise from inherited mutations. Second, except in certain neurodegenerative disease cases that are caused by an aberration of single genetic factor (e.g. a known mutation in a single disease-causing gene), neurodegenerative diseases may be affected by multiple genetic risk factors that act in concert, leading to the manifestation of disease-specific characteristics. For example, the intermediate-length of polyglutamine domain in the Ataxin-2 protein is a modifier of TDP-43 toxicity, a well-known hallmark of ALS (Elden et al., 2010), while Ataxin-2 is not known as a disease-causing genetic factor. Accordingly, we postulate that if multiple genetic risk factors in combination converge on specific disease-associated pathogenic mechanisms (e.g. mitochondrial dysfunction in PD or TDP-43 toxicity in ALS), then the analysis of ETAs will have a broader coverage of neurodegenerative disease than the analysis of known mutations. In other words, in addition to genetic mutations that directly cause neurodegenerative diseases, certain combinations of known (and potentially hidden) genetic risk alleles may be diagnosed using our strategy if this combination produces disease-representative ETAs.

In this review, we have presented our perspectives on the discovery of ETA-based biomarkers and their practical application to the early diagnosis of neurodegenerative disease based on recent advances in patient-derived organoid, patient-derived iPSC, and single-cell level analysis technologies. Although issues such as high cost may potentially prevent the widespread use of ETA-based biomarkers at this time, we are optimistic that our proposed strategy will be generally useful, especially considering the rapid pace at which technology continues to advance.

Declaration of competing interest

None.

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