From iPS Cells to Rodents and Nonhuman Primates: Filling Gaps in Modeling Parkinson’s Disease

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ABSTRACT: Parkinson’s disease (PD) is primarily known as a movement disorder because of typical clinical manifestations associated with the loss of dopaminergic neurons in the substantia nigra. However, it is now widely recognized that PD is a much more complex condition, with multiple and severe nonmotor features implicating additional brain areas and organs in the disease process. Pathologically, typical forms of PD are characterized by the accumulation of α-synuclein-rich protein inclusions known as Lewy bodies and Lewy neurites, although other types of protein inclusions are also often present in the brain. Familial forms of PD have provided a wealth of information about molecular pathways leading to neurodegeneration, but only to add to the complexity of the problem and uncover new knowledge gaps. Therefore, modeling PD in the laboratory has become increasingly challenging. Here, we discuss knowledge gaps and challenges in the use of laboratory models for the study of a disease that is clinically heterogeneous and multifactorial. We propose that the combined use of patient-derived cells and animal models, along with current technological tools, will not only expand our molecular and pathophysiological understanding of PD, but also assist in the identification of therapeutic strategies targeting relevant pathogenic pathways. © 2020 The Authors. Movement Disorders published by Wiley Periodicals LLC on behalf of International Parkinson Movement Disorder Society.

Key Words: iPS cells; animal models; α-synuclein; neurodegeneration; pathophysiology

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Parkinson’s disease (PD) is clinically recognized by the occurrence of movement manifestations collectively referred to as “parkinsonism,” consisting of bradykinesia, rigidity, resting tremor, and postural abnormalities (reviewed in reference 1). During the past 2 decades, it has become increasingly clear that PD patients are variably affected by a large number of nonmotor symptoms, the most common of which are
autonomic dysfunctions, hyposmia, sleep problems, and cognitive impairments. Along with a growing realization of these complex features, neuropathological studies have revealed that PD involves not only dopamine (DA) neurons in the substantia nigra, but also a variety of nondopaminergic neuronal systems in several brain regions. In 2003, a landmark study indicated that the deposition of Lewy pathology follows a characteristic spatiotemporal pattern during the natural history of idiopathic PD. Thus, Lewy bodies (LBs) and Lewy neurites (LNs) would first appear in some nuclei of the lower brain stem, then in the midbrain (where the substantia nigra is located), followed by the thalamus, lateral hypothalamus, basal forebrain, and other subcortical nuclei. Finally, in the most advanced disease stages, LBs and LNs would invade the cerebral cortex. Although the relationship between Lewy pathology and neuronal loss is quite unclear in most regions, and these neuropathological observations fit well with the notion that PD is associated with a variable degree of cholinergic, serotonergic, and noradrenergic cell loss, as well as degenerative changes in several subcortical nuclei. In addition, it has long been known that PD also affects both sympathetic and parasympathetic branches of the autonomic nervous system, as well as the enteric nervous system.

Owing to recent developments in PD genetics, in-depth clinical evaluations, neuropathological findings, and studies on patient-derived biological samples, we now have a deeper comprehension of the complexity of this disease and a new awareness of the difficulties facing the development of novel therapies. Therefore, the experimental modelling of PD has never been so challenging. However, model systems are instrumental for understanding the mechanistic underpinnings of any disease, and PD is not an exception. To create suitable model systems, we need to first understand the human disease and then reflect on what can and should be modeled in the laboratory for different purposes. Inevitably, existing gaps in our understanding of PD affect our ability to develop reliable models for either recapitulating the disease process or designing novel therapeutic strategies.

Here, we discuss possibilities, gaps, and controversies about creating experimental models of PD for translational research. In particular, we focus on patient-derived in vitro, rodent, and nonhuman primate models that are commonly used for preclinical validation of therapeutic principles.

What We Know and What We Can Model

The realization that PD is both clinically and pathologically heterogeneous has brought about an awareness that no single experimental model can capture the entire complexity of this nosological entity. PD is, after all, a human-specific disease, as it has not been observed in other animal species. In fact, even human PD-like conditions such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced parkinsonism or Mendelian parkinsonian disorders may not provide a sufficiently good “model” of the common age-related forms of PD that are referred to as idiopathic. Nevertheless, some key clinicopathological features of PD are possible to model in laboratory animals to explore their consequences on a functional, neurochemical, or molecular level.

Motor Features and Nigrostriatal DA Degeneration

A severe loss of dopaminergic afferents to the posterior part of the putamen is a prerequisite for the appearance of parkinsonian motor symptoms, and these are, in turn, essential for diagnosing PD. By the time parkinsonian motor features become manifest, more than 50% of putaminal DA contents are already lost, and a rapid loss of the residual putaminal DA occurs during the first 5 years after diagnosis. This degree and pattern of DA degeneration are reproduced in several animal models (see sections on rodent and nonhuman primate models).

One may argue that, although important, nigrostriatal degeneration mimics only 1 pathological aspect of PD. However, severe nigrostriatal DA denervation brings about pervasive secondary alterations to a variety of nondopaminergic neurons and their corresponding neurotransmitters and even to nonneuronal cells. Many of these secondary changes mimic functional and pathological features observed in human PD. Some prominent examples include, the dendritic regression of striatal projection neurons, oscillatory neuronal activities at specific frequency bands, and pervasive adaptations of most (if not all) nondopaminergic transmitter systems in the corticobasal ganglia network. Prominent changes to nonneuronal cells include reactive molecular-structural phenotypes of microglia and astrocytes, as detected in striatal and nigral tissue from both PD patients and DA-denervated animals.

Nonmotor Features and Multisystem Degeneration

The nonmotor features most consistently observed in PD patients are changes in mood and cognitive abilities, sensory impairments, sleep disturbances, cardiovascular dysregulation, gastrointestinal abnormalities, and bladder problems. On a technical level, most of these features can be modelled in mammalian species, as they affect well-conserved anatomofunctional structures. However, all these dysfunctions have also been reported in other neurodegenerative diseases, including frontotemporal dementia, amyotrophic lateral sclerosis, Alzheimer’s disease, and the parkinsonian-
This raises the question of what features are most important to mimic in a model of PD and based on what pathogenetic assumptions. To tackle such a question, one needs to first distinguish between nonmotor features that might occur even before the onset of clinical PD and those that are more prevalent in the advanced stages of the disease, being influenced by dopaminergic degeneration and potentially aggravated by DA replacement therapies (eg, psychosis, behavioral alterations, and cognitive disturbances). Among the early features, REM sleep behavior disorder (RBD) deserves particular mention as it holds high predictive value for a subsequent conversion to PD. The combination of RBD, olfactory loss, and gastrointestinal alterations in the absence of motor deficits fits well with the Braak model of pathology distribution during early disease stages (Braak et al, 2003). Accordingly, different combinations of these nonmotor features have been mimicked in bacterial artificial chromosome transgenic mice overexpressing human α-synuclein (partly reviewed in reference 16).

**Lewy Pathology**

The accumulation of intracellular inclusions known as LBs and LNs is a typical neuropathological alteration in the brains of people with PD, being used as a postmortem diagnostic criterion for PD along with detailed clinical information. LBs and LNs contain large amounts of α-synuclein (aSyn), an abundant protein in the brain that seems to be involved in synaptic vesicle biology. Importantly, Lewy pathology is also present in other diseases (referred to as LB diseases). Moreover, some of the most common genetic forms of PD (ie, those linked with leucine-rich repeat kinase 2 mutations) often lack Lewy pathology in the brain. Therefore, one cannot bluntly say that cells or animals displaying aSyn aggregation provide specific models of PD. Nevertheless, as the Braak staging theory gained popularity, aSyn aggregation became the subject of extensive investigations in model systems. Thanks to this research, we now have a reasonable understanding of the various steps involved in the protein aggregation process (Fig. 1). Yet despite tremendous efforts by the community, we still cannot say that we can fully recapitulate and model human LB formation in cells or animals, as the full molecular identity of LB is still not fully understood. Nevertheless, we can argue that using these experimental models, we can induce an accumulation of aSyn resembling some aspects of LB formation, such as the buildup of aSyn oligomeric and fibrillar species, their interaction with other proteins also present in LBs, the occurrence of certain posttranslational modifications of aSyn that are abundant in LBs, and the presence of certain membranes and cellular components that are also observed in LBs.18-21

**Gaps and Controversies**

Until any disease-modifying treatment for PD is successfully translated from the laboratory to the clinic, it appears unwarranted to either rate different models according to a degree of confidence or to claim that they hold a similar translational potential. However, past failures to achieve neuroprotection in PD urge critical reflection on how treatments are to be tested before undertaking expensive clinical trials. As preclinical
researchers, we strongly believe that the selection of interesting candidate interventions should be based on the soundest clinically driven preclinical validation and achieved through step-wise examination of the therapeutic principle and its target in the most appropriate experimental models.

In general, cell-based models of PD are particularly useful for molecular and mechanistic studies in which genes and pathways need to be readily manipulated. In addition, they are ideal for initial rapid, high-throughput screens (genetic or pharmacological) to define and characterize putative targets. Animal models are essential to test mechanistic hypotheses that lead to typical symptoms and to test therapeutic strategies at the whole-organism level (Fig. 2). This level of complexity cannot be replicated in vitro because of the large number of different cells, tissues, and circuits that make up a living animal. Although most investigators probably agree with these notions, the utility of animal models of PD has become a matter of controversy. For example, it was recently proposed that we should not “waste our time at studying imperfect models” but rather undertake experimental studies directly in PD patients. In addition to problematic ethical and logistic implications, this standpoint ignores the scientific necessity of using cells and animals to dissect the key elements of complex pathogenic cascades and demonstrate their causal interrelationships. Likewise, understanding the biological effects of candidate therapies requires their evaluation in experimental models that are fully accessible to in-depth investigations. Having said that, we should also acknowledge that major differences exist between laboratory animals and human subjects on several parameters of relevance to PD, such as longevity, age-related alterations, brain size, gene–environment interactions, and genotype–phenotype relationships. These are actual gaps that cannot be fully eliminated. Instead, we should understand and acknowledge these gaps and take them into account to optimize the use of existing models for translational purposes, as further discussed in the concluding section of this article. Below, we will specifically discuss the possibilities and limitations of models that are widely considered as relevant for translational PD research.

Patient-Derived iPS Cell Models

Somatic cells such as fibroblasts or peripheral blood mononuclear cells can be taken from a human subject, reprogrammed into inducible pluripotent stem cells (iPSCs), and differentiated into certain disease-relevant cell types that can be used for in-depth biological studies.

Although simple eukaryotic cells such as yeast and immortalized, highly proliferating cell lines such as HEK293, H4, or SH-SY5Y neuroblastoma cells have been widely used to study and manipulate many essential biological processes, iPSC-based models offer the advantage that they can be differentiated into virtually any (specialized) cell type in the human body and thus can be used to study processes specific to a cell type, such as for example, synaptic activity or axonal transport in neurons or the inflammatory response in microglia. In PD, the differentiation of patient-derived iPSCs carrying mutations associated with familial forms of the disease into dopaminergic (DA) neurons has provided important insights into the biological consequences of the identified mutations expressed at the endogenous level (Fig. 2). The transcriptomic profiles of purified iPSC-DA neurons are similar to human postmortem DA neurons. However, it is important to

**FIG. 2.** In vitro and in vivo models for studying PD. PD patients exhibit a variety of motor and nonmotor features that need to be modeled in various models. The iPSC cells derived from patients can be differentiated into neurons or other relevant cell types for in vitro studies. Nonhuman primates and rodents can be engineered to model PD-relevant features. Ultimately, it is essential to use and combine various models in a back-and-forth manner to model PD, to improve models, and to develop a better diagnosis and therapies. [Color figure can be viewed at wileyonlinelibrary.com]
consider a gap that occurs during the reprogramming process of primary cells into iPSCs: the epigenetic signatures of the original cell type are largely erased, and, consequently, the differentiation process into, for example, neurons results in cells that are similar to embryonic neurons and do not reflect many of the age-associated alterations.

With the development of gene-editing techniques, such as TALENS and CRISPR/CAS9, isogenic iPSC lines can be generated in which only the pathogenic mutation has been corrected to the wild-type sequence, thereby minimizing phenotypic variability because of differences in genetic background between patient and control cells, and therefore the observed phenotypes of the cells can be more clearly dissected and associated with the presence of the pathogenic mutation.28-30,33,34

The advantage of iPSC-based cellular models is obvious when we want to model familial forms of PD with a clear-cut pathogenic mutation. However, the vast majority of PD cases do not exhibit a clear Mendelian inheritance, and the risk for disease is a combination of genetic and environmental risk factors. Consistently, recent genetic studies have now identified more the 90 genetic risk factors that act in concert with different combinations of other factors.35 Therefore, patient-derived iPSC lines offer a realistic possibility to study the multitude of combinations of different risk alleles, but large numbers of iPSC lines generated from sporadic patients with different risk profiles need to be used. Although this enables the study of the biological consequences of different combinations of risk factors, this approach has the limitation that fully gene-corrected controls cannot be made for all the risk variants because the actual disease-associated variants have not been identified and because of the number of risk variants present. Although the scale of this approach is very large, it is already feasible with current technologies (see the Foundational Data Initiative for PD [FONUDIN-PD]; www.foundinpd.org). Although these 100 lines do not capture the full genetic complexity of sporadic PD, they contain multiple carriers of the strongest risk factors for PD, with 4 lines generated containing a Mendelian mutation in SNCA, the gene encoding for aSyn, and many lines that carry risk variants in LRRK2 (>20) or GBA (>20) or combinations of risk alleles at loci identified by genome-wide association studies, allowing the study of common and distinct effects between these major genetic risk groups. Initial data analysis is already showing that lines can be separated based on their genetic background and that scaling up the number of lines to include more risk profiles is indeed feasible. These lines may also prove powerful for testing personalized therapies, in which specific drugs or other treatments may be easily screened, opening novel perspectives into the era of precision medicine.

As with all model systems, iPSC-based models also have shortcomings and controversies. Working with iPSCs is labor intensive, time consuming, and expensive. Although the number of established protocols for differentiating iPSC into different cell types is quickly increasing, they often have a limited efficiency and result in mixed cultures of specialized cells with precursor cells.36-39 As a result, some phenotypes that are cell-type specific might be masked, and single-cell technologies such as single-cell RNA sequencing are important for dissecting expression data per cell type. In addition, automation protocols for iPSC growth and differentiation are being developed to overcome experimental variation and reduce labor.40

Despite these limitations, iPSC-based models offer exciting potential for developing scalable, screenable models of disease in vitro using disease-relevant cell types with patient-specific genetic backgrounds. In addition, iPSCs allow us to establish cocultures of neurons, astrocytes, and microglia from the same patient line to mimic more closely the cellular surrounding of a living tissue (especially when 3-dimensional [3-D] culture conditions are applied). In this context, one of the most exciting developments in iPSC-based modeling is the development of growing 3-D organoids41-43 that hold the promise of modeling a complete tissue within the context of the full genetic background of a human subject (patient or control).

**Brain Organoids**

Brain organoids are in vitro–derived structures that undergo some level of self-organization and resemble at least early stages of the developing brain in vivo. The transcriptional profiles of organoids cultured for up to 100 days are similar to those of developing human cortex in postconceptional weeks 17–24; however, they seem to present a more mature neuronal population than those of 2-D monolayer-derived neurons.44,45 A variety of protocols for brain organoid generation to model a range of human brain regions have been published.46-54 Important limitations of all these protocols are that a large part of the tissue remains undifferentiated and that organoids derived from the same iPSC line under the same conditions often produce tissues with different regional identities and spatial and cellular heterogeneity.45,55 Until the efficiency of the protocols is significantly improved and the variation reduced, single-cell RNA expression profiling can be used to separate the transcriptomes of undifferentiated versus differentiated cells.45,55,56

A current limitation is that organoids still lack some of the cell types present in the primary cortex, such as endothelial cells and microglia.57-59 However, combining brain organoids with nonneuronal cell types such as microglia-like cells into so-called assembloids can
model neuroimmunological interactions that might exacerbate or protect against neuronal pathology. However, the long culturing conditions and lack of vascularization within organoids remain major obstacles and need to be optimized. In addition, vascularization might enable the incorporation of the blood–brain barrier, which is relevant to many disease processes and pharmacokinetics.

**Rodent Models**

Mice and rats are the species most widely used to create models of PD. Several methodologies have been developed to induce nigrostriatal degeneration in these rodents (which is necessary for the appearance of L-dopa-responsive hypokinetic features). The best validated approaches include (1) intracranial injections of catecholamine-selective neurotoxins (such as 6-hydroxydopamine), proteasome inhibitors, or environmental toxicants (such as rotenone); (2) systemic administration of MPTP (although only effective in mice); (3) intranigral delivery of recombinant viral vectors coding for human aSyn; and (4) intrastriatal or intranigral inoculation of synthetic preformed fibrils of aSyn, which is sometimes combined with viral vector-mediated overexpression of the same protein (reviewed in reference 62). In both mice and rats, loss of dopaminergic innervation to motor striatal regions (the dorsolateral striatum) causes postural abnormalities, reduction in spontaneous forelimb use, slower motion in an open field, and increased muscle resistance to passive stimuli, which shares electromyographic features with parkinsonian rigidity. Phenotypes resembling resting tremor have been reported only from DA-depleted rats, consisting of tremulous forelimb movements that appeared when the limb was positioned off the floor in a non-weight-bearing posture. However, to the best of our knowledge, widely accepted models of parkinsonian tremor have not yet been characterized in rodent species. In addition to their motor features, DA-denervated rodents exhibit neuropsychiatric dysfunction, including motivational deficits, depressive-anxious traits, and cognitive executive deficits. Sleep pattern alterations and autonomic dysfunction have also been observed (reviewed in references 70 and 71). Whether these disturbances share the same causal mechanisms as those observed in PD is uncertain at this point, and this constitutes a gap that needs to be addressed with additional research (Fig. 3). Part of the difficulty in validating nonmotor phenotypes depends on the current lack of effective treatments against the equivalent symptoms in human PD, which precludes assessing the model’s predictive validity. Therefore, it is not surprising that the functional efficacy of candidate neuroprotective treatments in rodent models is still evaluated using motor end points only and that histopathological assessments are usually restricted to the substantia nigra and striatum. However, using a larger number of behavioral, histopathological, and molecular/neurochemical end points would provide a more robust scientific rationale to support or dismiss the relevance of an investigational treatment.

**Nonhuman Primate Models**

Studies modeling PD in nonhuman primates (NHPs) are still limited. The number of monkeys used in research is anecdotal. In the PD field, for example, studies in monkeys are extremely rare compared with studies in species such as rodents (less than 0.1% vs 80%, respectively). Of all animal models used in neuroscience research, the monkey is the animal whose brain is most similar to that of humans, not only in terms of morphology and wiring, but also in cellular biology and physiology, for example, the presence of neuromelanin in dopaminergic neurons (which rodents lack).

NHPs must be used in an ethically responsible manner, but they constitute an important asset in the field of PD research, with a history of successful translation from the bench to the clinic for the management of parkinsonian symptoms. The PD field is fortunate to have, since the 80s, the neurotoxin MPTP for modeling the consequences of the nigrostriatal denervation on behavior and also in the pathophysiology of the central nervous system in an animal species that is closest to humans.

MPTP-induced parkinsonism has dominated the field of the phenotypic models in cynomolgus (Macaca fascicularis) and rhesus (Macaca mulatta) macaques,
both of which display the most human-like motor and nonmotor symptoms associated with PD\textsuperscript{79,80} (but not gastrointestinal dysfunction, for example), as well as the hyperkinetic manifestations associated with dopaminergic replacement therapies\textsuperscript{81} (but not the motor fluctuations associated with PD progression).

However, further understanding of the mechanisms of PD etiology and cell death required the development of other true pathocpic models. Among the initial attempts was the local transgenesis, which has the advantage of inducing a prolonged effect and modeling disease pathophysiology by promoting the production of a pathological protein. Given the putative central role of aSyn in PD, aSyn overexpression models were generated based on the stereotactic delivery of adeno-associated virus or lentiviral vectors,\textsuperscript{82-87} encoding WT or A53T aSyn in adult monkeys. In monkeys, 50\% reduction in the number of nigral dopamine neurons and 50\% reduction in striatal dopamine were obtained independently of the age of the monkeys.

Attempts to achieve systemic expression of aSyn was based on viral administration on postnatal day 1,\textsuperscript{88} comparable to what was done in rodents. Although successful in terms of transfection,\textsuperscript{89} the resulting behavioral and pathological studies are still ongoing. Likewise, studies based on in utero delivery into the brain of macaque monkeys\textsuperscript{90} are underway, following procedures used for studying other conditions.\textsuperscript{91} Classic transgenesis, as for rodents, is also being developed but has not yet been applied to modeling PD. Thus far, it has been used almost exclusively for modeling Huntington’s disease.\textsuperscript{92-94}

Although parkinsonian NHP models have excellent face validity for many applications, the high costs and advanced organizational tools associated with NHP research are a matter of concern in an area such as PD neuroprotection, which requires undertaking studies of long duration on a sufficient number of animals. In addition, when the cost of preclinical therapeutic research becomes too high, the relevant stakeholders usually opt for direct transition to the clinical evaluation phase. However, this situation may change in the future if the putative disease-modifying treatment were proven to fill important gaps and target a uniquely primate-specific gene or protein variant.

**Conclusions**

How can one establish a confidence-rating system for different preclinical models of PD, and what variables should be integrated into such a system? We will not solve here an issue that is the cornerstone of pharmaceutical therapeutic development. However, in our opinion, a number of validity criteria should be fulfilled in the preclinical evaluation of candidate therapies. The first criterion is demonstration that the target mechanism is relevant to human PD, which can be obtained by studying human samples, whether they are postmor tem tissue or biological fluids. The chosen models in cells and animals should then exhibit comparable changes. It is striking to note that these basic considerations have not been fulfilled in many past studies. In addition, the vast majority of preclinical studies have involved only 1 animal model, raising an immediate question of generalizability. As a second criterion, we propose the use of several intrinsically different animal models to cross-validate a positive result. This should become standard praxis in a single laboratory as well as between independent laboratories. As to the models of aSyn pathology, we still need to “close the gap” in our ability to model LB formation. To this end, we need to understand how LBs form in the human brain, what they are composed of, and, ultimately, whether they are drivers of pathogenesis or simply bystanders of the neurodegenerative process. Nevertheless, we now have unique opportunities to use different cell and animal models of PD together with different inocula containing “disease-relevant” aSyn aggregates (as obtained from either recombinant or, even better, human brain-derived proteins).

The third criterion pertains to the appropriateness of the experimental design used to demonstrate efficacy of a neuroprotective strategy. Although PD is a progressive neurodegenerative disorder, a large majority of candidate neuroprotective treatments (whether targeting aSyn or other pathways) have been tested using a prophylactic exposure or concomitant administration. Thus, although PD patients are likely to receive a neuroprotective agent following diagnosis, that is, when the extent of dopamine neuron degeneration is already approximately 50\%,\textsuperscript{8,95} therapeutic candidates are tested in association with or long before the emergence of clear nigrostriatal pathology. With this type of study design, it is not at all surprising that, despite the strengths of the model at hand, it has not been possible to translate positive results from the laboratory to the clinic. Related to study design are also the methods of brain delivery (particularly in the case of gene therapy or trophic factor infusion) and the evidence of target engagement in vivo. In the booming field of synucleinopathies, we should build on past failures in other disorders (eg, Alzheimer’s disease) to minimize failures in future clinical trials. Therefore, keeping an open mind is essential.

The fourth criterion is a clear definition of the actual therapeutic objectives. Terms such as “neuroprotection” and “disease modification” raise questions about what a given strategy is meant to achieve, Truly protecting the neurons from degenerating? Potentiating the function and plasticity of residual neurons? Slowing down the prion-like
spreading of aSyn aggregates? Decreasing the load of monomeric aSyn? Resolving already existing aSyn pathology? Many studies are unclear about the true therapeutic objective and, importantly, about how a positive result obtained in the experimental model can be translated into an exploitable clinical trial end point.

In conclusion, models are indispensable tools for the study of PD, and understanding the strengths and limitations of each model will help investigators to ask the right question with the right experimental approach. Among all models, patient-derived in vitro models, rodents, and nonhuman primate models are important and complementary tools in our quest to understand, diagnose, and treat neurodegenerative disorders. We argue that in the field of translational PD research, the rigorous utilization of several of these models currently represents the best strategy to assess the potential validity of disease-modifying strategies at multiple levels.

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