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The potential of induced pluripotent stem cells in models of neurological disorders: Implications on future therapy

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Abstract
There is an urgent need for new and advanced approaches to modeling the pathological mechanisms of complex human neurological disorders. This is underscored by the decline in pharmaceutical research and development efficiency resulting in a relative decrease in new drug launches in the last several decades. Induced pluripotent stem cells represent a new tool to overcome many of the shortcomings of conventional methods, enabling live human neural cell modeling of complex conditions relating to aberrant neurodevelopment, such as schizophrenia, epilepsy and autism as well as age-associated neurodegeneration. This review considers the current status of induced pluripotent stem cell-based modeling of neurological disorders, canvassing proven and putative advantages, current constraints, and future prospects of next-generation culture systems for biomedical research and translation.

Keywords
cells, stem, pluripotent, therapy, future, implications, induced, disorders, potential, neurological, models

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The potential of induced pluripotent stem cells in models of neurological disorders: implications on future therapy

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Summary

There is an urgent need for new and advanced approaches to modelling the pathological mechanisms of complex human neurological disorders. This is underscored by the decline in pharmaceutical research and development efficiency resulting in a relative decrease in new drug launches in the last several decades. Induced pluripotent stem cells represent a new tool to overcome many of the shortcomings of conventional methods, enabling live human neural cell modelling of complex conditions relating to aberrant neurodevelopment, such as schizophrenia, epilepsy, and autism, as well as age-associated neurodegeneration. This review considers the current status of induced pluripotent stem cell-based modelling of neurological disorders, canvassing proven and putative advantages, current constraints, and future prospects of next-generational culture systems for biomedical research and translation.

Keywords

induced pluripotent stem cells, neurological disorders, modelling, biomaterials, drug screening, tissue engineering, regenerative medicine, therapy.

Induced pluripotent stem cells (iPSCs) proffer new opportunities to research complex neurological disorders, extant therapies, and develop treatments with improved and personalised efficacy including *in vitro* detection of neurotoxicity. As stem cells engineered from readily obtained somatic cells, iPSCs are able to be derived from patients of all ages with disorders of development and/or neurodegeneration, and provide insight to etiopathology previously unattainable by conventional approaches. That is not to say that iPSC-based research supersedes other methods such as animal-based modelling or human brain tissue studies, but rather it enables a complimentary line of research to advance understanding and treatment of human disease-related neural-phenotypes using living cells and derivative tissues. For example, where analyses of post-mortem tissues have for the most part provided insight to the advanced phases of aberrant development and degeneration[1], and despite dramatic progress in experimental methods for using mice to study monogenic and polygenic traits with relevance to human disease, mouse modelling encompasses a minority of neurological diseases, frequently fails to express every trait of a disorder, while human iPSCs can be derived with the specific genetic traits of any disease from any patient during their entire lifetime. Cells can therefore be used to recapitulate the different stages of a disease and model singular or cumulative effects of defective genes. Additionally, since most diseases involve interaction with environmental risk factors, more sophisticated iPSC-based modelling can incorporate relevant physical and chemical stimuli able to be rigorously controlled and investigated[2]. This approach will be especially useful for studying sporadic or idiopathic forms of a disease to understand gene-environment interactions and disease pathogenesis.

iPSC-based models of neurological disorders

Human neurological disorders can involve the central and peripheral structures of the nervous system and be due to traumatic injury (TI), aberrant neurodevelopment (NDv) and neurodegeneration (NDg). Whereas TI is caused by a focal impact with primary damage at the time of injury and secondary damage in the days thereafter, NDv disorders relate to pre- and post-natal anomalies of the developing nervous system, and NDg disorders are characterised by prolonged neural deterioration due to disease progression. Both NDv and NDg disorders are often associated with specific genetic lesions but can involve non-hereditary stimuli such as environmental stressors. In any event, the short- or long-term outcome will typically be aberrant neuronal morphology, branching and connectivity, with TI and NDg disorders also associated with cell death.

Notwithstanding the potential utility of iPSCs for modelling TI *in vitro*, unlike NDv and NDg disorders, the significance is for the most part limited to providing an alternative source of neural cells and tissues to evaluate the effects of TI and develop strategies to improve cell survival after trauma and endogenous neural stem cell mobilisation to form new functional neurons at the site of injury. Other stem and progenitor cells able to be differentiated to neural lineage can fulfil the same role and animal models for *in vivo* research and development (R&D) are available. Predictably, to the best of our knowledge, no iPSC-based modelling of TI has been described to date. By comparison, disease-specific iPSCs provide new prospects for disease-related R&D by enabling screening for genes and disease processes potentially modifiable by drugs identified through *in vitro* screening. Consequently, iPSCs have been successfully derived from patients with NDv disorders including schizophrenia[3-11], Down's syndrome[12-21], autism spectrum disorders (ASDs) including fragile X, Rett and Timothy syndromes[22-35], and epilepsy[36-39], as well as NDg disorders such as

Alzheimer's disease[40-48], Parkinson's disease[49-64], Huntington's disease[65-71], spinal muscular atrophy (SMA)[72-75], amyotrophic lateral sclerosis (ALS) [76-86], and Friedreich's ataxia [87-89].

As the field moves beyond proof-of-concept for the utility of patient-specific iPSCs, modelling is growing exponentially, with increasingly sophisticated culture systems, cell lines, and characterisation for more informative readouts. A topical study by Bilican *et al* (2012) of iPSCs derived from patients with ALS report an increased sensitivity to a stressor measured by a lactate dehydrogenase (LDH) release assay to measure neuronal survival[86]. The difference between ALS and control cells was not apparent under basal culture conditions, underscoring the need for innovative modelling paradigms to identify potentially significant disease-associated phenotypes.

Another recent study of iPSC-derived neurons from PD patients, demonstrates the importance of selecting appropriate control iPSCs beyond conventional "healthy" cell-lines by showing the need for isogenic gene-corrected hiPSC lines to detect changes specifically associated with mutant Leucine-rich repeat kinase 2 (LRRK2) gene[90]. The mutant phenotypes were not evident using iPSC lines derived from age- and sex-matched control patients.

It is ever more apparent that different disorders will have different requirements for optimal modelling. Similarly, modelling complex and heterogeneous disorders (such as ALS, schizophrenia and ASDs) will undoubtedly benefit from selecting donor cohorts of patients with similar clinical phenotypes, case histories, therapeutic responses and, wherever possible, common genotypes, in addition to tailoring cell culture conditions to account for putative relevant non-hereditary environmental triggers for disease onset.

Neural differentiation of iPSCs: Quality and quantity

The ability to differentiate iPSCs to *bone fide* neurones and supporting cells that accurately imitate the form and function of cells and tissue of the developing and diseased nervous system is a fundamental requirement for modelling. For instance, refinement of differentiation methods to develop specific neuronal subpopulations that are preferentially impaired in a disease will enable more specific and informative mechanistic studies[30]. In spite of a myriad of methods published to date, many based on early protocols for human embryonic stem cell (hESC) differentiation[30,91-95], their application is ordinarily hindered by low and variable efficiency[95]. Although the problem may in part relate to the differentiability of different iPSC lines, incomplete reporting and poor standardisation of process and reagents are likely contributing factors. The former is more difficult to address, however, the latter should theoretically be easily remedied through use of quality controlled cells, processes and reagents, as well as detailed, accurate and transparent reporting of old and new methods employed for publication.

While operators within academic and other publically or privately funded laboratories are presumed to systematically and meticulously develop and execute first-rate protocols for repeatable and precise in-house experimentation, translation for application by the wider field can be hindered by inadvertent or intentional omission of seemingly cursory actions from published documentation, and constrained research budgets can favour the use of cheaper lower grade consumables for R&D. While not the whole solution, advocating standards for the quality and disclosure of materials and methods used to maintain, culture and differentiate iPSCs, including both their strengths and limits, will benefit both research and translation of modelling[96-98]. To this end, influential bodies such as granting agencies, publishing houses and perhaps even regulatory bodies have important roles to play by requiring compliance with

standards in order for a research laboratory to obtain funding, publish and gain approval for clinical trials or therapeutic goods/products[97,98].

In spite of the above mentioned challenges, improvements for iPSC differentiation to neural cells and tissues are being made through the development of better defined, optimised and efficient protocols[30,99-103], bolstered by increased availability of superior stem cells attributable to improved somatic cell reprogramming, stem cell culture, banking and distribution[104-107]. A major advance from traditional differentiation methods is the circumvention of embryoid body (EB) formation for more efficient and direct induction of neural progenitor cells (NPCs) and expansion of neurospheres[99-102]. For example, Lie *et al* proffers high yield production of NPCs from feeder-free iPSC aggregates cultured in mTeSR™1(Stem Cell Technologies)[99]. Intermediate steps include sequential differentiation over 15 days of stem cell aggregates to monolayer neural rosettes that are expanded into free-floating neurospheres[99]. NPCs can be further differentiated into a variety of neuronal subtypes, including dopaminergic neurons within 21 days.

A more protracted method by Shi *et al* induces iPSCs over 90 days to excitatory "cortical projection" neurons, with intermediate "cortical primary" stem/progenitor cells formed within 2 weeks, followed by "early-born" neurons produced between 2-3 weeks, and "last-born" neurons arising as late as day 90 [100]. The method is based on a much earlier protocol of SMAD signalling inhibition[93], and is purportedly highly efficient and less variable among different cell lines due to replacing noggin with SMAD inhibitor dorsomorphin[100]. In addition to modelling cortical cell function and dysfunction, the authors assert the utility of their approach for cortical tissue engineering for transplantation.

Shofuda *et al* propose a three stage protocol for generating neurospheres from human iPSCs by initially using human recombinant noggin medium and poly-L-lysine/laminin substrate to induce NPCs, followed by neurosphere formation with FGF2/heparin medium in low-attachment polyethylene glycol coated microwell plates, and finally neurosphere expansion with EGF/FGF2/LIF medium in flasks [101]. The use of microwell plates ostensibly facilitates quicker, efficient, reliable and more standardised production of neurospheres, and draws from the use of microwell systems for more standardised culture of stem cells and other cells including EBs[101].

A fourth and most recent method by Musah *et al* represents a different approach to neuronal induction by using substratum mechanics rather than soluble signalling factors to regulate neuronal specification from iPSCs[103]. Consistent with advances in biomaterials based cell support and tissue engineering (see below section), whereby physical and other non-chemical stimuli are increasingly being applied to regulate cell fate, Musah and colleagues use hydrogels with elasticity similar to brain tissue to rapidly and efficiently differentiate iPSCs to neurons. Surprisingly, neuronal induction is achievable with mTeSR™1-based culture medium (with or without medium components FGF2, TGF-β or GABA) or basal (DMEM/F12) medium. The protocol underscores the importance and utility of cell substratum for stem cell differentiation, and highlights unconventional cell signalling pathways such as transcriptional co-activator Yes-associated-protein (YAP) as useful targets for controlling neural induction in conjunction with ubiquitous soluble factor signalling (eg. SMAD).

Modelling with biomaterials: The way for the future

Traditional protocols for iPSC maintenance and differentiation rely on methods originally devised for hESCs using two-dimensional (2-D) culture on smooth and

inflexible surfaces such as glass or plastic, with growth media, biochemical supplements such as recombinant growth factors, and either a feeder layer of mitotically-inactivated mouse or human fibroblasts, or feeder free[108-113]. Feeder free platforms are preferable for both R&D and clinical product development (ie. to simplify process and facilitate scale-up from laboratory-based research to industrial-scale biomedicine) and incorporate specialist media, for example mTeSR™ [110,111], StemPro® (Life Technologies), and Essential 8™ (Life Technologies)[114], with more or less complex substrates such as Matrigel™ (Becton-Dickenson; a solubilized basement membrane preparation extracted from mouse Engelbreth-Holm-Swarm sarcoma), single or combinations of extracellular matrix (ECM) proteins such as laminin, fibronectin, vitronectin, and collagen, cell adhesion proteins including E-cadherin, or synthetic peptide coatings such as Synthemax™ (Corning). Matrigel™ comprises variable levels of proteins and growth factors, including laminin, collagen IV, heparan sulfate proteoglycans, and entactin (nidogen), as well as substantial amounts of sarcoma derived growth factors such as TGF-β, fibroblast growth factor, and insulin-like growth factor. Other more defined culture surfaces include Primorigen's StemAdhere™ and Vitronectin XF™, both of which are xenobiotic-free.

Although useful, the classical approaches described above fall short of recapitulating the complex and dynamic environment of cells *in vivo* (ie. the cell niche), with conventional flat-bed culture on a dish or in a flask predictably resulting in markedly different cell behaviour[115]. There is, therefore, scope for newer systems that provide biomimetic environments to create conditions for cells to better mimic their *in vivo* counterparts. Initial strategies have focused on using biocompatible materials with properties of ECM that support cell growth, including ECM stiffness and related mechanical signals for improved and directed cell migration, proliferation and

fate. More recently, electrical stimulation using conductive materials has been shown to effect proliferation, differentiation, migration, and changes in cell adhesion. Not surprisingly, the mechanical and electrical properties of tissues are altered in many disease states, resulting in cellular dysfunction and disease progression. Also, endogenous electric fields occur in body tissues as transepithelial cellular potentials or neuronal field potentials, and are important for tissue regeneration following injury and during embryonic and fetal development, with disturbances to environmental electric fields causing aberrant development[116-119]. Accordingly, elastic modulus and surface properties (eg. topography/roughness) of cell culture substrates and electrical stimulation are being used to control stem cell behaviour and function for basic research and future translational application. To this end, natural and synthetic biomaterials are being identified with different mechanical, chemical, electrical, and physical features of micro- and nanoscale proportions to control cell fate and function for *in vitro* modelling of neural tissues and disease phenotypes [120,121].

Natural biomaterials include Polysaccharides as important components of extracellular matrix (ECM) that can be formulated to rapidly gel for 3-dimensional (3-D) bioprinting, and have been used in various combinations for culture and differentiation of pluripotent stem cells [122-127]. Commonly employed polysaccharide-based 3-D scaffolds include collagen, gelatin, agarose, hyaluronic acid, elastin, alginate and chitosan, each having the potential for use in combination with iPSCs for neural tissue engineering. Not surprisingly, ECM has informed the development of platforms and constructs based on natural biomaterials. Advantages include biocompatibility (essential for *in vitro* cell interfacing, transplanted cell support *in vivo* and related endogenous/host tissue compatibility) and bioactivity with materials supporting cell adhesion and survival, induction of iPSC differentiation, and structural support of

engineered tissues. Disadvantages relate to quality control with variable purity and biological activity from one lot to another, and limited mechanical properties. Synthetic biomaterials, on the other hand, have the advantage of being more defined and controllable (through, for example, fabrication) so as to conform to required specifications concerning nanotopography, chemical composition, stability and functionality, stiffness, adhesiveness and binding affinity, degradability and related by-products[128].

Biomaterials can therefore be tailored to support and regulate iPSCs and derivative neuronal cells, carry and release drugs and other compounds, and degrade over a set period of time so as to meet the rigorous requirements for pharmaceutical drug screening and clinical use. While disadvantages can include poor biocompatibility resulting in poor cell adhesion, survival, self-renewal, differentiability and transplantability, the inherent capacity for refinement through design and reengineering provides opportunities to systematically optimise performance and application (FIGURE 1).

Although there have been few reports to date of biomaterial based iPSC culture for neural induction, the ability for materials to interface with hESCs for neural differentiation is indubitably applicable. Nevertheless, a recent landmark report involving both hESCs and iPSCs describes 3-D poly(*N*-isopropylacrylamide)-co-poly(ethylene glycol) (PNIPAAm-PEG) hydrogel support of stem cell expansion and differentiation [125](Table 1). PNIPAAm-PEG is a synthetic thermoresponsive hydrogel that is liquid at low temperature for cell loading, which solidifies at 37^{o}C for subsequent 3-D cell culture, including directed differentiation to neuronal progenitors (NPs). The system ostensibly enables defined, good-manufacturing practice-compatible and large-

scale expansion of both stem cells and NPs for translational application, including commercial-scale drug screening and clinical-scale use.

A second report of significance relates to the use of 3-D conductive carbon nanotube (CNT) composites as substrates to support and differentiate NPs derived from human iPSCs[129](Table 1). The CNTs were incorporated by vacuum-driven impregnation to electrospun poly(lactic-*co*-glycolic acid) (PLGA) membranes and shown to enhance differentiation of iPSC derived NPs, further augmented by electrical stimulation. Similar to Lei and Schaffer described above, the authors proffer their platform for drug discovery, disease modelling and *in vivo* transplantation including facilitation of exogenous cell delivery and integration.

In recognising the important role of biomaterials in next generation stem cell technology including tissue engineering and regenerative medicine, like others, we at the ARC Centre of Excellence for Electromaterials Science are undertaking R&D in the additive manufacturing or 3-D bioprinting (BP) space to reproducibly interface natural and synthetic materials with human iPSCs towards solving the many and unique challenges in neural tissue engineering and disease modelling. To this end, we are developing optimal and novel bioinks primarily for extrusion printing, comprising iPSCs, biocompatible gel composites, and other components for supporting cell growth and differentiation to neural lineage. While currently we are printing single cell types for *in situ* differentiation, we will progress to more complex multicellular printing and placement for more efficient and germane construct design, with the holy-grail for developing multidimensional “live” constructs being able to support vascularisation towards formation of clinical-scale tissues and whole-organ substitutes[130]. Incorporation of vascular networks will also benefit metabolically active neural constructs, currently limited to millimetre thickness.

Expert commentary

`Based on the remarkable complexity of the human nervous system, and in particular the brain, it is the least understood body system and is difficult to model with conventional technology. Short of modelling the human CNS as a whole, models must ideally be humanised, diverse, complimentary and be explicitly defined in terms of what they simulate. To this end, human patient/disease-specific iPSC modelling provides an opportunity to unravel the complexity behind neurological function and disease in a way that has never been possible before. As cells containing the actual genetic information of the patients from which they are derived and able to be differentiated to mixed and subtype-specific neuronal populations both in 2-D and 3-D configurations, iPSCs are suited to modelling NDv disorders, enhanced by their presumed early developmental status, as well as NDg disorders by providing a pathological context to elucidate aberrant biological processes for therapeutic targeting including halting endogenous disease progression without neurotoxicity, and where necessary concomitant tissue regeneration. Importantly, toxicity testing is vital to determining the clinical efficacy of a drug or device, and relates to both chemical and physical impairment[131,132]. The developing brain is particularly sensitive to chemical perturbations. *In vitro* iPSC-based systems potentially offer a cost and time effective approach to identifying and characterising neurotoxicity, being amenable to mechanistic studies at both cellular and molecular levels, as well as ranking neurotoxicants for toxic potency. Therefore, neural-specific endpoints for screening putative neurotoxicants would include neuro-chemical, -morphologic, and –transmission functions.

In recognising the value of iPSC modelling though, there are a number of challenges required to be overcome before their potential as clinically relevant *ex vivo* models of neurological disorders can be fully realised. A critical requirement is to demonstrate robust and reproducible cell phenotypes relating to both normal and aberrant function. This will depend on overcoming shortfalls in knowledge about iPSC biology including the effects of cell reprograming, transcriptional memory of primary cells, cell-line variability in pluripotency and differentiability, impact of donor age and associated cell line maturation, as well as related standardisation of cell culture and characterisation. In addition, complex genetic conditions such as schizophrenia with heterogenous clinical etiologies and symptoms will benefit from developing iPSC study cohorts of patients with common clinical manifestations and/or genomic mutations[7]. This is particularly important for small donor-cohorts characteristic of iPSC-based modelling.

Through better understanding of the various modalities, more controllable systems with properties that are tailored to modelling specific neural cells and disorders of interest can be developed. This should ideally extend to being compatible with necessary characterisation tools and minimally incorporate key components of the *in vivo* cellular microenvironment as critical stimuli of normal and anomalous cell behaviour. The latter will likely require a biomaterials-based approach whereby synthetic, natural and functionalised materials will interface with iPSCs and iPSC-derived neural tissues *via* inherent and engineered physical and chemical properties. The ability to spatially modulate composition and function using emerging approaches such as 3-D printing provides an unprecedented opportunity to systematically probe and control cellular behaviour. Ironically, the use of material properties to dictate clinically relevant cell phenotypes *ex vivo* will be paralleled by material-mediated

correction of aberrant cellular function as a potential therapeutic strategy for TI's, NDds and NDgs. The materials may be fabricated into scaffolds, encapsulating gels or probes to generate healthy autologous tissues *in vitro* from diseased tissues for *in vivo* grafting, and/or optimised as medical devices to modify endogenous cells and tissues. While the jury is still out, it is hoped that in addition to generating more efficacious systems, harnessing the inductive capabilities of biomaterials will circumvent current cost-barriers caused by inefficient and expensive bioprocessing, including reducing or omitting the need for biochemical reagents[128].

Five-year view

The next five years will see a rapid transition from first generation iPSC-based modelling using simple 2-D study paradigms to more sophisticated and clinically-relevant second-generation systems that incorporate, for example, extracellular stimuli with patient-specific cells and 3-D tissues using "smart" biomaterials and microfluidics[133,134]. Although there are challenges, a number of which are highlighted above, there is sufficient evidence for being able to recapitulate with iPSCs the neuropathologies of various neurological disorders to further elucidate underlying cellular and molecular mechanisms that have heretofore been unknown. Combined with increasing recognition of the importance of standards for modelling, including iPSC culture, differentiation and characterisation, the body of knowledge will continue to increase exponentially, ultimately benefiting progress in therapy.

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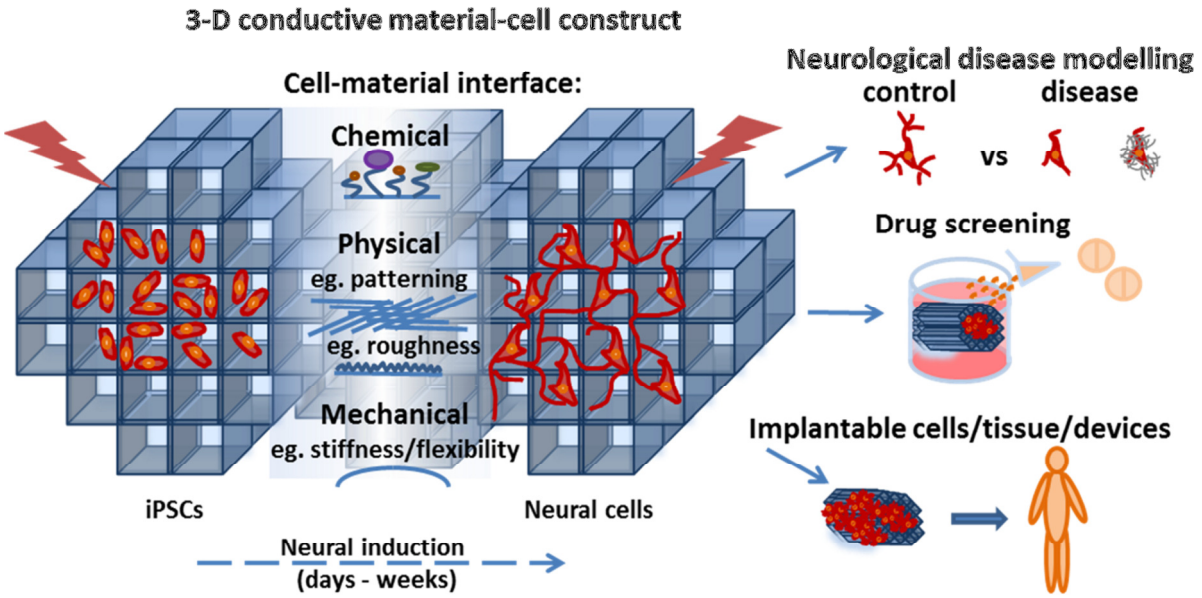

Figure 1. Schema of biomaterials-based iPSC culture and differentiation for advanced neurological disease modelling, early-phase drug screening, and development of implantable neural cells, tissues and medical devices. Biomaterials with suitable chemical, physical, mechanical, and electrical properties are being interfaced with iPSCs for expansion and to ameliorate neural induction for improved modelling of neural tissues and disease phenotypes.