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Modelling of Neurological Diseases Using Induced Pluripotent Stem Cells

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1. Introduction

Human embryonic stem cells (hESCs) were first established as an *in vitro* culture system (Thomson et al., 1998). hESCs are pluripotent cells that are able to self-renew and can differentiate into the three primary germ layers: endoderm, ectoderm and mesoderm. Specifically, hESCs can be used to generate gut epithelium, cartilage, bone, muscle, neuroepithelium, and embryonic ganglia (Zhang et al., 2001, Itskovitz-Eldor et al., 2000, Sottile et al., 2003, Thomson et al., 1998, Green et al., 2003). Since these hESC cell lines can be maintained for months in an undifferentiated state, they can be used as a stable resource to model human development *in vitro*. The conversion of hESCs to neural progenitors and subsequently to the three main neural lineages: neurons, astrocytes and oligodendrocytes was first demonstrated in 2001 (Reubinoff et al., 2001).

The recent advent (Takahashi and Yamanaka, 2006) of induced pluripotent stem cells (iPSCs) makes it possible to derive pluripotent stem cells from somatic tissue. iPSCs are derived by transfecting somatic cells (e.g. skin fibroblasts) with a select group of transcription factors; Sox2, Oct4, Myc and Klf4 to induce reprogramming of the genome over a period of 3-4 weeks. This breakthrough by Yamanaka and colleagues enables the modelling of human disease by, for example, taking a patient's skin cells, converting them to iPSCs and then differentiating it to any desired cell-type. Thus iPSC technology opens a new era of patient-specific disease modelling. Here, we review diseases of the nervous system that have been modelled using iPSC which includes; RETT syndrome (RTT), Familial dysautonomia (FD), Parkinson's disease (PD), Huntingtons (HD) and Amyotrophic Lateral Sclerosis (ALS). To provide some background to the neurological disease modelling studies by iPSCs we begin by reviewing protocols for neural induction (differentiation) of hESC.

2. Neural induction and neuronal differentiation of human embryonic stem cells (hESCs)

2.1 Neural induction of human ESCs

Several protocols, based on knowledge from developmental studies of embryogenesis and neurogenesis, have been developed to optimize yield and purity of neural progenitors from hESCs (Fig1). They can be categorized into two broad approaches. The first involves a

stepwise change of culture medium components followed by expansion of neural progenitors (Reubinoff et al., 2001). This protocol involves the long-term culture (3 weeks) of hESCs without replenishing the mouse embryonic fibroblast layer. These cells rapidly undergo morphological changes, forming neural rosettes. They are subsequently dissociated and replated in medium permissive for the growth and maintenance of NSCs (DMEM/F12, B27 supplement, EGF [epidermal growth factor], bFGF [basic fibroblast growth factor]). Similar to mouse ESCs, hESCs can also be differentiated as suspension cultures where they form cystic embryoid bodies (EBs). Formation of these three-dimensional spheres can recapitulate the microenvironment during embryo development, and are preferred over monolayer formats because it allows for up-scaling of differentiation cultures to a greater extent. However, differentiation of hESCs in this sphere format gives rise to issues such as heterogeneity (containing cell types pertaining to the three germ layers) and differential access to soluble factors, which translates to difficulties in optimizing the purity of the desired differentiated progenitors.

The second approach to induce neural lineage cells, is to co-culture hESCs with a variety of stromal cell types, such as the PA6 and MS5 (Kawasaki et al., 2000, Hong et al., 2008, Chinge and Bayarsaihan, 2010). Both PA6 and MS5 stromal cells were derived from murine bone marrow. This induction method is based on the knowledge that signals from mesodermal cells of the Spemann organizer (which develop into the notochord) can induce overlying ectoderm to neuroectodermal fate (Harland and Gerhart, 1997, Londin et al., 2005). This neural induction protocol was reported to generate up to 92% NCAM [neural cell adhesion molecule]-positive cells in 12 days. Given the ease and efficiency of this protocol it is not surprising that it is widely used. However, the neural-inducing effect of the stromal cells is not fully understood. PA6 cells can induce neural differentiation of hESCs in the absence of physical contact, but the conditioned medium of PA6 cells was unable to induce neural differentiation (Kawasaki et al., 2000). In addition, paraformaldehyde-treated PA6 cells continued to exhibit neural-inducing activity, suggesting that the viability of these cells is not vital. In order to fine-tune the procedure to exclude animal products, a matrix material from the human amniotic membrane was found to support neural induction of hESCs with similar efficiencies to that of the mouse stromal cells (Ueno et al., 2006).

Given the caveats in the EB and co-culture methods, strategies were developed to maximize the induction of hESCs into neural lineage, using differentiating factors and monolayer subculture systems. The secretion of the bone morphogenic protein (BMP) antagonists, noggin and chordin, from the epidermal ectoderm of the *Xenopus* embryo was demonstrated to be essential for neural induction (Sasai et al., 1996). In addition, follistatin, an inhibitor of Activin signaling, was found to promote neural induction (Hemmati-Brivanlou and Melton, 1994). Hence, strategies used for increasing the yield of neural derivatives from hESCs include using inhibitors of BMP signaling such as Noggin (Itsykson et al., 2005), and the inhibitors of Activin/Nodal signaling such as the pharmacological inhibitor of Nodal signaling, SB431542 (Smith et al., 2008). Since both signaling pathways converge to downstream SMAD proteins, subsequent work further optimized the induction of neural lineage cells by using the inhibitors of both BMP and Nodal signaling (Chambers et al., 2009). However, Noggin, as a protein is expensive and may exhibit batch-to-batch variability. Recent findings report that substituting noggin with a small-molecule inhibitor of BMP, Dorsomorphin, can efficiently promote neural differentiation of hESCs and iPSCs (Morizane et al., 2010, Zhou et al., 2010). Other methods rely on genetic manipulations such

as generating nestin-EGFP hESC reporter lines to allow purification of hESC-derived neural progenitors (Noisa et al., 2010).

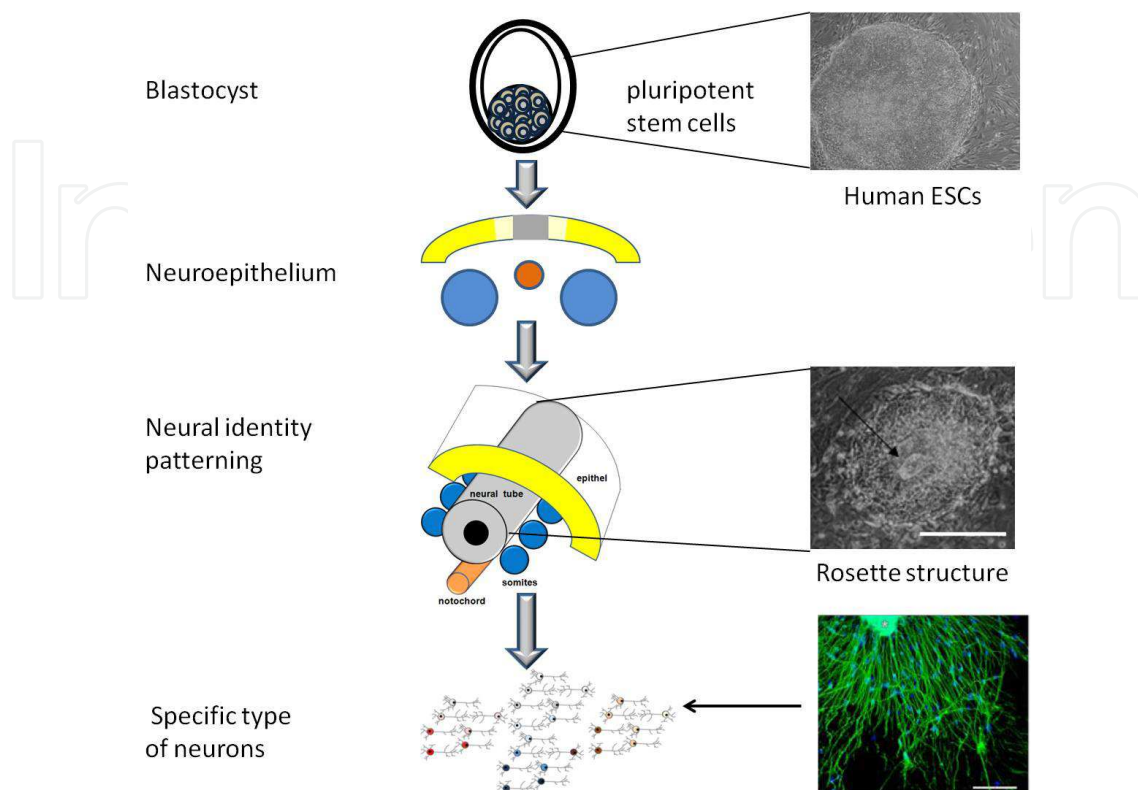


Fig. 1. Schematic representation of neural induction and patterning in *in vivo* and *in vitro*. 1) At early gastrulation, the notochord (orange) secretes BMP-antagonists, such as Noggin and Chordin to create a *gradient of BMP activity* (yellow). 2) Neuroepithelial (NE) cells that are proximal to the notochord become neural precursors (grey) as a consequence of low BMP activity; and the rest of the ectoderm becomes epithelial (yellow) or neural crest (not shown). The NE is patterned by many signals including Shh, RA, Noggin and Chordin (orange) that are secreted from the notochord; BMP (yellow) from the epithelium and FGF (blue) from the somites. 3) The cells in the NE differentiate to specific subtypes of neurons depending on the location in the neural tube and the factors that influence the differentiation. Neural induction and patterning of human ESCs can be induced *in vitro* as shown in the right panels (bright field micrograph or GFP labeled neuronal sub type).

The next aim, upon obtaining neural progenitors/stem cells, is to coax these cells into differentiation of various neuronal subtypes including the cholinergic neurons, dopaminergic neurons, motoneurons, and peripheral sensory neurons. Several methods using various combination of protein factors, co-culture systems and small molecules have successfully been utilized to obtain individual neuronal subtypes. The basic strategy for developing culture cocktails to obtain neuronal subtypes is to mimic signals from early developmental events during specification of the body axis of the embryo. Factors frequently used to induce neural progenitor differentiation include retinoic acid (RA), FGFs and Wingless-Int (Wnts) that can poise cells to differentiate to the neural lineage along the the anterior/posterior (A-P) axis (Hendrickx et al., 2009, Onai et al., 2009). Similarly, Sonic Hedgehog (Shh) and BMPs secreted from the ventral and dorsal neural

tube respectively are important for the precise patterning of multiple neuronal progenitors (Furuta et al., 1997, Litingtung and Chiang, 2000). Hence, the efficiency of deriving specific neuronal subtypes can be influenced by the mode of neural induction. The selection of the appropriate neural differentiation protocol is pertinent for the maximization of desired neuronal subtype.

2.2 Neuronal differentiation of human ESCs

Many neuronal subtypes have successfully been derived from hESCs (Fig1). Amongst them are the dopaminergic neurons, serotonergic neurons, peripheral sensory neurons, and cholinergic neurons (Bissonnette et al., 2011). Dopaminergic neurons are progressively lost in Parkinson's disease (PD), while the motoneurons are lost in amyotrophic lateral sclerosis (ALS). The factors and conditions used for successful derivation of dopamine neurons, motoneurons as well as other neuronal subtypes can be found in Table 1.

Shh and FGF8 were identified to be crucial for the specification of dopaminergic neurons *in vivo* (Ye et al., 1998) but were not directly effective *in vitro* (Stull and Iacovitti, 2001). This led to the search for other signals in promoting dopaminergic neuron differentiation. Currently, differentiation of human ESCs into midbrain dopaminergic neurons can be achieved through the formation of EBs and the use of various factors such as serum-free conditioned-medium from human hepatocarcinoma cell line HepG2, brain-derived neurotrophic factor (BDNF), ascorbic acid, transforming growth factor (TGF), and glial cell line-derived

Neurotrophic factor (GDNF) (Cho et al., 2008a, Cho et al., 2008b, Park et al., 2004, Schulz et al., 2004, Yan et al., 2001). Particularly, TGF β can cooperate with Shh and FGF8 to increase the yield of dopaminergic neurons by enhancing their survival (Roussa et al., 2004); and GDNF was found to be important for the maintenance of motoneurons in the striatum proper (Nevalainen et al., 2010). Large-scale production of dopaminergic neurons from hESC was reported using the EB format with addition of Shh, FGF8 and ascorbic acid. 86% of totally differentiated cells were dopaminergic neurons. Secretion of factors by PA6 cells is important for dopaminergic differentiation (Vazin et al., 2008). PA6 secreted factors include stromal cell-derived factor 1 (SDF-1/CXCL12), pleiotrophin (PTN), insulin-like growth factor 2 (IGF2), and ephrin B1 (EFNB1).

The protocols used for the specification of spinal motoneurons from hESCs were derived from developmental studies. Here motoneurons were found to be induced by signals such as retinoic acid from the caudal paraxial mesoderm (Guidato et al., 2003). Shh signaling from the notochord is also required for the induction of motoneurons (Lewis and Eisen, 2001). Neural induction is predominantly performed through the formation of EBs, using defined medium conditions. Because neuroectodermal cells differentiated from hESCs generally have a rostral character, motoneuron differentiation is then achieved by the caudalizing factor retinoic acid followed by the ventralizing factor Shh (Li et al., 2005). Small molecules that can activate the Shh signaling, purmorphamine and SAG (a chlorobenzothiophene-containing Hh pathway agonist), have been shown to be effective as substitutes of Shh in motoneuron differentiation (Li et al., 2008, Hu and Zhang, 2009, Wada et al., 2009). The use of Hb9 (homeobox gene selectively expressed in motoneurons) promoter driven GFP hESCs allows isolation of a fairly pure population of motoneurons (Singh Roy et al., 2005). Enrichment of derived motoneurons, up to 80%, was also shown to be achieved by gradient centrifugation in Biocoll (Wada et al., 2009), alleviating the need for genetic manipulations.

Neuronal Subtypes	Neural Induction Method	Factors for Final Differentiation	Done with iPS?	Reference
Dopaminergic neurons	50%-MedIIconditionedmedium, bFGF or DMEM/N2	DMEM/N2, GDNF, BDNF, 5% serum		(Schulz et al., 2004)
	EB with RA and bFGF	BDNF, TGF- α		(Park et al., 2004)
	EB, followed by bFGF and N2 supplement	Shh, FGF8		(Cho et al., 2008)
	PA6 co-culture	PA6, Shh, FGF8, GDNF		(Vazin et al., 2008)
	PA6 co-culture	PA6 co-culture or SDF-1/CXCL12, PTN, IGF2, EFNB1		(Vazin et al., 2009)
	MS5 co-culture	RA, Shh, FGF8, Wnt1	√	(Cooper et al., 2010)
Motoneurons	EB, then adherent in F12/DMEM, N2 supplement, heparin and bFGF	RA, then Shh		(Li et al., 2005)
	EB, bFGF	RA in DMEM/F12,N2, heparin, cAMP. Then Shh/purmorphamine, then GDNF, BDNF, IGF1		(Li et al., 2008)
	EB	RA, then Shh/purmorphamine, then BDNF, GDNF, IGF, cAMP	√	(Hu and Zhang, 2009)
Peripheral sensory neurons	PA6 co-culture	Co-culture with PA6 stromal cells		(Pomp et al., 2008)
	MS-5 co-culture, followed by Shh, FGF8, BDNF, ascorbic acid	BDNF, GDNF, NGF (nerve growth factor), Dibutyryl-cAMP	√	(Lee et al., 2009)
Cholinergic neurons	Free-floating aggregates	BDNF, NT3, CNTF, NGF		(Nilbratt et al., 2010)
	RA, followed by free-floating aggregates	FGF8, Shh, BMP9		(Bissonnette et al., 2011)
Serotonergic neurons	RA with EB formation	5-HT, forskolin, acidic FGF, BDNF, GDNF		(Kumar et al., 2009)

Table 1. Factors used in differentiation of hESC-induced NSCs into the various neuronal subtypes.

2.3 Glial differentiation of hESCs

The two glial cell types of the nervous system, the astrocytes and oligodendrocytes, play supporting roles in the brain and peripheral nervous system. Astrocytes secrete various neurotrophic factors, are known to modulate oligodendrocyte myelination of neuronal axons (Moore et al., 2011), and modulate neurotransmitter levels through re-uptake and release mechanisms (Voutsinos-Porche et al., 2003). Evidence is emerging that implicates astrocytes in the pathogenesis of neurological disorders such as Rett syndrome (Maezawa et al., 2009) and ALS (Nagai et al., 2007). A protocol to obtain astrocytes includes exposure of adherent hESCs to cyclopamine, an inhibitor of hedgehog signaling, and subsequent culture in human astrocyte medium to generate a high percentage of nestin and glial fibrillary acidic protein (GFAP)-expressing cells (Lee et al., 2006).

Oligodendrocytes are cells that produce myelin sheaths that insulate axons of neurons, enabling saltatory conduction between the Nodes of Ranvier for rapid propagation of action potential. These cells are targets of severe developmental diseases such as Pelizaeus-Merzbacher disease, and demyelinating diseases such as multiple sclerosis and Charcot-Marie-Tooth (Bramow et al., 2010, Garbern, 2007, Sargiannidou et al., 2009). Dysfunctional oligodendrocytes in these diseases lead to disruptions in axonal transport. In spinal cord injury, demyelination of nerve fibres attributes to functional loss of neurons. So, myelin formation and insulation of neurons is crucial to restore functional network. Hence, it is important to source for pure population of glial (oligodendrocyte precursor) cells that can restore function of neurons by remyelination. A few reports and studies from Geron trial have shown the feasibility of using functional OPCs or neurotrophin expressing GRPs from directed differentiation of hESCs and their therapeutic potential at early time points after spinal cord injury (Keirstead et al., 2005, Cao et al., 2005).

Oligodendrocytes undergo multiple stages of differentiation, from oligodendrocyte progenitors, to pro-oligodendrocytes, to non-myelinating oligodendrocytes, and finally myelinating oligodendrocytes (Reviewed in Miller, 2000). A protocol by Hu *et al.* 2009 provides an excellent example of the procedure for obtaining oligodendrocyte progenitors. Briefly, hESC-derived neural progenitors are treated with RA and Shh for patterning into the ventral spinal progenitors, followed by FGF2 for inhibition of differentiation into motoneurons. Oligodendrocyte progenitors were induced with the addition of factors promoting for survival and proliferation of oligodendrocytes – transferrin, progesterone, sodium selenite, putrescine, triiodothyronine (T3), neurotrophin 3 (NT3), platelet-derived growth factor (PDGF), cyclic adenosine-monophosphate (cAMP), insulin growth factor-1 (IGF-1) and biotin. Other factors shown to promote oligodendrocyte differentiation of hESCs include hepatocyte growth factor (Hu et al., 2009) and extracellular matrix protein vitronectin (Gil et al., 2009). Oligodendrocytes have also been shown to be derived from iPS cells (Czepiel et al., 2011).

3. iPSC technology and implications for neurodegenerative diseases

Neurological diseases are conditions that affect the central and peripheral nervous system. At present pharmacological interventions for many neurological diseases, especially the degenerative conditions, are limited and predominantly restricted to alleviation of symptoms. Finding drugs for treatment of neurological disorders represents one of the critical goals of medical research today. The recent discovery of iPSC technology (Takahashi and Yamanaka, 2006) opens the possibility to generate patient

specific models of human disease. The generation of patient specific cells using iPSC technology will be a powerful resource for both cell therapy and drug screening (Fig2). iPSC is particularly important for neurological diseases as there are limited cellular models of the nervous system.

3.1 X-linked diseases

A disproportionately large number of disease conditions have been associated with the X chromosome because the phenotypic consequence of a recessive mutation is revealed directly in males for any gene that has no active counterpart on the Y chromosome. Thus, although the X chromosome contains only 4% of all human genes, almost 10% of diseases with a mendelian pattern of inheritance have been assigned to the X chromosome (307 out of 3,199). 168 X-link diseases have been explained by mutations in 113 X-linked genes (Ross et al., 2005).

While males carry a single X chromosome, females have two and hence two copies of each gene. Yet, as one of their X chromosomes is inactive in each cell, females also have only one working X chromosome in each cell. Thus, females have a mosaic of cells that express either the paternal X allele or the maternal X allele. This cellular mosaicism gives females a big advantage over males in the context of X-linked diseases. When an X-linked gene is mutated, the normal cells can partly compensate for the cells that express the mutant allele as a result from cell elimination or by functional compensation (Migeon, 2007). In males, on the other hand, all cells express the mutant gene and therefore usually show much more severe symptoms.

Because the process that inactivates X chromosomes is random with respect to parental origin of the X, usually half of the female cells contain a working X chromosome from the father while the other half contain a working X chromosome from the mother. By reprogramming fibroblasts from female with X-linked disease one can generate both the perfect pair of control (expressing the normal allele) and experimental (expressing the mutant allele) cell types for investigation of the disease phenotype (see schematic Fig. 3). These isogenic control and mutant iPS-derived neurons represent a promising source for modelling X-linked diseases.

3.2 Rett syndrome (RTT)

RTT is one of the most common causes for mental retardation in females, affecting 1 in 10,000 live female births. It was first reported in 1966 by the neurophysiologist Dr. Andreas Rett (Rett, 1966). The large majority of RTT cases are caused by sporadic or from germline mutations within the coding sequence of the X-linked methyl CpG binding protein 2 (MeCP2) gene (Amir et al., 1999) and therefore the mutation cannot be detected by simple screening of the parents. Females with classic RTT appear normal from birth until 6–18 months of age, but then they fail to acquire new milestones and enter a period of regression during which motor and language skills are lost. These females show a large diversity of symptoms, that appear progressively (reviewed in Chahrour and Zoghbi, 2007) until they reach a plateau, suggesting that the condition does not involve progressive neurodegeneration (Sun and Wu, 2006). The postnatal onset of symptoms might be explained by the increase in MeCP2 levels in cortical neurons throughout normal development (Akbarian et al., 2001, Balmer et al., 2003, Jung et al., 2003).

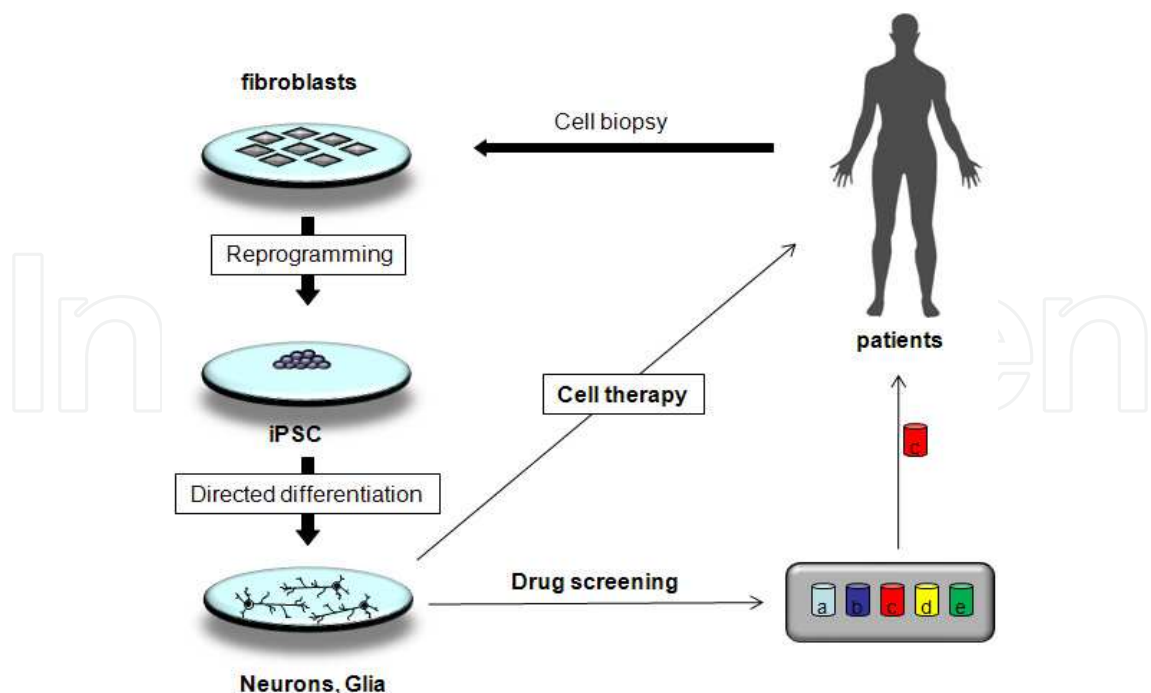


Fig. 2. The potential of iPSCs technology. Cell sample can be easily obtained from patients and cultured *in vitro*. These cells can then be reprogrammed into iPSCs and further be differentiated into the afflicted cells. Cellular phenotype is assessed by measuring cell properties (i.e. neural morphology, maturation of synapses, cell survival under stress, etc). Once a distinct disease related phenotype is identified, drug screening platform can be developed to test their potential to reverse these phenotypes (in this example, component ‘c’ is a potential candidate (see Fig. 3) and can be used for further tests *in vivo*). Another usage for this technology is cell therapy - transplantation of the cells back to the patient.

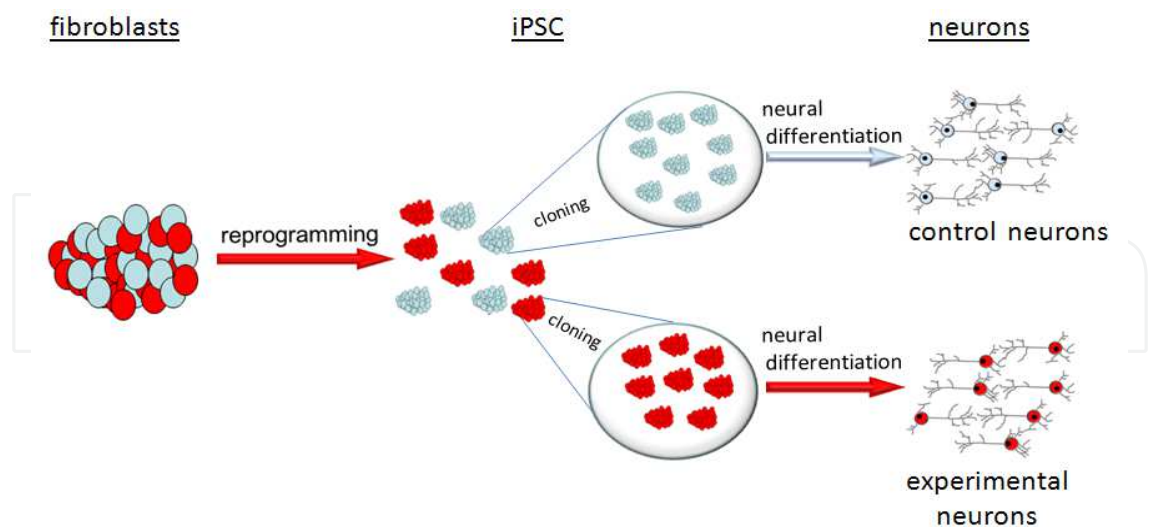


Fig. 3. *In vitro* disease modeling for X-linked diseases. Schematic explaining reprogramming of mosaic cell population from a female with X-linked disease, such as RTT, both iPSC clones expressing the mutant allele ($Xa^{mut} Xl^{wt}$) and iPSC clones expressing the wild-type ($Xa^{wt} Xl^{mut}$) can be expanded and further differentiated into the afflicted cells (i.e. neurons). The wild type neurons, can be used as a perfect control for the mutant neurons for further studies, such as screening of molecules that might potentially reverse the symptoms.

Although MeCP2 is expressed in a large variety of cells, the most afflicted cells in RTT are neurons. In agreement, a specific knockout in the central nervous system revealed the same spectrum of symptoms as the full mouse knockout. Several specific neurons were studied in the context of RTT. For example, pyramidal glutaminergic neurons, which show obvious morphological differences when they express the mutation (see Fig. 3), can account for the mental retardation seen in RTT; dopaminergic neurons can account for the motor function; neurons in the Amygdala can affect learning and memory; and hypothalamus neurons can affect feeding, aggression and stress.

In females, the major source for phenotypic variability is the pattern of X chromosome inactivation (XCI) (Bourdon et al., 2001). Females with classical RTT are usually a balanced mosaic with regard to MeCP2 expression (Adler et al., 1995), whereby half of their cells express the wild-type MeCP2 allele and the other half express the mutant MeCP2 allele (Shahbazian et al., 2002). However, sometimes, by chance, the XCI pattern favors expression of the wild-type allele. Such 'lucky' females might show milder symptoms and even to be asymptomatic (Sirianni et al., 1998). Another source for phenotypic variability is the type of mutation in the MeCP2 gene. Over 300 different mutations in the gene MeCP2 were identified, which are the major source of the phenotypic variability in males with RTT. Thus generation of iPSCs from multiple patients might shed more light on the correlation between mutation type and symptoms as well as the function of different domains in MeCP2 protein. In addition, there are important implications for drug screening, as some drugs might treat patients with certain mutations but not others. For example, aminoglycosides antibiotics, such as gentamicin, can increase wild type MeCP2 expression levels in affected neurons by skipping a premature stop codon by bind to the 16S rRNA, impairing ribosomal proofreading (Kellermayer, 2006, Marchetto et al., 2010). Thus, disease modeling for multiple patients will allow us to tailor specific drugs for each patient.

Initially, RTT was thought to be a neurodegenerative disease, however, the decrease in axondendritic arborization and impaired development of dendrites (Fig. 4) suggest that RTT is a disorder featuring an arrest in neuronal development (Hagberg, 1985, Belichenko et al., 1994, Armstrong, 1992, Armstrong, 2001). Furthermore, no obvious cell death is seen in RTT, and this therefore begs the question of whether restoring MeCP2 expression would restore normal neuronal function and reverse the resulting disease phenotypes. In a seminal work, Guy et al (2007) provide evidence supporting the feasibility of disease reversibility in mouse models of RTT (Guy et al., 2007). They created a mouse in which endogenous MeCP2 is silenced by insertion of a Lox-Stop cassette and can be conditionally activated through Cre-mediated deletion of the cassette. The MeCP2 lox-Stop allele behaved as a null mutation, and its activation was controlled by a tamoxifen-inducible Cre transgene. Gradual tamoxifen injection reversed the late-onset neurological phenotype of adult MeCP2-lox-Stop/+; cre heterozygotes, indicating that MeCP2-deficient neurons are not permanently damaged, since MeCP2 activation leads to robust abrogation of advanced neurological defects in both young and adult animals. This work establish that consequences of MeCP2 loss of function are reversible, and suggest that the neurological defects in RTT, and other MeCP2 disorders, are not impervious to therapeutic possibilities. Indeed, several molecules, have delayed the onset of RTT-like symptoms in animal models, and enhanced survival rates (Chang et al., 2006, Ogier et al., 2007). Potential molecules for treatment of RTT are BDNF, IGF-1 and NGF. A molecule with promise in RTT therapy is BDNF. There are phenotypic similarities between MeCP2- and BDNF-null mice, including a reduction in brain weight and hindlimb

clasping. Over expression of BDNF in MeCP2-null mouse brains delayed the onset of RTT-like symptoms, and enhanced survival rates (Chang et al., 2006, Ogier et al., 2007). More specifically, BDNF overexpression rescued the hypoactivity in wheel running exhibited by MeCP2 knockout mouse, and the low frequency of action potential firing observed in their cortical neurons (Chang et al., 2006); and treatment of MeCP2 null mice with AMPAkinases (which increases BDNF mRNA and protein levels) rescued the irregular respiratory patterns exhibited by MeCP2. However, the therapeutic utility of BDNF is hampered by its poor efficiency at crossing the blood-brain barrier. Nevertheless, a therapeutic intervention in humans might thus arise from identifying an agent similarly capable of stimulating synaptic maturation.

An *in vitro* disease modeling for RTT was established by Marchetto et al (2010). In this study they reprogrammed fibroblasts from RTT patients into iPSCs, and further differentiated them into neurons. When analyzed, these neurons showed lower synaptic density, simpler morphology with less branching and smaller cell body. Furthermore, by using electrophysiological methods they showed that RTT neurons have a significant decrease in frequency and amplitude. This is in agreement with studies on RTT mice models and on postmortem brain tissues from patients. This RTT disease model was used to screen molecules, such as IGF-1. Like BDNF, IGF-1 is widely expressed in the CNS during normal development (D'Ercole et al., 1996), strongly promotes neuronal cell survival and synaptic connections. Indeed, IGF-1 treatment leads to a partial rescue of RTT (Tropea et al., 2009).

In their study, Marchetto et al (2010) investigated the use of IGF1 and gentamicin in iPSC-derived neurons carrying a MeCP2 mutation. While IGF1 treatment increased synapse number in some clones, it stimulated glutamatergic RTT neurons above normal levels. Gentamicin was used to rescue neurons derived from iPSCs carrying a nonsense MeCP2 mutation by increasing full-length MeCP2 levels in RTT neurons, rescuing glutamatergic synapses. Thus, this *in vitro* disease model can be used to screen therapeutic candidate molecules.

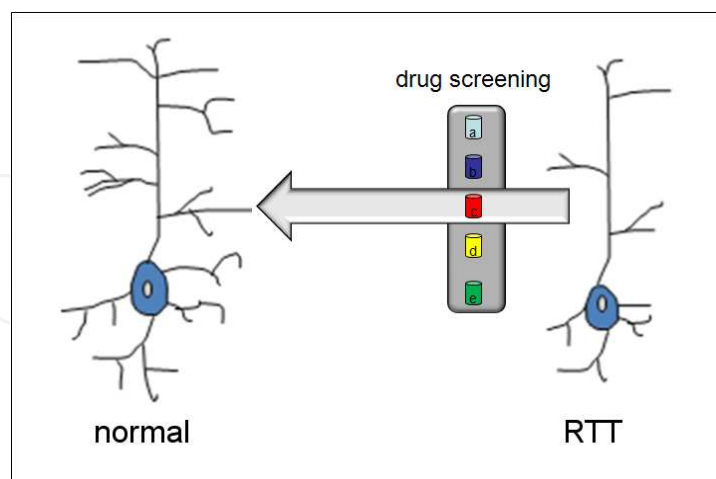


Fig. 4. Schematic representation of drug screening for pyramidal neurons carrying MeCP2 mutation. In RTT, the cell body is small and there is reduced dendritic branching (Armstrong 1995), fewer synapses, and reduced spine density compared to the control. These morphological differences can be an indicator for drug screening. In this example, component 'C' reverses the impaired morphology of the RTT neurons and therefore can be further studied in RTT animal models as a potential drug to reverse the RTT symptoms.

3.3 Familial dysautonomia

Familial dysautonomia (FD), also known as Riley-Day syndrome or HSAN-III is a neurodevelopmental disorder, caused by a mutation in the I-k-B kinase complex-associated protein (*IKBKAP*) gene on chromosome 9. This condition affects 1 in 3,700 Eastern European Jewish ancestry (Ashkenazi Jews), but rare in the general population. The disease was first reported by Conrad Milton Riley and Richard Lawrence. It is a fatal autosomal recessive disease characterized by the degeneration of sensory and autonomic neurons (Slaugenhaupt et al., 2001, Axelrod et al., 2002), resulting in variable symptoms including: insensitivity to pain, inability to produce tears, poor growth, and labile blood pressure (episodic hypertension and postural hypotension). Future parents can be screened for the mutation and if both parents are shown to be carriers by genetic testing, there is a 25% chance that the child will produce FD. The point mutation in the *IKBKAP* gene results in a tissue-specific splicing defect with various levels of exon 20 skipping and reduced levels of normal IKBAP protein (Slaugenhaupt et al., 2001, Anderson et al., 2001). The ratio between the normal and mutant transcripts affects the severity of the disease.

An iPSC-based model for FD was established by Lee et al (Lee et al., 2009). FD-iPSC-derived neural crest showed reduced migration as well as decreased rate of neurogenesis. Furthermore, when they screened the afflicted cells with candidate drugs, they found that kinetin resulted in a marked reduction of the mutant *IKBKAP* splice form, associated with an increase in normal *IKBKAP* levels. This treatment increased the percentage of differentiating neurons but it did not affect the migration potential of the neural crest. Thus FD-iPSC model is an example of gaining insight about early progression of a neurodevelopmental disease.

3.4 Parkinson's disease

Parkinson's disease (PD) is one of the most widely studied neurodegenerative disease caused by the progressive degeneration of midbrain dopaminergic neurons. Although the aetiology of PD remains enigmatic, partially attributed to complex environmental factors, and mutations in several genes (*LRRK-2*, *Parkin*, *DJ-1*, *PINK1*, *ATP13A2*, *alpha-synuclein*, *GBA*) have been found to give rise to PD-like pathogenesis. However, these genetic factors contribute to only a small fraction of PD cases, impeding significant progress in understanding PD pathogenesis completely. Moreover, it was found in PD patients who had received neuronal grafts through transplantation that the cells which were previously young and healthy developed α -synuclein and ubiquitin-positive Lewy bodies after more than a decade. This led to the proposal of several mechanisms such as inflammation, oxidative stress, excitotoxicity and growth-factor deprivation that may have substantial impact on the propagation of PD (Brundin et al., 2008). Thus, it would be necessary to re-examine the disease pathology by mimicking characteristics of the disease under these varied conditions or in combination with known genetic defects since the benefits of cell transplantation in PD trials is still uncertain. This objective may soon be achievable as Soldner et al (2009) demonstrated that iPSCs could be derived from patients with idiopathic PD. Furthermore, the iPSCs could be directed to differentiate into dopaminergic neurons through EB formation in the presence of FGF2, FGF8 and Shh. Coupled with the possibility of deriving large-scale functional dopaminergic neurons; via the generation of homogenous spherical neural masses (Cho et al., 2008), the ultimate goal of creating patient-specific neuronal cells for transplantation therapy may soon be in the pipeline. It is reassuring to note that neurons

reprogrammed from mouse fibroblasts were able to integrate functionally into the fetal brain and improve the locomotor behavior in at least a rat model of PD upon transplantation into the adult brain (Wernig et al., 2008). However, whether these transplanted cells will succumb to the same fate in the long-term as observed in the post-mortem human PD trails remains to be investigated.

3.5 Huntington's disease

Huntington's disease (HD) is caused by CAG repeats in the N-terminus of the gene encoding Huntingtin protein. Like Lewy proteins in Parkinson's disease patients, the expression of aberrant polyglutamine-containing molecules in HD leads to massive loss of medium spiny neurons in the striatum and neurons in the cortex as the disease progresses. Despite substantial efforts invested in various cellular and animal models to understand the disease, the mechanisms implicated in the selective cell death of neurons remains unclear and there is no cure at present. One major hurdle is the lack of appropriate human samples carrying the genetic mutation for HD which would offer the most ideal system for investigating the process of neurodegeneration. With the advent of iPSC technology, HD-specific iPSCs were generated (Park et al., 2008). Thereafter, HD specific NSCs (nestin+/PAX6+/SOX1+/OCT4-) were obtained and these were subjected to differentiation conditions combining morphogens (SHH and DKK1) and neurotrophins (BDNF) to induce neurons of the striatal lineages (Zhang et al.). Importantly, these neurons contained the same CAG expansion as the mutation in the HD patient in which the iPSC line was established. Thus, a valuable resource is now available to search for drugs that can reduce the toxicity of polyglutamine.

3.6 Amyotrophic lateral sclerosis

Another seminal study to interrogate a debilitating disease, arising from the progressive degeneration of motor neurons of the spinal cord using the iPSC technology is amyotrophic lateral sclerosis (ALS) (Dimos et al., 2008). In this landmark study, skin fibroblasts produced directly from an 82-year-old elderly woman patient, diagnosed clinically with ALS and a SOD1 mutation were reprogrammed successfully into the pluripotent state. These patient-specific iPSCs showed strong alkaline phosphatase activity and exhibited markers (SSEA-3, SSEA-4, TRA1-60 and TRA1-81) and a transcriptional profile (*REX1*, *FOXD3*, *TERT*, *NANOG* and *CRIPTO*) that are comparable to pluripotent hESCs. Furthermore, these cells could form EBs and were capable of differentiating into cells of the germ layers. Most importantly the iPSCs were directed specifically towards both motor neurons and glia fates, enabling further exploration of either a cell autonomous (such as the amount of SOD1 in motor neurons) or non-cell-autonomous (such as the role of neighboring astrocytes, microglia and oligodendrocytes) function of the disease as implicated in rodent models (Boillee et al., 2006, Yamanaka et al., 2008). Thus, the iPSC technology essentially reversed the patient's history to allow the onset and progression of ALS to be captured in culture for drawing mechanistic insights.

In addition to ALS, Park and co-workers (Park et al., 2008) managed to single-handedly generate reprogrammed cells from patients with a range of genetic diseases that were either Mendelian or complex in inheritance. The diseases in which iPSCs were derived include: adenosine deaminase deficiency-related severe combined immunodeficiency (ADA-SCID), Shwachman-Bodian-Diamond syndrome (SBDS), Gaucher disease (GD) type III, Duchenne

(DMD) and Becker muscular dystrophy (BMD), Parkinson disease, Huntington disease, juvenile-onset, type 1 diabetes mellitus (JDM), Down syndrome (DS)/trisomy 21 and the carrier state of Lesch-Nyhan syndrome. Similar to the set of assays used in the characterization of the ALS iPSCs, these disease-associated iPSC lines were all confirmed to be pluripotent and capable of multi-lineage differentiation. Moreover, these human iPSCs produced teratomas in immunodeficient Rag2-/- γ C-/- mice, the golden standard for testing pluripotency. Taken together, these efforts undoubtedly demonstrated the feasibility of the iPSC technology to reprogram somatic cells from a variety of diseased patients. And in the process, provided valuable source of material that pave the way for unraveling disease mechanisms and customized cellular therapies tailored for the individual.

4. Conclusion

Research on hESCs has allowed the development of specific protocols for generation of neural progenitors and differentiated neural lineages. These protocols can now be applied to iPSCs to enable the generation of specific neurons, astrocytes and oligodendrocytes. The availability of samples from relevant donors carrying different mutations will also facilitate modelling of neuronal diseases by reprogramming somatic cells. A 'mutation library' for a particular disease can then serve as a platform for the tailoring of specific drugs for specific patients. Furthermore, disease modelling from multiple patients might provide additional insights on the pathogenesis of the disease; particularly RTT in which specific mutations are associated with variable severity in clinical symptoms. Another advantage in reprogramming is that the clinical history of every donor is known. This will allow us to model diseases in which the genetic component is not known such as Alzheimer and Parkinson's. Thus the use of iPSCs technology will herald a new era in the study of neurological diseases.

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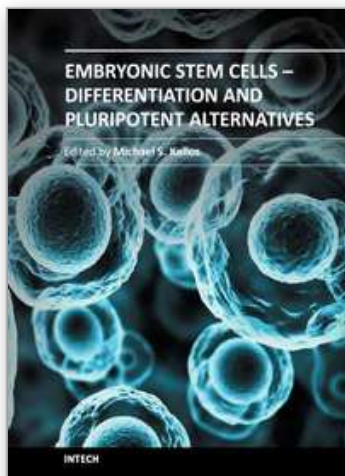
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The ultimate clinical implementation of embryonic stem cells will require methods and protocols to turn these unspecialized cells into the fully functioning cell types found in a wide variety of tissues and organs. In order to achieve this, it is necessary to clearly understand the signals and cues that direct embryonic stem cell differentiation. This book provides a snapshot of current research on the differentiation of embryonic stem cells to a wide variety of cell types, including neural, cardiac, endothelial, osteogenic, and hepatic cells. In addition, induced pluripotent stem cells and other pluripotent stem cell sources are described. The book will serve as a valuable resource for engineers, scientists, and clinicians as well as students in a wide range of disciplines.

How to reference

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