

# Stem Cells as *In Vitro* Models of Disease

Guest Editors: Mirella Dottori, Mary Familiari, Stefan Hansson,  
and Kouichi Hasegawa





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Stem Cells International

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## Contents

**Stem Cells as *In Vitro* Models of Disease**, Mirella Dottori, Mary Familiari, Stefan Hansson, and Kouichi Hasegawa  
Volume 2012, Article ID 565083, 2 pages

**Prospective *In Vitro* Models of Channelopathies and Cardiomyopathie**, Nanako Kawaguchi, Emiko Hayama, Yoshiyuki Furutani, and Toshio Nakanishi  
Volume 2012, Article ID 439219, 10 pages

**Extrafollicular Dermal Melanocyte Stem Cells and Melanoma**, James D. Hoerter, Patrick Bradley, Alexandria Casillas, Danielle Chambers, Carli Denholm, Kimberly Johnson, and Brandon Weiswasser  
Volume 2012, Article ID 407079, 10 pages

**Stem Cells as *In Vitro* Model of Parkinson's Disease**, Patricia L. Marti'nez-Morales and Isabel Liste  
Volume 2012, Article ID 980941, 7 pages

**Skin-Derived Precursor Cells as an *In Vitro* Modelling Tool for the Study of Type 1 Neurofibromatosis**, Araika Gutiérrez-Rivera, Haizea Iribar, Anna Tuneu, and Ander Izeta  
Volume 2012, Article ID 646725, 9 pages

**The Potential of iPS Cells in Synucleinopathy Research**, Leonhard Linta, Marianne Stockmann, Tobias M. Boeckers, Alexander Kleger, and Stefan Liebau  
Volume 2012, Article ID 629230, 6 pages

**Endothelial Progenitors as Tools to Study Vascular Disease**, Reinhold J. Medina, Christina L. O'Neill, T. Michelle O'Doherty, Sarah E. J. Wilson, and Alan W. Stitt  
Volume 2012, Article ID 346735, 5 pages

## Editorial

# Stem Cells as *In Vitro* Models of Disease

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At the present time, for many human diseases, medical research predominantly relies on having appropriate model systems to study disease states and in order to develop therapies. Nonhuman animal models, particularly transgenics, have allowed us to recreate disease states *in vivo*. However, there is still a need to have corresponding *in vitro* model systems to study pathogenesis at the cellular level as well as for fast-tracking discoveries of therapeutic compounds. Ideally, an *in vitro* disease model would be established from human diseased tissue that can demonstrate relevant degenerative mechanisms. The tremendous advances within the field of stem cell biology over the last decade may now make *in vitro* models possible for certain diseases that were previously thought unlikely.

This special issue in *Stem Cells International* is a collection of review articles that describe various ways in which stem cells have been utilized to create *in vitro* disease models.

The Noble-prize winning discoveries in reprogramming have given scientists the opportunity to generate stem cells from any disease type. Whilst this technology has opened new doors for creating *in vitro* model systems, challenges still remain including how to differentiate pluripotent stem cells to generate stage-specific (whether progenitor or mature stage) and homogeneous cell types that are relevant to the disease. A significant aspect of that relies on the development of *in vitro* assays to study the cellular function. N. Kawaguchi et al. highlight these issues with respect to using pluripotent stem cells to model cardiac disease and channelopathies.

Reprogramming technology is highly useful for creating *in vitro* disease models that are caused by known genetic

mutations. The review by L. Linta et al. describes how neurons derived from pluripotent stem cells carrying mutations in alpha-synuclein or *LRRK2* genes show pathological characteristics of synucleinopathies. However, for many diseases, the causes are idiopathic and interrogation of specific cell types in isolation may not elucidate other potential environmental triggers. When there are multifactorial causes, such as those for Parkinson's disease as reviewed by P. L. Martínez-Morales and I. Liste, then it is advantageous to establish multiple stem cell lines from different patients, including both idiopathic and specific genetic mutant conditions.

Parallel to the development of reprogramming technologies, the field of stem cell biology has also rapidly expanded through the identification of stem cell niches within most adult tissues. Isolation, maintenance, and expansion of stem cell/progenitor pools within a tissue provide an alternative source of cells that can be used to model disease conditions, particularly of that tissue. The review by R. J. Medina et al. describes how a subpopulation of endothelial progenitor cells isolated from human blood can be used to model vascular disease.

For some diseases, especially certain cancers, pathological mechanisms may begin within the stem cell population, perhaps due to DNA damage. In these scenarios, isolation of tissue-specific stem cells is used to model disease genesis. The review by A. Gutiérrez-Rivera et al. explains how skin-derived precursor cells may be the cells of origin in neurofibromatosis type 1 tumours. J. D. Hoerter et al. also provide a thorough review on how melanomas may arise from damaged extrafollicular melanocyte stem cells.

In summary, this special issue provides a broad spectrum of how stem cells can offer innovative and novel approaches to study disease mechanisms. We would like to thank all of the authors, reviewers, Guest Editors, and Editor-in-Chief for their great contributions and support towards this special issue.

*Mirella Dottori*  
*Mary Familari*  
*Stefan Hansson*  
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## Review Article

# Prospective *In Vitro* Models of Channelopathies and Cardiomyopathies

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An *in vitro* heart disease model is a promising model used for identifying the genes responsible for the disease, evaluating the effects of drugs, and regenerative medicine. We were interested in disease models using a patient-induced pluripotent stem (iPS) cell-derived cardiomyocytes because of their similarity to a patient's tissues. However, as these studies have just begun, we would like to review the literature in this and other related fields and discuss the path for future models of molecular biology that can help to diagnose and cure diseases, and its involvement in regenerative medicine. The heterogeneity of iPS cells and/or differentiated cardiomyocytes has been recognized as a problem. An *in vitro* heart disease model should be evaluated using molecular biological analyses, such as mRNA and micro-RNA expression profiles and proteomic analysis.

## 1. Introduction

Most of the genes responsible for congenital heart diseases have been identified with genetic studies, where healthy individuals and patients' genes sequences were compared to find mutations. The responsible genes were then subjected to functional analyses, using knock-out mouse and/or other animals to make a disease model which possessed the mutated genes [1, 2].

Since their establishment [3–5], iPS cells have been used to make *in vitro* disease models because of the difficulty in using a patient's cells or tissues, especially from the heart [6–9]. Transfection of mutated genes into a normal parent cell prior to formation of iPS cells has also been used to make an *in vitro* disease model. Thus, iPS cells or differentiated cells containing the mutated gene can be compared with parent cells that do not have the mutated gene. ES cells and iPS cells differentiate into heart cells more easily than adult cardiac stem cells in both mice and humans because of their multipotency and pluripotency characteristics. Therefore, these cells have been used in regenerative medicine studies [10–12]. Although cardiac stem cells have advantage for *in vivo* regenerative medicine [13, 14], heterogeneity was

observed in long-term cultures in our *in vitro* cultures [15]. A previous report showed that immature cardiomyocytes were obtained *in vitro* differentiation [16], suggesting the limitations of using adult stem cells as a cell source for *in vitro* disease model. Taken together, ES cells/iPS cells provide a better cell source of cardiomyocytes required for *in vitro* disease models.

In heart disease, iPS cells from Long-QT-syndrome-(LQTS-) type1 [17] and LQTS-type2 [18] patients were made and differentiated cardiomyocytes were obtained from these iPS cells. These cardiomyocytes worked as *in vitro* heart disease models since they possessed similar characters to patients' cardiomyocytes. LQT1 and LQT2 are caused by missense mutations of the *KCNH1* and *KCNH2* gene, respectively. These mutations in potassium channels lead to QT interval prolongation [19]. Interestingly, the differentiated cardiomyocytes also showed marked arrhythmogenicity and early afterdepolarizations [18]. Potassium channel activators, such as PD118057, cured prolonged action potentials of LQT2-hiPS cell-derived cardiomyocytes [20]. Cardiomyocytes derived from patients' fibroblasts, or other somatic cells, are gaining attention as promising models to discover drug targets for disease.

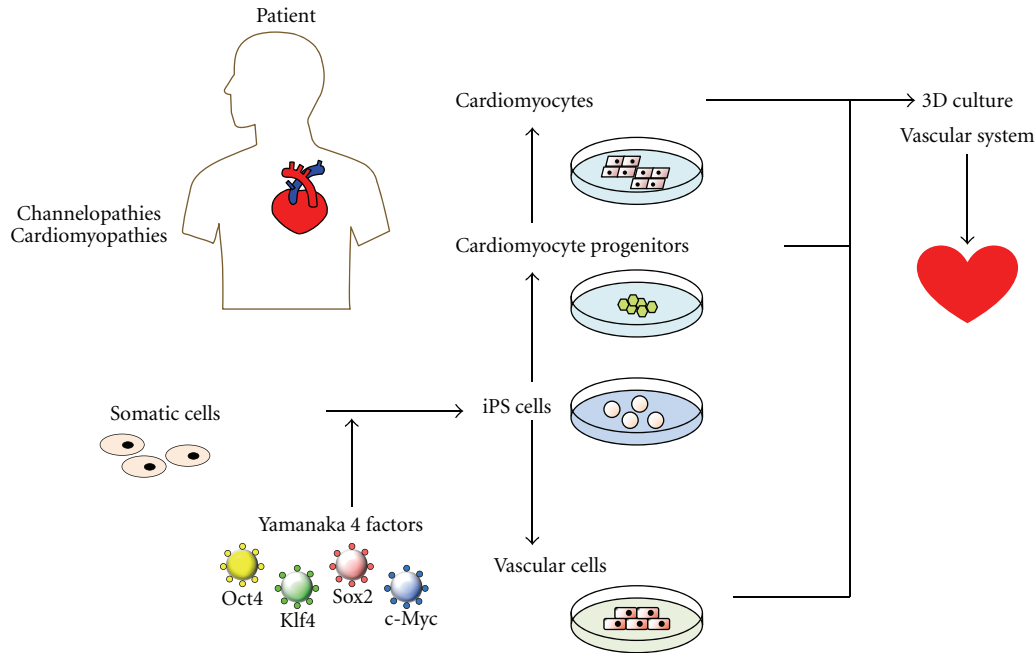


FIGURE 1: Whole concept of *in vitro* disease model for heart disease (channelopathies and cardiomyopathies).

The differentiated cardiomyocytes from murine iPS cells, mutated with the LQT3 gene (Scn5a $\Delta^{+/+}$ ), showed prolonged action potentials because of a Na channel dysfunction mutation in an LQTS-type3 patient [21], suggesting even murine iPS-derived cardiomyocytes can be used for an *in vitro* disease model. iPS cell-derived cardiomyocytes from Timothy syndrome showed irregular contractility consistent with the disease phenotype [22]. At least 13 LQTS genes have been reported so far, and similar abnormalities in iPS-derived cardiomyocytes from patients can be anticipated.

Channelopathies have been currently used as *in vitro* disease models because of the development of systematic current measurements. Another recent model from channelopathy was catecholaminergic polymorphic ventricular tachycardia (CPVT), carrying a novel mutation (S406L) of the ryanodine receptor (RyR) 2 which reduced sarcoplasmic reticular (SR) Ca $^{2+}$  content to levels lower than control myocytes. In this case, Dantrolene is a drug rescued arrhythmogenic phenotype [23]. LEOPARD (lentiginos, electrocardiographic abnormalities, ocular hypertelorism, pulmonary valve stenosis, abnormal genitalia, retardation of growth, and deafness) syndrome is caused by a different missense mutation of the *PTPN11* gene (T468M and Y279C are the most recurrent). Differentiated cardiomyocytes from these patients were larger than wt-iPS-cell- or ES-cell-derived cardiomyocytes, which correspond to the disease phenotype of LEOPARD cardiac hypertrophy [24].

Another attractive method can be direct programming into stem cells/progenitors/cardiomyocytes from patients' somatic cells. iPS cells induced from adult neural stem cells with only one transcription factor (TF), Oct4, were similar to ES cells [25]; therefore, primitive cells may be more suitable than differentiated cells to make iPS cells

with only one factor introduction. Transient introduction of Yamanaka 4 factors (Oct3/4, Sox2, Klf4, and c-myc) and immediate growth factors, mainly bone morphogenetic protein 4 (BMP4), to cultured cells adequately directed cardiomyogenesis [26]. Interestingly, direct reprogramming from fibroblasts into cardiomyocytes was successful using 3 TFs, which are associated with cardiomyogenesis [27], which is another possible method of producing cardiomyocytes.

## 2. Generation of iPS Cells from Patients

Although a retrovirus was originally used, recently there have been several methods of reprogramming developed to introduce Yamanaka 4 factors (Figure 1). The Sendai-virus [28, 29], transient transfection of mRNA [30, 31], is more attractive than conventional retroviral infections because of safety, which is important for regenerative medicine and also *in vitro* models. If reprogramming vectors are integrated into the host genome, tracking the location can be difficult. Moreover, additional artifacts are also a concern. Recent studies show that epigenetic modulators such as the histone deacetylase inhibitor, valproic acid (VPA) can affect reprogramming efficiency [32]. In this way, only two factors (Sox2 and Oct4) efficiently induce iPS cells [33]. The butyrate [34] DNA methyl transferase inhibitor, RG108 [35], improves the efficiency of skeletal myoblast reprogramming. Interestingly, cardiomyocytes differentiated from these skeletal myoblast-derived iPSs (SiPS) improved the cardiac function of an infarcted heart without tumorigenesis [35, 36]. Epigenetic studies of reprogramming and stemness have attracted the interest of many researchers [37–40]. Indeed, hot spots are investigated that are difficult to methylate [41]. Therefore,

more efficiency is expected by identifying and modifying these spots.

Congenital heart diseases, modified from the work of Ackerman et al. [42], are summarized in Table 1. The diseases of channelopathies and cardiomyopathies are listed and summarized with experts evaluation, “is recommended” or “not is recommended,” according to the present characterization of gene mutations. Currently, channelopathies have been well characterized because of systematic measurements of cardiomyocytes or beating embryonic bodies (EBs). These diseases are candidates for *in vitro* models from iPS cells. Recently, iPS cell-derived cardiomyocytes from Pompe disease, known as a glycogen storage disease, were established and were revealed to have higher glycogen contents than hESC and control iPS-derived cardiomyocytes [43]. The generation of iPS-derived cardiomyocytes from these patients is expected to provide important information about these diseases.

### 3. Generation of Cardiomyocytes from iPS Cells

The differentiation method from iPS cells into cardiomyocytes basically follows the protocol of embryonic stem (ES) cells, using embryonic bodies (EBs, see Figure 2). Yang et al. showed that KDR<sup>low</sup>/C-KIT<sup>neg</sup> EBs differentiated into cardiomyocyte lineages and became NKX2.5, ISL1, TBX5 positive but not KDR<sup>low</sup>/C-KIT<sup>pos</sup> or KDR<sup>neg</sup>/C-KIT<sup>pos</sup> [44]. The combination of activin A, BMP 4, basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), and Dickkopf homolog 1 (DKK1) in a serum-free media was necessary for cardiomyogenesis. Likewise, addition of Wnt inhibitors to BMP 4 enhanced cardiomyogenesis [45]. These activin/nodal and BMP signaling pathways promote cardiac differentiation in a stage-specific manner [46]. The role of c-kit may be different even in the embryonic stage, since c-kit high-expressing cells became cardiomyocytes and other cardiac cell lineages near birth [47]. The level and timing of c-kit expression can change its role [48]. Flk-1<sup>+</sup> cells from EB clusters are produced in ES cell cultures without LIF, and cardiac progenitors and cardiovascular cells were also formed from these EB clusters [49, 50].

Cardiomyocytes obtained from iPS cells were functionally similar to ES cells-derived cardiomyocytes [51], and multiple type action potential (nodal, atrial, and ventricular) phenotypes were observed [52]. Overall, the gene expression profiles of iPS cells were similar to ES cells, but differentiation direction and efficiency were variable [53, 54]. Overall, iPS-cell-derived cardiomyocytes have similar contractile behaviors to ES cell-derived cardiomyocytes but are significantly different from native tissues from comparable ages [52]. However, the drug effect on iPS-cell-derived cardiomyocytes is similar to cardiomyocytes derived from hESC cells [55]. As a cell source, ventricular cardiomyocytes produced more cardiomyocytes than somatic cells such as tail-tip fibroblasts [56]. The variability of differentiation among the cell lines has been previously reported [57]. The heterogeneity of iPS-derived cardiomyocytes is a problem for establishing good models [58]. One of the solutions is to obtain extremely

pure cardiomyocytes to eliminate heterogeneity as much as possible. Ma et al. selected highly purified iPS cell-derived cardiomyocytes using blastocidin resistance gene expression controlled from the cardiac-specific endogenous MYH6 promoter and investigated drug electrophysical properties [59]. Another method used to eliminate heterogeneity was to establish a systematic protocol which produced highly purified cardiomyocytes (more than 90%) by optimization of the culture condition [60]. Cao et al. reported that ascorbic acid robustly enhanced cardiomyogenesis of all 11 lines so that differences were smaller [61]. Ascorbic acid functioned to proliferate cardiomyocyte progenitors. Ribosomal S6 kinase [62] and mitogen-activated protein kinase (MAPK) activities [63] affected cardiomyogenesis. Some small molecules had been known to have effect on cardiomyogenesis. Previously investigated effects of 36 small molecules using ES cells were summarized [64]. In addition to that, recently, small molecule, dorsomorphin, an inhibitor of BMP signaling [65], and XAV929, an inhibitor of Wnt/ $\beta$  signaling [45, 66], promoted cardiomyogenesis. Cyclosporin-A [67], sulfonyl hydrazone-1 [68], and even a simple dissociation of EBs [69] enhanced cardiomyogenesis. These molecules will help to accelerate cardiomyogenesis. However, a more concise profiling of molecular signatures is necessary to evaluate maturity and function.

Recently, a unique method to purify cardiomyocytes using the high number of mitochondria within cardiomyocytes was reported [70, 71]. In this method, genetic engineering is not required, and damage to cells should be decreased. On the other hand, another method was established using the signal-regulatory protein alpha (SIRPA), which can select immature cardiomyocytes which have fewer mitochondria [72].

### 4. Future Model of Heart Disease Composed of the iPS-Cells-Derived Cardiomyocytes

Very recently, in a genomic mutation heterozygous for polycystic kidney disease 1 (Pkd1), the deletion is restored by spontaneous mitotic recombination [73]. Indeed, the frequency of genetic repair events by spontaneous mitotic recombination in pluripotent stem cells is higher than that in somatic cells [74]. Interestingly, from the RT-PCR data from Cheng et al., not only wild-type iPS cells but also  $-/-$  iPS were detected [73]. These results are also important to heart diseases, especially for dominant mutation. Comparison of these  $(+/+)$  and  $(-/-)$  cells can be perfect because there is no genetic background difference, since they are derived from the same person.

Currently, several multielectrode array systems for *in vitro* extracellular electrophysiology are available for QT prolongation screening with iPS cell-derived myocytes. In order to screen the function of mutated channels located on subcellular organelles such as the RYR2, fluctuations in intracellular  $Ca^{2+}$  concentrations should be measured. Development of a user-friendly detection system for stimulation and recording of such channels in patient cardiomyocytes is

TABLE 1: HRS/EHRA Expert Consensus Statement on Genetic Testing (Heart Rhythm 2011; 8 : 1308–1339).

Cardiac Channelopathy /Cardiomyopathy	Diagnostic implications of genetic testing	Class I “is recommended”	Class IIa “can be useful”	Class IIb “may be considered”	Class III “is not recommended”	Testing genes	Common disease genes Genes % of disease
Long QT syndrome (LQTS)	Patient in whom a cardiologist has established a strong clinical index of suspicion for LQTS	○					
	Asymptomatic patient with QT prolongation in the absence of other clinical conditions that might prolong the QT interval on serial 12-lead ECGs defined as QTc >480 ms (prepuberty) or >500 ms (adults)	○				Comprehensive or LQT1-3	KCNQ1 (LQT1) KCNH2 (LQT2) SCN5A (LQT3) LQT4-13
	Asymptomatic patient with otherwise idiopathic QTc values >460 ms (prepuberty) or >480 ms (adults) on serial 12-lead ECGs			○			30–35% 25–40% 5–10% >5%
	Family members and other appropriate relatives subsequently following the identification of the LQTS-causative mutation in an index case	○				Mutation-specific	
Catecholaminergic polymorphic ventricular tachycardia (CPVT)	Patient in whom a cardiologist has established a clinical index of suspicion for CPVT	○				Comprehensive or CPVT1 and CPVT2	RYR2 (CPVT1) 60%
	Family members and appropriate relatives following identification of the CPVT-causative mutation in an index case	○				Mutation-specific	CASQ2 (CPVT2) 3–5%
Brugada syndrome (BrS)	Family members and appropriate relatives following identification of the BrS-causative mutation in an index case	○				Mutation-specific	
	Patient in whom a cardiologist has established a clinical index of suspicion for BrS The setting of an isolated type2 or type3 Brugada ECG pattern		○		○	Comprehensive or SCN5A —	SCN5A (BrS1) 20–30%

TABLE 1: Continued.

Cardiac Channelopathy /Cardiomyopathy	Diagnostic implications of genetic testing	Class I “is recommended”	Class IIa “can be useful”	Class IIb “may be considered”	Class III “is not recommended”	Testing genes	Common disease genes Genes % of disease
Progressive cardiac conduction disorders (CCD)	Family members and appropriate relatives following the identification of the CCD-causative mutation in an index case Patients with either isolated CCD or CCD with concomitant congenital heart disease, especially when there is documentation of a positive family history of CCD	○				Mutation-specific  <i>SCN5A</i> and <i>TRPM4</i>	<i>SCN5A</i>  5%
Short QT syndrome (SQTS)	Family members and appropriate relatives following the identification of the SQTS-causative mutation in an index case Patient in whom a cardiologist has established a strong clinical index of suspicion for SQTS based on examination of the patient's clinical history, family history, and electrocardiographic phenotype.	○		○		Mutation-specific  Comprehensive or SQT1-3	<i>KCNH2</i> (SQT1) <i>KCNQ1</i> (SQT2) <i>KCNJ2</i> (SQT3)  >5% >5% >5%
Atrial fibrillation (AF)	Genetic testing is not indicated for atrial fibrillation at this time.				○	—	None of the known disease-associated genes has been shown to account for >5% of this disease.
Hypertrophic cardiomyopathy (HCM)	Patient in whom a cardiologist has established a clinical diagnosis of HCM  Family members and appropriate relatives following identification of the HCM-causative mutation in an index case	○				Comprehensive or targeted ( <i>MYBPC3</i> , <i>MYH7</i> , <i>TNNI3</i> , <i>TNNI2</i> , and <i>TPM1</i> )  Mutation-specific	<i>MYBPC3</i> <i>MYH7</i> <i>TNNI2</i> <i>TNNI3</i> <i>TPM1</i>  20–45% 15–20% 1–7% 1–7% >5%
Arrhythmogenic cardiomyopathy (ACM)/Arrhythmogenic right ventricular cardiomyopathy (ARVC)	Family members and appropriate relatives following identification of the ACM/ARVC-causative mutation in an index case Patients satisfying task force diagnostic criteria for ACM/ARVC Patients with possible ACM/ARVC (1 major or 2 minor criteria) according to the 2010 task force criteria (European Heart Journal) Patients with only a single minor criterion according to the 2010 task force criteria	○	○	○	○	Mutation-specific  Comprehensive or targeted ( <i>DSC2</i> , <i>DSG2</i> , <i>DSP</i> , <i>JUP</i> , <i>PKP2</i> , and <i>TMEM43</i> )  —	<i>PKP2</i> <i>DSG2</i> <i>DSP</i> <i>DSC2</i> <i>TMEM43</i>  25–40% 5–10% 2–12% 2–7% >5%

TABLE 1: Continued.

Cardiac Channelopathy /Cardiomyopathy	Diagnostic implications of genetic testing	Class I “is recommended”	Class IIa “can be useful”	Class IIb “may be considered”	Class III “is not recommended”	Testing genes	Common disease genes Genes % of disease
Dilated cardiomyopathy (DCM)	Patients with DCM and significant cardiac conduction disease (i.e., first-, second-, or third-degree heart block) and/or a family history of premature unexpected sudden death	○				Comprehensive or targeted ( <i>LMNA</i> and <i>SCN5A</i> )	
	Family members and appropriate relatives following the identification of a DCM-causative mutation in the index case	○				Mutation-specific	<i>LMNA</i> <i>SCN5A</i> >5% >5%
	Patients with familial DCM to confirm the diagnosis, to recognize those who are at highest risk of arrhythmia and syndromic features, to facilitate cascade screening within the family, and to help with family planning		○			Mutation-specific	
Left ventricular noncompaction (LVNC)	Family members and appropriate relatives following the identification of an LVNC-causative mutation in the index case	○				Mutation-specific	<i>LBD3</i> ~5%
Restrictive cardiomyopathy following the identification of an RCM-causative mutation in the index case	Patients in whom a cardiologist has established a clinical diagnosis of LVNC		○			<i>LBD3</i> , and so forth	
	Family members and appropriate relatives following the identification of an RCM-causative mutation in the index case	○				Mutation-specific	<i>β-MHC</i> ~5%
Out-of-hospital cardiac arrest survivors	Patients in whom a cardiologist has established a clinical index of suspicion for RCM			○		<i>MYH7</i> , <i>TNNI3</i> , <i>TNNI2</i>	<i>TNNI3</i> ~5%
	The survivor of an Unexplained Out-of-Hospital Cardiac Arrest Routine genetic testing, in the absence of a clinical index of suspicion for a specific cardiomyopathy or channelopathy	○			○	Appropriate genes following diagnosis of the survivor —	<i>RYR2</i> <i>KCNQ1</i> <i>KCNH2</i> 10–15% 5–10% ~5%
Postmortem genetic testing in sudden death cases (SUD/SIDS)	For all SUDs and SIDS cases, collection of a tissue sample	○				comprehensive or targeted ( <i>RYR2</i> , <i>KCNQ1</i> , <i>KCNH2</i> , and <i>SCN5A</i> )	<i>RYR2</i> <i>KCNQ1</i> <i>KCNH2</i> <i>SCN5A</i> SCN5A : 3–5%
	In the setting of autopsy negative SUDS			○			
Family members and other appropriate relatives following identification of a SUDS-causative mutation in the decedent		○				Mutation-specific	

HRS: the Heart Rhythm Society, EHRA: European Heart Rhythm Association. We summarized their tables with permission.



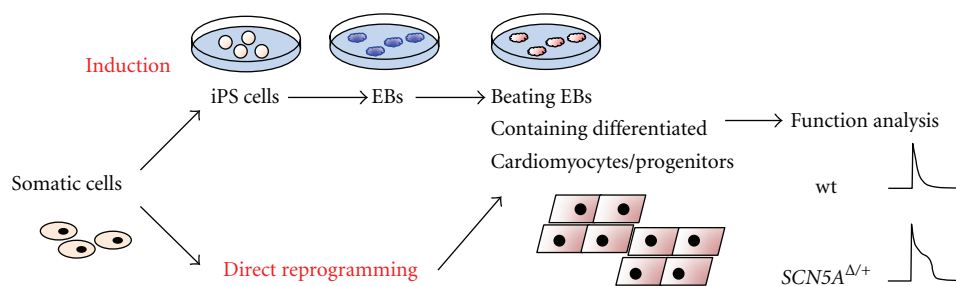


FIGURE 2: The methodology for *in vitro* cardiomyocyte differentiation.

expected [75], as well as sensor techniques and bioanalytical approaches for cardiotoxicity testing [76].

Because tissues are three dimensional, 3D *in vitro* models can be made using scaffolds [77] or cell sheets [78, 79] in the near future. The process of tissue formation can be observed and compared with normal tissue formation. For this purpose, not only cardiomyocytes, but also other cardiac cells should be developed. Hearts contain a vascular system, which is difficult to constitute using a 2D model; however, it may be possible using a 3D model [80].

Gene expression levels [81, 82] and protein profiles [83] can be analyzed similarly to other cell culture systems. Recent progresses in the investigation of micro-RNA have provided information on the process to disease. Micro-RNA can be biomarkers for cardiovascular diseases [84] and have gained attention as regulators for cardiac injury and protection [85]. Cardiac differentiation by BMP from cardiac progenitors was mediated by micro-RNA [86]. In fact, micro-RNA is associated with cell fate decision [87]. In cardiomyocyte differentiation, miR-1 and miR-133 are upregulated, and miR-499 promotes cardiomyogenesis [88]. Thus, the state of the disease can be more precisely assessed by micro-RNA expression. Networks of mRNA and micro-RNA to determine human cardiomyocytes differentiation were investigated [89], and such attempts should be required, and analytical development is also required to fit this. Not only gene expression, but also global methylation analysis of CpG islands and the identification of non-CpG islands by next generation sequences is also useful. Other epigenetic approaches should make progress in this field [90]. Because some differences were reported between iPS-cell-derived cardiomyocytes and tissue-derived cardiomyocytes, where iPS cell-derived cardiomyocytes were more immature than tissue derived cardiomyocytes, further studies should be performed to evaluate their quality.

## 5. Conclusion

Using iPS cells for *in vitro* heart disease models is a promising method for evaluating drug effects. Many disease models should be constructed. However, further studies are necessary to evaluate cardiomyocytes in terms of heterogeneity using molecular biological analyses derived from the patient's tissues.

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## Review Article

# Extrafollicular Dermal Melanocyte Stem Cells and Melanoma

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Recent studies suggest that extrafollicular dermal melanocyte stem cells (MSCs) persist after birth in the superficial nerve sheath of peripheral nerves and give rise to migratory melanocyte precursors when replacements for epidermal melanocytes are needed on the basal epidermal layer of the skin. If a damaged MSC or melanocyte precursor can be shown to be the primary origin of melanoma, targeted identification and eradication of it by antibody-based therapies will be the best method to treat melanoma and a very effective way to prevent its recurrence. Transcription factors and signaling pathways involved in MSC self-renewal, expansion and differentiation are reviewed. A model is presented to show how the detrimental effects of long-term UVA/UVB radiation on DNA and repair mechanisms in MSCs convert them to melanoma stem cells. Zebrafish have many advantages for investigating the role of MSCs in the development of melanoma. The signaling pathways regulating the development of MSCs in zebrafish are very similar to those found in humans and mice. The ability to easily manipulate the MSC population makes zebrafish an excellent model for studying how damage to MSCs may lead to melanoma.

## 1. Introduction

Cutaneous malignant melanoma is the most serious form of skin cancer with the highest mortality rate. The rate of incidence for melanoma has been rising around the world with the USA reporting about 60,000 cases and 8,000 deaths every year [1]. It is a complex disease with multifaceted etiology that involves both genetic and environmental factors. Most melanomas arise *de novo*, but some are known to arise in preexisting nevi or moles [2]. Melanomagenesis is often medically described as a gradual transformation of a cutaneous melanocyte in the basal layer of the epidermis, enabling the stepwise metamorphosis from nevus to radial-growth phase, to vertical growth, and finally to metastatic malignant melanoma [3].

The identity of the original target cell that acquires the requisite DNA lesions for transformation to melanoma still remains elusive. The traditional hypothesis is that cutaneous melanocytes progressively accumulate mutations in oncogenes and tumor suppressor genes over a long period of time during exposure to UV, leading to uncontrolled proliferation, acquisition of invasive properties, and ability to metastasize

[4, 5]. An alternative hypothesis is that melanoma begins in an extrafollicular dermal melanocyte stem cell (MSC). Although MSCs have not been definitively isolated, they are likely to exist based on the presence of stem cell markers on putative melanocyte precursors in the dermis [6] and on multipotent stem cells in the dermis that have been isolated and shown to differentiate into melanocytes in human skin constructs [7–9]. Understanding the mechanisms controlling self-renewal, expansion, and differentiation of the extrafollicular MSCs will provide greater insight into the possible mechanisms for the neoplastic development of malignant melanoma. UV-induced mutation may alter the normally tightly controlled process of self-renewal, expansion, and differentiation of MSCs as well as their exit from the stem cell compartment. Different forms of melanoma may reflect the stage in the melanocytic differentiation pathway where transformation occurs.

The purpose of this review is to summarize the data supporting the existence of extrafollicular dermal MSCs and to explain how the neoplastic development of malignant melanoma is better understood when it is viewed as having its earliest origins in an extrafollicular MSC or in

a melanocyte precursor derived from it, rather than in a mature, fully differentiated cutaneous melanocyte in the basal layer of the epidermis [9–11].

## 2. Results and Discussion

**2.1. Epidermal Melanocytes.** In humans melanocytes are melanin-producing cells that comprise only about 5–10% of all of the body's skin cells. Each melanocyte is located on the basal layer of the epidermis and immediately surrounded by approximately five keratinocytes. Through multiple dendritic extensions, melanocytes establish contact with an additional 35–40 keratinocytes to form an epidermal melanin unit. The epidermal melanin unit is thought to be a symbiotic relationship between the melanocyte and the pool of associated keratinocytes. The melanocyte maintains this homeostatic ratio throughout its lifespan [12, 13]. When a keratinocyte is exposed to sunlight, it relays signals to its connecting melanocyte and turns on the pathways leading to melanin synthesis and the formation of melanosomes. The melanosomes are then transferred from the melanocytes to the keratinocytes through the dendritic connections, collectively forming a temporary microparasole over the skin. Melanin serves to absorb UV photons and to quench highly reactive oxygen radicals, providing the skin with a first line of defense from the damaging rays of UV radiation [14].

Although melanocyte mitosis has been rarely observed *in vivo* under normal physiological conditions, this is not to suggest that melanocytes have lost their ability to divide. A small percentage of melanocytes appear to divide during normal skin homeostasis [15]. When melanocytes are separated from their normal physiological niche and cultured in isolation, they divide continuously with doubling times of 48–96 hrs [16, 17]. The precise mechanisms that control the organization and the number of melanocytes in the epidermis are unknown. Several studies strongly suggest that keratinocytes interact with melanocytes via growth factors, cell surface molecules, or other factors related to proliferation and differentiation of the epidermis [18–20]. Continuous crosstalk between keratinocytes and melanocytes is required to maintain a ratio of approximately 5:1 of keratinocytes to melanocyte on the basal epidermal layer [21, 22]. When melanocytes become transformed, they escape the controlling influences of the surrounding keratinocytes and develop new cellular connections with fibroblasts and endothelial cells to support their growth and invasion [23].

**2.2. Evidence for Extrafollicular Dermal MSCs.** Even though extrafollicular dermal MSCs have not yet been isolated in human skin, it is plausible that they do exist based on indirect evidence of stem cell markers, tissue culture studies, and repigmentation patterns observed in patients with vitiligo. Although it is always possible that existing melanocytes could be stimulated to divide and to replace damaged melanocytes, given the vital role that melanocytes play in constantly protecting the skin against UV radiation, the skin is likely to have a pool of MSCs in a well-protected area of the dermis to draw upon to replace any damaged melanocytes in the basal

layer of the epidermis. The signal to replace a melanocyte may originate from the surrounding keratinocytes that comprise the epidermal melanin unit. Fully differentiated melanocytes, like other cells of the skin, are constantly exposed to solar UV. After a sufficient number of lesions, a melanocyte that is damaged beyond the capability to repair itself is likely to be eliminated via an apoptotic pathway. Following apoptosis the homeostatic balance between the melanocyte and surrounding keratinocytes will be disrupted thus signaling a melanocyte stem cell in the dermis to differentiate and migrate to the epidermis to reestablish the melanocyte: keratinocyte ratio. For example, this may occur after a severe sunburn or during body growth from childhood to adult stature to accommodate increased skin surface area.

It was suggested many years ago that extrafollicular dermal melanocytes may be derived from pluripotent cells that migrate from the neural crest to the skin via the paraspinal ganglia and their peripheral nerves [24–26]. Here they give rise to melanocyte precursors when replacements for epidermal melanocytes are needed in the postnatal skin [27]. Many studies support the hypothesis that peripheral nerves may function as a MSC niche from which cutaneous melanocytes are recruited during skin regeneration and repair [28–32]. The most convincing evidence comes from the isolation of human multipotent stem cells from the dermis of glabrous skin that are capable of self-renewing and expressing the neural crest stem cell markers NGFRp75 and nestin. When these stem cells are placed in human skin reconstructs, they migrate to the basal epidermis, establish communication with keratinocytes, and differentiate into melanocytes [7, 9]. Other supporting evidence for extrafollicular MSCs in the dermis comes from the observation that Schwann cells, the principal glia of peripheral nerves, display a highly unstable phenotype that can be reversed or induced to transdifferentiate while in culture, indicating that these cells, like stem cells, are permissive for phenotypic changes [33].

The presence of MSC markers KIT and BCL-2 in the basal layer of human epithelia suggests the presence of melanocyte precursors [6]. Another possible marker of a MSC, human CD133 antigen, has been found in stem cell niches comprising the basal layer of human neonatal epidermis [34]. Other evidence for dermal MSC comes from clinical studies in patients with a skin disorder, vitiligo. Vitiligo is an acquired disorder of pigmentation, characterized by depigmented, cutaneous macules resulting from the loss of functioning melanocytes. When a patient with vitiligo was administered an oral dose of 8-methoxypsoralen in combination with UVA therapy to stimulate repigmentation, tyrosinase-positive melanocytes were detected along the basement membrane in the previously depigmented palms. This provides clinical evidence supporting the existence of a MSC reservoir in glabrous skin [35, 36].

The evidence for the presence of extrafollicular dermal MSCs in association with the peripheral nerves is derived from a variety of vertebrates including quail, chicken and zebrafish, suggesting that the developmental pathways regulating MSCs are conserved and that the use of these animal



TABLE 1: Evidence for the presence of extrafollicular dermal MSCs in human skin and in association with peripheral nerves of other vertebrates.

Source	Origin	MSC marker	Marker type
Human	Dermis	kit (+), trp-1(-), bcl-2 (+)	Cytokine receptor, tyrosinase apoptosis regulator [6]
Human	Dermis	NGFRp75, Oct-4	Nerve growth factor [7–9]
Quail	Schwann cells	ETR-B	Endothelin receptor [29]
Chicken	Spinal ganglia	Melanin	Pigment [31]
Zebrafish	Dermis	trp-1 (-)	Tyrosinase [37–40]
Zebrafish	Nerve	foxd3 and sox10	Transcription factors [41]

models will likely yield important insights into the origin, regulation and control of MSCs in humans (Table 1).

The presence of stem cell markers which are indicative of cells in the melanocyte lineage, the isolation of multipotent stem cells from the human dermis capable of differentiating into melanocytes, and the capability of melanocytes to regenerate provide substantial evidence that the vertebrate dermis contains an extrafollicular reservoir of MSCs.

There is some evidence to suggest that the bulge region of the hair follicle might serve as a supplemental reservoir of MSCs to replenish melanocytes in the basal epidermal layer of the skin [42]. This study is supported by the finding that MSCs in the bulge region of the murine hair follicle are capable of producing transient amplifying cells with the potential to migrate into empty niches including the skin [43].

**2.3. Regulation of Extrafollicular Dermal MSCs.** The microphthalmia-associated transcription factor (MITF) is the master regulator of melanocyte differentiation, development and survival [44]. It plays a central role in the complex network of interacting genes regulating the migration, survival and proliferation of melanocytes [45–47]. Because of its crucial importance in regulating the development of melanocytes, it is not surprising to find that at different stages of melanocyte development, MITF expression is regulated by an array of cooperating transcription factors that all influence how the MITF promoter responds to developmental signals [45]. MITF is also required to establish the MSC in the follicular niche [48, 49] and for this reason, it is thought to play a role in regulating extrafollicular dermal MSCs.

Two important transcription factors, PAX3 and SOX10, are involved in the regulation of the MITF promoter and thus may also play critical roles in regulating the extrafollicular MSCs [50–52]. Synergistically with SOX10, PAX3 strongly activates MITF expression. These two transcription factors physically interact and bind directly to the MITF promoter [53, 54]. PAX3 is a member of a highly conserved family of transcription factors essential during the early development

TABLE 2: Genes involved in the development of melanocytes in humans, mice, and zebrafish.

Gene	Description	Humans	Mice	Zebrafish
MITF	Transcription factor	[62, 63]	[64]	[65]
PAX3	Transcription factor	[66]	[67]	[68]
SOX10	Transcription factor	[50, 52]	[69]	[70]
Wnt	Signaling protein	[71]	[72]	[73]
Notch	Membrane protein	[74, 75]	[76]	[77]
KIT	Cytokine receptor	[6, 78]	[79]	[5]

of many different tissue types and to the preservation the stem cell state. It affects melanocyte proliferation, resistance to apoptosis, migration, lineage specificity and differentiation [55, 56]. This transcription factor functions as a nodal point for coactivators and inhibitory proteins involved in regulating the MSC, as well as its differentiation into a mature melanocyte. Although the PAX3 protein is required for melanocyte development during embryogenesis, it is not found in normal mature melanocytes. PAX3 expression preserves pluripotency and its repression induces differentiation [57].

Wnt and Notch signaling pathways also play key roles in regulating MSCs. WNT signaling regulates quiescence, expansion and differentiation of MSCs by modulating the levels of PAX3, SOX10 and MITF [56–58]. NOTCH signaling pathways are part of an evolutionary conserved complex of signaling pathways and are essential for the maintenance of the immature status of MSCs. In the hair follicles of mouse skin, NOTCH signaling controls the spatial distribution of MSCs and the timing of their differentiation into melanocytes [59, 60]. These signaling pathways may be equally important in regulating both quiescent (out of cell cycle and in a lower metabolic state) and active (in cell cycle and not able to retain DNA labels) MSC subpopulations that may coexist in the dermis in separate yet adjoining locations [61].

Genes regulating the development of MSCs are highly conserved in humans, zebrafish and mice, providing confidence that experimental findings involving MSCs in zebrafish can be translated to humans for understanding the origins of melanoma (Table 2).

**2.4. Melanoma Begins in an Extrafollicular Dermal MSC.** Solar ultraviolet (UV) radiation is the prominent environmental physical carcinogen involved in melanoma. Decades of epidemiologic studies link solar UV radiation to the development of malignant melanoma [80]. Solar UV reaching Earth's surface is a continuum of electromagnetic radiation and is divided into UVA (320–400 nm) and UVB (290–320 nm) wavelengths for purposes of describing the biological effects associated with long- and short-wave UV radiation. The specific contribution of UVA and UVB in the etiology of melanoma is controversial. However, there

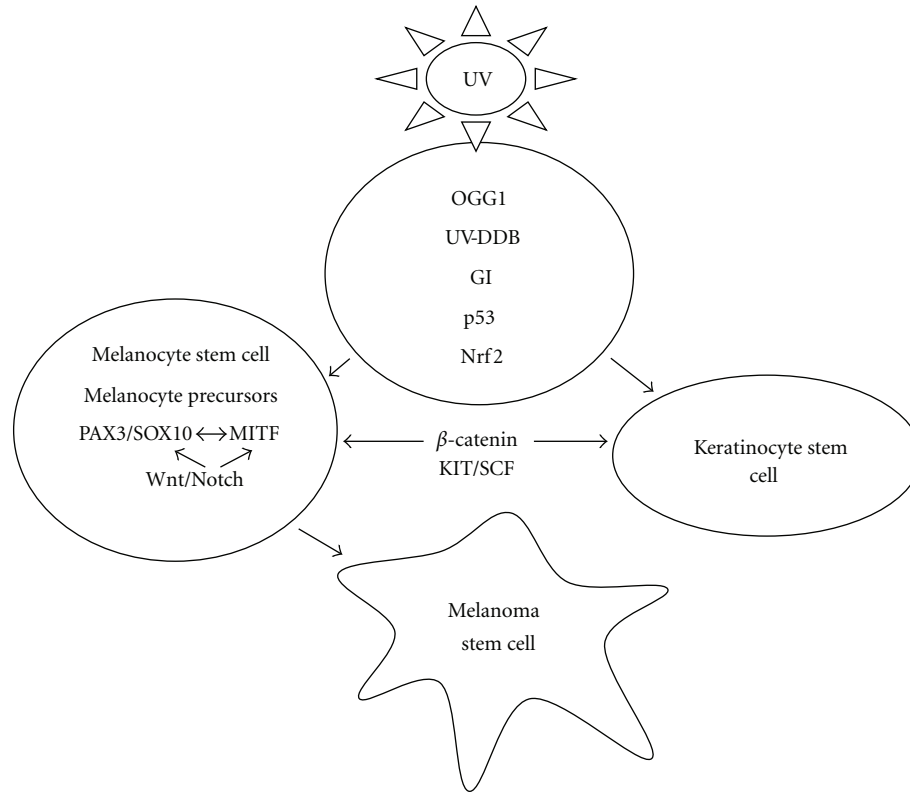


FIGURE 1: A model summarizing how important signalling pathways, and some DNA repair and transcription factors in melanocyte and keratinocyte stem cells or in their derivatives, might be impaired by UV irradiation, leading to the development of melanoma stem cells. Lower levels of the repair enzyme, human 8-oxoguanine-DNA glycosylase (OGG1), UV-damaged DNA-binding protein (UV-DDB), along with an attenuated p53 apoptotic response will increase survival of cells in the melanocyte lineage with mutational loads and genetic instability (GI). Increased expression of Nrf2 will further prevent UVA-induced apoptosis and thus promote survival of cells, increasing the retention and accumulation of mutations. Mutations in critical genes for transcription factors regulating melanocyte stem cell proliferation and differentiation (MITF, PAX3 and SOX10) or in signaling pathways (Notch and Wnt) will have profound and cascading effects on those pathways regulating the quiescence, expansion and differentiation of melanocyte stem cells. Increased  $\beta$ -catenin stimulate proliferation, abnormal differentiation, and self-renewal of melanocyte stem cells. Alterations in c-kit tyrosine kinase receptor (KIT) and its ligand, (stem cell factor (SCF)), will alter the homeostatic balance between keratinocytes and melanocytes. All of these factors may interact and contribute to the transformation of an epidermal MSC into a melanoma stem cell.

is adequate evidence to suggest that both UVA and UVB radiation act together and sometimes synergistically to promote the development and progression of malignant melanoma [81]. UVB radiation is less penetrating and is directly absorbed by DNA, causing several mutagenic DNA lesions; UVA radiation on the other hand, penetrates deeper in the skin and indirectly produces its effects by the generation of reactive oxygen species (ROS), leading to oxidative damage of DNA and protein [82]. That fact that ROS is implicated in all stages of multisteps carcinogenesis suggests that UVA may play an important role in the development of melanoma [83]. However, the relative roles of UVA and UVB radiation in the development of melanoma are far from being resolved. One of the most important factors in the cellular ROS defense machinery is the transcription factor Nrf2. It induces the production of a variety of ROS detoxifying enzymes and antioxidants such as glutathione and plays an important role in the protection of the skin against UVA-induced apoptosis [84]. Nrf2-mediated ROS cytoprotection

is also thought to be partially responsible for decreased UVB-induced apoptosis in the skin [85, 86]. We speculate that this increased level of protection against apoptosis via Nrf2 may be a double-edged sword. By decreasing apoptosis of damaged cells after exposure to intense and intermittent UV radiation of tanning beds, it may well promote the retention and accumulation of UV-induced damage in quiescent and active MSCs in the dermis.

Our proposed model (Figure 1) for the origin of malignant melanoma is based on the hypothesis that cutaneous melanoma has its earliest origins in an extrafollicular MSC residing in the dermis of the skin. Under normal environmental conditions, cellular DNA in the MSC will be frequently exposed to various doses and fluence rates of UVA and UVB radiation from sunlight, and in some cases, from exposure to high fluences delivered by tanning beds. UVA radiation because of its longer wavelength will penetrate deeper into the dermal layer of the skin than the shorter UVB wavelengths [87]. Therefore, our model places greater

emphasis on the effects of UVA radiation on dermal MSCs in the beginning stages of melanoma. UVA has the potential to inflict DNA damage to the MSC residing in the superficial nerve sheath of peripheral nerves. This scenario is similar to what is found in squamous cell carcinomas where UVA fingerprint mutations are most abundant in the basal germinative layer, suggesting that UVA- rather than UVB-induced DNA damage is an important carcinogen in the stem cell compartment of the skin [88]. Damage to DNA and repair proteins in MSCs will occur via the production ROS, hydrogen peroxide, and superoxide anion [89]. ROS may also give rise to the very reactive hydroxyl radical via Fenton reactions when ferritin, which is known to restrict the availability of iron after UVA irradiation, is impaired or fails to be induced [90, 91]. Short-term exposure to high fluences of UVA in tanning beds provokes an immediate increase in intracellular labile iron before defense mechanisms have time to remove the iron. This provides an ideal environment for generating oxidative reactions leading to increased damage to DNA and proteins [92]. An important repair enzyme, human 8-oxoguanine-DNA glycosylase (OGG1), known to be involved in repairing UVA-induced oxidative DNA damage, is much lower in skin cells of the basal layer of the human epidermis including melanocytes [93]. This suggests that oxidative DNA mutations may also be less efficiently repaired in MSCs. A study showing that epidermal stem and progenitor cells in murine epidermis are prone to the accumulation of cyclobutane pyrimidine dimers (CPDs) despite nucleotide excision repair (NER) proficiency suggests that human MSC will also accumulate more DNA damage in the form of CPDs during chronic UV irradiation [94, 95]. UVA irradiation has been shown to promote a greater number of oxidative DNA lesions in melanocytes than in keratinocytes, supporting the role that UVA may play in promoting DNA damage in cells of the melanocyte lineage including MSCs or melanocyte precursors [96].

Our extrafollicular dermal MSC model for the origin of melanoma predicts that any MSC residing in the dermis will accumulate DNA damage over the lifetime of an individual when protective and repair mechanisms are impaired due to the cumulative effects of UVA exposure from the sun or artificial sources such as tanning beds. Emerging evidence indicates that both quiescent (out of cell cycle and in a lower metabolic state) and active (in cell cycle and not able to retain DNA labels) stem cell subpopulations may coexist in several tissues in separate yet adjoining locations [61]. This may provide opportunities for MSCs to accumulate mutations when repair mechanisms are impaired. Severe sunburn in early childhood poses a significant risk for the development of melanoma well into adulthood [97, 98]. Damage to repair pathways will make MSCs more susceptible to subsequent radiation. When this occurs earlier in life, the MSCs will have a longer period of time to accumulate additional mutations [10]. Through the years, if they evade DNA repair and escape apoptosis due to defects in ROS defense mechanisms and damage response signaling pathways, MSCs will progressively accumulate genetic and epigenetic changes in their genome. We speculate that over time the dermal MSCs in different areas of the skin will accumulate a vast array of

mutations due to exposure to different intensities of UVA from natural and artificial sources.

The importance of DNA repair in preventing the development of melanoma is illustrated in patients with xeroderma pigmentosum that have a defect in the nucleotide excision repair gene XPA and develop tumors with a high frequency on sun-exposed areas of the skin [99]. This suggests that DNA repair capacity plays an important role in preventing MSCs from accumulating UVA-induced DNA lesions that will tend to make any adult melanocyte developmentally derived from them to be more vulnerable to subsequent UVB-radiation when they migrate to the basal layer of the epidermis. Early onset of tumors and malignancy due to unrepaired DNA lesions, mutations or chromosomal modifications will then occur more often in sun-exposed areas of the skin.

MSCs ensure that adequate numbers of melanocytes are maintained so that keratinocytes receive enough melanin to protect the skin against the damaging rays of the sun. They regenerate melanocytes in response to damage and replace senescent melanocytes that no longer function. Age related loss of DNA damage repair pathways, through accumulated mutations from increased oxidative stress imposed by UV radiation, poses a significant threat to stem cell survival and function. Normal MSCs have strict control of gene expression and DNA replication whereas MSCs with loss of DNA repair may have altered patterns of proliferation, quiescence, and differentiation. Aging MSCs with loss of DNA repair may be more susceptible to malignant transformation upon subsequent exposure to intermittent UV [100]. Activation of DNA repair involves the participation of p53 [101]. Hair graying is a visible manifestation of aging MSCs and loss of self-renewal in the niche [102]. Thus, it is quite reasonable to suspect that the extrafollicular dermal MSC population in the skin is susceptible to UV-induced breakdown of DNA repair, bringing about increased genomic instability in these cells over time.

Mutated MSCs within the nerve sheath of peripheral nerves associated with skin may remain quiescent for years before they are called upon to replace severely damaged cutaneous melanocytes eliminated through apoptosis. How long the mutated MSCs remain quiescent will depend on the need of the skin to replace damaged cutaneous melanocytes. The extent of damage will depend on the nature and extent of UV radiation received by any given area of the skin over a certain period of time. For example, the intensity and frequency of both natural and artificial doses of UVA/UVB radiation experienced due to the lifestyles of any one individual, will certainly affect the lifespan of an epidermal melanocyte and thus how often the skin will need to signal a MSC to provide an replacement.

The duration as well as the precise stages of differentiation that a melanocyte precursor will go through as it migrates to a permanent residence on the basal layer of the epidermis is largely unknown. It may take several months or more to complete all four stages of melanocyte differentiation starting from the MSC (nerve-sheath precursor stage) and proceeding through the dermal and junctional migratory stages ending with the dendritic stage on the basal

layer of the epidermis [24]. It is likely that transformation of an MSC itself or of a precursor during any of the stages leading up to a fully differentiated melanocyte will involve disruption of the normally tightly controlled process of self-renewal, stem cell expansion, differentiation and migration. Any factors which disrupt the normal physiology of the niche may trigger cascading effects and disrupt the normal homeostatic mechanisms regulating MSCs. Many of the signaling pathways regulating the regeneration of organ and tissues from stem cells overlap with those involved in pathways leading to carcinogenesis. This is consistent with the hypothesis that the earliest origins of melanoma begin in an extrafollicular dermal MSC when its molecular pathways regulating cell-cycle status are altered, leading to uncontrolled proliferation and abnormal differentiation [103].

Our model for the development of melanoma is based on the premise that a melanocyte precursor will be more vulnerable to UV-induced lesions than a fully differentiated melanocyte as it progresses through the different stages of maturation before reaching permanent residence on the basal layer of the epidermis. As a dermal MSC enters the melanocytic differentiation pathway and begins its migration to the epidermis to establish contact with the surrounding keratinocytes, it will gradually become more exposed to the less penetrating but more energetic UVB rays of sunlight. UVB radiation is directly absorbed by DNA and indirectly damages proteins and lipids by the formation of ROS. UVB-induced DNA modifications can lead to deleterious mutations, while oxidation of proteins and lipids may impair cell signaling pathways [85]. This continuum of possible target cells in the layers of the skin, created by the existence of melanocyte precursors at all stages along the differentiation pathway, may explain the different degrees of malignancy commonly seen in melanoma in different areas of the skin [104]. For example, aggressive forms of melanoma may reflect carcinogenic action in melanocyte precursors that were in the more primitive stages of the differentiation pathway [105]. Melanocyte precursors that have accumulated more mutations in critical repair and defense pathways but not yet transformed will have a higher probability of being transformed at later stages when they experience additional UV radiation from natural or artificial sources during their journey to the basal layer of the epidermis. Some melanocytes may reach the epidermis but will have compromised genomes, signaling proteins and antioxidant pathways making them more susceptible to transformation when exposed to high fluences of UVA/UVB irradiation.

Keratinocyte-derived growth factors and molecular crosstalk mediated by E-cadherin are likely to play a major role in regulating the activation and proliferation of abnormal melanocyte precursors immediately derived from extrafollicular dermal MSCs. Mutations in any of the genes regulating growth factors or crosstalk pathways may contribute to the transformation of a MSC at any stage of differentiation. The  $\beta$ -catenin gene is a likely candidate since it is generally involved in the self-renewal of stem cells and mutations of this gene have been found in patients with melanoma [106].  $\beta$ -catenin dissociates from E-cadherin at the epithelial membrane and translocates to the nucleus

where it activates transcription of WNT target genes [107]. Impaired  $\beta$ -catenin signaling is known to be linked to increased proliferation, abnormal differentiation and increased self-renewal of MSCs [108]. Increasing the frequency of MSC renewal will provide greater opportunities for accumulated DNA lesions to be converted to mutations in apoptotic and cell-cycle pathways, increasing the probability that either the MSC or its immediate melanocyte precursor will have a greater chance to transform into a melanoma stem cell.

**2.5. Melanocyte Stem Cells in the Zebrafish.** The zebrafish is becoming an ideal vertebrate system to study the interplay of those variables known to play a role in the development of melanoma. The melanocyte is common to both zebrafish and humans. A wealth of zebrafish pigmentation mutants are available that affect melanocyte specification, differentiation and function. Many of these genes have conserved roles in mammals and are nearly identical to humans [109]. Fish skin biology has major relevance to mammalian skin and offers a convenient animal model to gain molecular insights into regeneration and regulation of MSCs [110].

Our laboratory is currently utilizing the zebrafish model to investigate the effects of UVB/UVA radiation on MSCs. We are taking advantage of the recent discovery that the copper chelator, neocuproine (NCP), ablates adult melanocytes but not MSCs in the zebrafish. Following NCP washout, melanocytes regenerate from MSCs [38]. This is providing us with a very powerful technique to control the development of the entire melanocyte population in the zebrafish and synchronize their regeneration. Using this drug we can irradiate the entire stem cell population at one time. The drug-induced ablation of melanocytes can be done multiple times, permitting us to study how repeated rounds of UVA/UVB irradiation of MSCs affect the development of melanoma.

The regeneration of the adult zebrafish caudal fin is offering another opportunity to determine the effects of UVA/UVB irradiation on MSCs. Melanocytes in the regenerated fin arise from MSCs rather than from migration of previously differentiated melanocytes [111]. Furthermore, ontogenetic and regeneration melanocytes not only come from the same MSCs that colonize the fin, but also from the same MSCs responsible for growth and maintenance of the melanocyte pattern [112]. Studies of single progenitors or MSCs reveal no transfating or transdifferentiation between other lineages in the regenerating fin, showing that MSCs retain fate restriction when passed through the blastema [113]. This assures us that any damage to melanocyte pattern or proliferation that persists after additional amputations will be due to permanent genetic lesions in one or more MSCs. We stop the division and progression of melanocyte precursors from MSCs at different stages after amputation of the fin by using small-molecule inhibitors to achieve transient, reversible suppression of Wnt/ $\beta$ -catenin pathway [114, 115]. Thus, we have the capability to study the effects of UV irradiation on synchronized populations of melanocyte precursors at different stages of differentiation leading to a mature melanocyte. This is helping us to determine specific



stages of increased sensitivity to UV and how increased UV at any one stage contributes to the development of melanoma.

### 3. Conclusion

Identifying the cell of origin for melanoma has a direct bearing on prognosis and chemoprevention strategies for melanoma. If melanoma has its origins in a MSC or in one of the melanocyte precursor stages that eventually leads to a fully differentiated melanocyte on the basal layer of the epidermis, it will be far better to target the pathways in these cells to keep self-renewal in check [116]. Identifying the molecular pathways and signaling molecules involved in MSC self-renewal and how these pathways are dysregulated by solar UV to produce a melanoma stem cell will be important for the development of more effective drugs for melanoma prevention and intervention [117]. If a damaged MSC or one of its precursors can be shown to be the cellular origin of melanoma, targeted identification and eradication by antibody-based therapies will be the best method to treat melanoma and a very effective way to prevent its recurrence [118]. The zebrafish model offers some powerful methods for investigating the role of MSCs in the development of melanoma.

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## Review Article

# Stem Cells as *In Vitro* Model of Parkinson's Disease

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Progress in understanding neurodegenerative cell biology in Parkinson's disease (PD) has been hampered by a lack of predictive and relevant cellular models. In addition, the lack of an adequate *in vitro* human neuron cell-based model has been an obstacle for the uncover of new drugs for treating PD. The ability to generate induced pluripotent stem cells (iPSCs) from PD patients and a refined capacity to differentiate these iPSCs into DA neurons, the relevant disease cell type, promises a new paradigm in drug development that positions human disease pathophysiology at the core of preclinical drug discovery. Disease models derived from iPSC that manifest cellular disease phenotypes have been established for several monogenic diseases, but iPSC can likewise be used for phenotype-based drug screens in complex diseases for which the underlying genetic mechanism is unknown. Here, we highlight recent advances as well as limitations in the use of iPSC technology for modelling PD “in a dish” and for testing compounds against human disease phenotypes *in vitro*. We discuss how iPSCs are being exploited to illuminate disease pathophysiology, identify novel drug targets, and enhance the probability of clinical success of new drugs.

## 1. Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder, characterized by a large number of motor and nonmotor features that can affect function in a variable degree.

The main pathological hallmark in PD is the loss of midbrain dopaminergic (DA) neurons in the substantia nigra pars compacta (SNpc) projecting to the striatum and abnormal cytoplasmic inclusions enriched in  $\alpha$ -synuclein, the Lewy bodies, deposited in surviving neurons of the brain [1–3].

There is no effective test for the diagnosis of PD; the disorder must be diagnosed based on clinical criteria. The main clinical features are tremor at rest (unilateral, prominent in the distal part of an extremity), rigidity (increased resistance to move), akinesia or bradykinesia (slowness of movement), postural instability, and other motor abnormalities. Other symptoms include secondary motor symptoms such as dystonia and dysphagia and nonmotor symptoms including cognitive abnormalities, sleep disorders, and pain [3].

Despite the research efforts in this area, with new and intriguing findings constantly being reported, at present, PD is still an incurable disease, but treatment can improve quality of life and functional capacity. To date, L-dopa in combination with a peripheral dopa decarboxylase inhibitor (benserazide or carbidopa) is the most effective therapy as an initial treatment option. However, not all symptoms respond equally to the drug; while tremor may be only marginally reduced, bradykinesia and rigidity respond better. Unfortunately, the treatment's success is reduced over time, and side effects increase, leaving the patient helpless [2]. Deep brain stimulation of the subthalamic nuclei is an additional therapeutic option for PD patients but requires surgical intervention.

Although all of these treatments provide symptomatic relief, none of them is able to stop or reverse the progression of the disease [1, 4]; for this reason there is a need for novel therapeutic approaches. One alternative strategy is cell-replacement therapy; in fact, clinical trials with intrastriatal transplantation of human embryonic mesencephalic tissue have shown that grafted DA neurons reinnervate the

striatum, restore the striatal dopamine release, and, in some patients, induce a major clinical benefit [5–7].

## 2. Molecular and Cellular Mechanism of Parkinson's Disease

The cause of PD is still unclear but most people suffering this disorder have idiopathic PD (around 90%). A small proportion of cases (approximately 10%), however, can be attributed to known genetic factors that contribute to PD complex pathogenesis.

Our understanding of the mechanisms underlying the initiation and progression of PD began with the identification of mutations in the gene encoding  $\alpha$ -synuclein (SNCA) and the demonstration that  $\alpha$ -synuclein is the major component of Lewy bodies, present in the disease. Since then, at least 16 loci (designed as *PARK1* to *PARK16*) and 11 genes have been associated with inherited forms of parkinsonism, including, for example, *PARK1*, *PARK4/SNCA*, *PARK2/parkin*, *PARK5/ubiquitin COOH-terminal hydrolase L1 (UCHL1)*, *PARK6/PTEN-induced kinase 1 (PINK1)*, *PARK/DJ-1*, and *PARK8/Leucine-rich repeat kinase 2 (LRRK2)*.

*SNCA* is an autosomal dominant gene that encodes the protein  $\alpha$ -synuclein, expressed abundantly in presynaptic terminals of the neurons [8]. Several evidences support the physiological functions of  $\alpha$ -synuclein in the regulation of vesicle dynamics at the presynaptic membrane [9]. Mutations in *SNCA* increase in the self-assembly and fibrillization of the protein that might lead to the formation of the pathogenic inclusion bodies [9]. Another autosomal dominant gene implicated in PD disease is the leucine-rich repeat kinase 2 (*LRRK2*) [8, 10]. *LRRK2* encodes a large protein with multiple domains, including a Ras-like GTP binding domain and a serine, threonine kinase domain [10]. Mutations within these two functional domains have been associated with PD [8, 10]. In normal conditions, the function of *LRRK2* kinase had been implicated in the regulation of the cytoskeleton architecture [10]. In contrast, *Parkin* is an autosomal recessive gene involved in PD [11]. This gene encodes the Parkin protein with an ubiquitin-like sequence E3, which acts as a substrate for target proteins bound to degrade by the ubiquitin proteasome system (UPS) [11]. Inactivation of Parkin leads to reduction in UPS-mediated degradation of target proteins [11] that could result in protein accumulation. In addition, some data suggest a possible function of Parkin in mitochondria, where the protein is localized and promotes gene transcription [8, 11]. *PINK1* is another autosomal recessive gene, whose mutations might cause PD [11]. *PINK1* encodes a protein localized in the mitochondria membrane and its function is associated with protection of cells from stress-induced mitochondrial dysfunction [8, 11]. Interestingly, mutants of *Drosophila melanogaster* lacking *PINK1* display phenotypes similar with those *Parkin* mutants; moreover the forced expression of Parkin1 is able to rescue the mitochondrial dysfunction caused by the absence of *PINK1*, suggesting their interaction [9, 11]. Likewise, DJ-1 is a protein localized in

the mitochondria membrane and mutations in this gene may cause autosomal recessive early-onset PD [8, 11]. Its functions are related to the resistance of oxidative stress [11].

The knowledge acquired of these proteins has revealed pathways of neurodegeneration that may be shared between inherited and sporadic PD. A set of data in different model systems strongly suggest that mitochondrial dysfunction plays a central role in clinically similar, early-onset autosomal recessive PD forms caused by *parkin* and *PINK1* and possibly *DJ-1* gene mutations [12, 13]. Further comprehension of molecular and cellular mechanisms and interaction between these proteins that causes PD with others is essential to identify crucial and potential targets to improve the treatment.

## 3. The Importance of *In Vitro* Models of PD

Most of the current knowledge about neurological diseases, including PD, is gathered from postmortem studies due to the limitations of live brain tissue. This restricts the understanding of the disease progression and development, since postmortem samples only represent the end-stage of the disease. In addition, aspects of the exhibited pathology in these samples could be secondary and not faithfully reflect the exact disease phenotype on a cellular level. Besides, interspecies differences make it difficult to accurately simulate human neurological diseases in animal models. Therefore, disease modelling by recapitulating the diseases phenotype *in vitro* and in defined cell populations is an important advancement and would make it possible to understand cellular and molecular mechanisms of the neurodegenerative disorder [14, 15]. Consider that investigation of a multifactorial disease, such as PD, is more challenging than monogenic disorders due to their complex genetic backgrounds and because they are usually influenced by environmental factors [15].

A progressive loss of substantia nigra DA neurons is the main pathological hallmark of PD. Understanding the mechanism of neuronal cell death involved in PD may be of value in developing neuroprotective therapies. However studying neuronal cell death in human brains is extremely difficult by several (methodological, practical) reasons. Development of *in vitro* models of DA neurons can be powerful, as they would allow the study of neurodegeneration as well as novel therapeutic strategies [16]. Nevertheless, availability of human DA neurons derived from fetal material is extremely limited, and it has been difficult to examine directly toxicity and/or protective effects of multiple factors in these neurons.

In this context, stem cells, particularly pluripotent stem cells and neural stem/progenitor cells, are an excellent source of cells, because of their availability, unlimited proliferation, and plasticity to differentiate into other cell types. Moreover, stem cells are an excellent alternative to *ex vivo* primary cultures or established immortalized cell lines that can contribute to our understanding of neuronal neurodegenerative process and our ability to analyze the cytotoxic or neuroprotective effects of chemicals, drugs, and so forth (Figure 1).

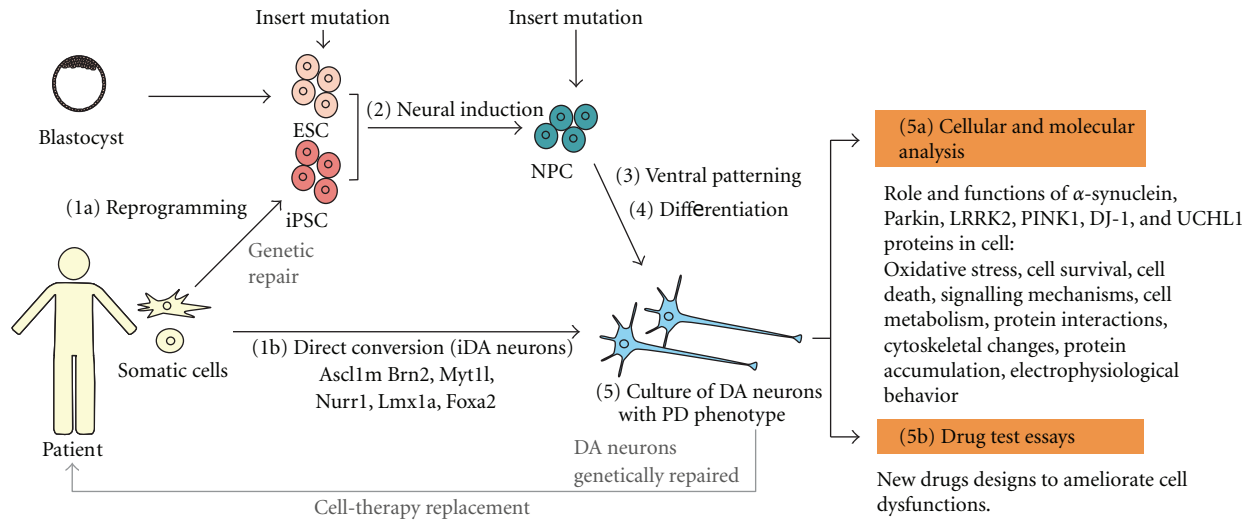


FIGURE 1: Possible cellular sources for modeling Parkinson's disease *in vitro*. Somatic cells from patient with PD can be reprogrammed into iPSC and differentiated into mesencephalic dopaminergic neurons (1a)–(5). ESC and NPC can be genetically modified by inserting specific mutations related with PD and be differentiated. Alternatively, somatic cells can be directly converted into dopaminergic neurons (1b).

#### 4. Stem Cell Types and Properties

Stem cells are characterized by the ability to renew themselves through mitotic cell division and differentiate into a diverse range of specialized cell types. They can be classified according to their potential to differentiate into specialized cells. The first type is totipotent stem cells that can give rise to an entire viable organism, including placental cells. The zygote and the cells at the very early stages following fertilization (i.e., the 2-cell stage) are considered totipotent.

The second type is pluripotent stem cells, which have the capacity to develop into specialized cells of the three germ layers (ectoderm, mesoderm, and endoderm) except extraembryonic tissues, such as placenta. The first and best described are Embryonic Stem Cells (ESCs), derived from the inner cell mass of the blastocyst [17]. Theoretically, because of their properties, these cells may constitute an optimal source of DA neurons for cell-replacement therapies and drug screening experiments; however, to achieve this aim, it is essential to have an efficient protocol for differentiation into functional midbrain DA neurons. In fact, cultures enriched in human DA neurons have been generated from ESC using a variety of methods, such as the use of the coculture with stromal cells, growth factors, secreted factors, transcription factors, and morphogens, with some beneficial effects having been demonstrated after transplantation of these cells in animal models of PD [18–21].

Recent advances in stem cell biology have led to technologies to reprogram somatic cells from the adult human to a state of pluripotency [22–24]. The first reported lines of reprogrammed cells, termed induced pluripotent stem cells (iPSCs), were originally generated by introducing four transcription factors: *Oct3/4*, *Sox2*, *Klf4*, and *c-myc* (or *Nanog*, *Lin28*) into adult fibroblasts. These cells are similar to hESC in morphology, gene expression profile, and differentiation potential. The induced iPSC technology

offers new possibilities for biomedical research and clinical applications, as these cells could be used as an *in vitro* cellular model of PD, and for autologous transplantation (theoretically, no immunosuppressive therapy would be necessary). For this reason, it is essential to obtain an efficient and strict differentiation protocol of hiPSC into midbrain DA-like neurons. In addition, hiPSC do not raise ethical concerns since they are derived from somatic cells, following routine tissue donation procedures.

The third type of stem cells is multipotent stem cells that only generate specific lineages of cells, like Neural Stem Cells (NSCs) that are derived from neural tissues. These cells are self-renewing and differentiate into lineage-specific neural precursor or progenitor cells (NPCs) that can give rise to all cell types (neurons, astrocytes, and oligodendrocytes) of the nervous system [25]. However, although sometimes not evident from the literature, hNSCs—particularly those derived from the ventral mesencephalon (vm)—grow poorly in culture, their properties change over time (passages), and they lose their ability to generate neurons, particularly DA neurons, thus making them difficult to use on a large-scale approach [25, 26].

#### 5. Directed Differentiation of Pluripotent Stem Cells into DA Neurons

The necessary first step towards PD modeling is the production, in enough number, of disease neuronal phenotypes, that is, DA neurons, from differentiated human pluripotent stem cells *in vitro*. Current *in vitro* differentiation from either ESC or iPSC includes protocols based on embryoid body formation or the use of stromal feeder coculture [18–20, 27–34]. Efficient generation of DA neurons needs the combined actions of factors such as Noggin, FGF8, Sonic Hedgehog, Retinoic Acid, Wnt1, BNDF, GDNF, Ascorbic Acid, cyclic-AMP, and Wnt5 [18, 20, 27, 29], similar to those secreted



factors present during development [35]. *In vitro*, early exposure to Noggin [20, 27], antagonist of the BMP signaling, or to inhibitors of Lefty/Activin/TGF $\beta$  pathways allows a highly efficient feeder-free neural induction in adherent cultures and permits a dopaminergic and motoneuronal potential [18, 20]. In contrast, neural induction can be obtained in the absence of factors and coculturing ES with stromal feeder cell lines [28]. Subsequently, dopaminergic patterning is established by the combined action of FGF8, FGF2, SHH [18, 20], GDNF [28], BDNF, and ascorbic acid. Finally, terminal differentiation is accomplished after withdrawal of SHH and FGF8 and promoted by the presence of ascorbic acid, GDNF, TGF $\beta$ -1, cyclic-AMP, and Wnt5 [29].

Alternatively, DA neurons can be obtained by the forced expression of transcription factors crucial for ventral midbrain identity [36–40]. Thus overexpression of *Lmx1a* induces DA neurons from murine ESC [36, 37], hESC, and hiPSC [41]; moreover, neuron precursors derived from hESC overexpressing *Lmx1a* are able to survive and differentiate when grafted into the brain of adult mice [41].

Although iPSC and ESC differ in their origin, differentiation of both cell types into DA neurons seems to use similar cues and signals. The analysis by transcriptome revealed no differences in the level of expression of genes involved in dopaminergic differentiation such as *EN1*, *Nurr1*, *TH*, *AADC*, and *Girk2*; moreover analysis of genes involved in imprinting, cell cycle regulation, and reprogramming revealed no significant differences [34].

## 6. Induced Pluripotent Stem Cells as *In Vitro* Model of PD

Derivation of pluripotent stem cells from somatic tissues has provided researchers with a source of patient-specific stem cells. In addition, iPSC technology renders a good model *in vitro* for diseases and drug treatment essays [42].

So far, some groups have developed protocols to increase the yield of DA neurons generation from iPSC from either human or mice [22, 30–32, 42, 43]. In murine models, following protocols developed for ES cells, neural precursor cells and DA neurons were obtained from healthy iPSC [33]. Moreover, derived cells transplanted into the developing brain are able to integrate, migrate, differentiate, and display electrophysiological functions showing spontaneous action potential currents in the host brain. Cells derivatives included glutamatergic, GABAergic, and DA neurons. Importantly, grafts derived from iPSC are capable to restore motor function in animal models for PD [33] suggesting that dopaminergic neurons derived from iPSC are functional *in vivo*.

In humans, DA neurons derived from iPSC can be obtained from healthy donors [34] or patients with PD caused by idiopathic conditions [32, 42] or by mutation [30, 31]. For instance, DA neurons can be generated from iPSCs that carry a mutation in *LRRK2* gene (p.G2019S), the most common PD-related mutation [8, 10]. After reprogramming, culture differentiation protocol using feeders, iPSC-p.G2019S generated a significant number of DA

neurons up to 55 days of differentiation; furthermore, these neurons show properties of mature neurons, including the expression of synaptotagmin-1, a protein localized to synaptic vesicles, and the ability of fire action potentials in response to depolarizing current injections and produce spontaneous synaptic activities. Moreover these DA neurons are able to synthesize and release dopamine in response to stimulation with high potassium [30]. A detailed phenotypic characterization related with the PD at day 35 reveals that iPSC-p.G2019S expressed higher levels of genes involved in oxidative stress pathways than controls; indeed trials testing the peroxide-induced cell death show that G2019S-iPSC-derived DA neurons may be more susceptible to oxidative stress and show significantly more cell death than controls. Due to the phenotypes in iPSC-p.G2019S resemblance to the PD phenotype that provides a good model for the *in vitro* disease, this system has been used to test some potential drugs for the treatment of PD [30].

In contrast, other models of PD based on triplication of  $\alpha$ -synuclein locus had been generated [31]. This mutation causes a fully penetrant and aggressive form of PD with dementia [8–10] compared with the homozygous G2019S mutation of *LRRK2* that has incomplete penetrance, even with homozygous conditions [44]. Using a feeder-free monolayer differentiation method, iPSC differentiated efficiently into midbrain DA neurons after 20 to 31 days when  $\alpha$ -synuclein protein could be detected and secreted to media [31]. In addition to this model, fibroblasts obtained from a patient carrying the A53T (G209A)  $\alpha$ -synuclein mutation have been reprogrammed into iPSC and successfully differentiated into DA neurons [43], which could serve as a good model for the *in vitro* analysis; nevertheless, further phenotypic characterization of cells related with PD remains to be studied.

Other models include those DA neurons derived from iPSC and obtained from patient with idiopathic conditions [32, 42]. After reprogramming patient iPSC, cells were differentiated into DA neurons using the stromal feeder cell-based differentiation protocol. At 42 days, these cells, including DA and non-DA neurons, were transplanted into the striatum of healthy animals. 8 weeks after implantation DA neurons marked with the nigral marker Girk-2 were found into the viable grafts. Moreover, transplantation experiments by engrafting DA neurons derived from iPSC on animal models of PD showed functional effects, although only a few of them sent their axons toward the DA-depleted host striatum. Analysis of behavior in animal models of PD exhibited a significant improvement of motor dysfunction [32, 34]. In summary, several evidences suggest that DA neurons from pluripotent stem cells are functional in both *in vitro* and *in vivo* conditions. Hence, some of the DA neurons derived from PD patients that exhibit some characteristic phenotypes of the disease could provide a valuable cellular source to study *in vitro* the PD. For instance, iPSCs derived from PD patients carrying a nonsense (c.1366C>T; p.Q456X) or missense (c.509T>G; p.V170G) mutations in the *PINK1* gene have been used to examine the role of endogenous PINK1 in dopaminergic neurons [45]. *PINK1* encodes a kinase localized on the outer mitochondrial membrane and



TABLE 1: Examples of *in vitro* models to study Parkinson's disease derived from patient iPSC.

Gene	Genetic disease	Genetic alteration	References
<i>SNCA</i>	Autosomal dominant	Triplication of $\alpha$ -synuclein locus	[31]
		G209A mutation	[43]
<i>LRKK2</i>	Autosomal dominant	p.G2019S mutation	[30]
<i>PINK1</i>	Autosomal recessive	p.Q456X and p.V170G mutation	[45]
Idiopathic condition	Idiopathic		[32, 42]

is implicated in the regulation of mitochondrial degradation [11]; mutations in *PINK1* have been associated with PD [8, 11]. In contrast, Parkin proteins function as an E3 ubiquitin ligase and are localized in the cytosol [11]. In addition, Parkin can be translocated to damaged mitochondria in a *PINK1*-dependent manner [44]. Thus, experiments on DA neurons from PD patients exhibit impairment in the translocation of Parkin of mutant *PINK1* iPSC cell-derived DA neurons compared to controls. Moreover, rescue experiments by overexpressing wild type *PINK1* in *PINK1* mutant neurons restore the translocation of Parkin to mitochondria [45]. In conclusion, DA neurons obtained *in vitro* from PD patients are a suitable model to study the pathogenesis of PD at cellular level (Figure 1; Table 1).

However, several challenges must be overcome before successful implementation of iPSC-based drug screening and pathway discovery can be achieved (Figure 1). The most critical issue is whether the PD phenotype can be reproduced *in vitro*, and if so, whether it can accurately predict disease behavior *in vivo*. PD can be difficult to model, since it occurs late in life and is caused by complex environmental and genetic factors. In fact, in one study that generated DA neurons from iPSC derived from patients with sporadic PD, no obvious abnormalities could be detected [42], indicating that additional stressors may be required to reveal the disease phenotype. Nevertheless, the study of rare family forms of the disease that are associated with specific gene mutations can provide valuable information on the general disease mechanisms. It would be interesting to study whether the iPSC generated from familial PD patients could exhibit disease genotypes and phenotypes *in vitro*.

Other additional limitations are related to the low efficiency and high variability of the reprogramming process and the heterogeneity of the maturation stage and cellular phenotypes obtained after differentiation of iPSC into DA neurons. Even though great progress has been made, our understanding of the factors controlling the induction and specification of DA neuronal fate is far from complete. Further advances in the field will facilitate the generation of clinically relevant DA neurons at least *in vitro*.

## 7. Direct Conversion of Somatic Cells to DA Neurons

Recent reports have demonstrated that human somatic cells can be directly converted to functional neurons, named induced neurons (iNs) by using combined expression of defined factors (*Ascl1*, *Brn2*, and *Myt1l*) [39]; the same

authors showed that these neurons can be directed toward dopaminergic phenotype by overexpression of *Lmx1a* and *FoxaA2* (two genes involved in DA neuron generation during development). A different cocktail of factors, with only three transcription factors (*Mash1*, *Nurr1*, and *Lmx1a*), were used by other group for direct generation of functional DA neurons (iDA, induced dopamine neurons) from adult fibroblasts from healthy donors and PD patients [38]. Reprogrammed cells were similar to brain DA neurons in gene expression and dopamine release. However the possible PD phenotype of the generated iDA from PD patients remains to be demonstrated.

This strategy opens new possibilities for regenerative therapies and diseases modelling of PD. Cells generated via direct conversion do not pass through a pluripotent or progenitor state, are probably not tumorigenic, and may serve as an interesting alternative to iPSCs for generating patient and/or disease-specific neurons. However, to be clinically relevant, the overall cell conversion process needs to be highly efficient in order to obtain enough amounts of cells available to study the disease or grafting studies. Both iPSC and iDA cells circumvent the ethical concerns related to embryonic stem cell derivation and potential issues of allogeneic rejection in cell-replacement therapy studies.

## 8. Future Prospects

Many questions that define the underlying genesis of the neuronal death in disorders like PD remain unanswered, with evidence suggesting a key role for mitochondrial dysfunction. In this sense, stem cells, in general, and mainly pluripotent stem cells can provide an unlimited source of human DA neurons for *in vitro* studies of neurotoxic and neuroprotective processes that might be related to PD.

iPSC technology has been shown to be of specific interest in monogenic diseases, providing innovative models to understand disease pathology. Modelling late-onset and multifactorial diseases, such as PD, may be more difficult and probably will require additional advances. However, the study of rare forms of PD, associated with specific gene mutations, can provide valuable information on the general disease mechanism. Importantly, patient cell donors can be genetically modified in order to correct mutations. This modification permits the generation of healthy and mutated DA neurons from the same donor, improving the comparative analysis between both cell types in isogenic conditions. Indeed, genetic repairment in iPSC could provide also a good tool in the advances toward iPSC-based cell-replacement therapies.

Even though the large body of current research iPSC technology is still in its infancy, several limitations need to be solved in the near future, for example, standardization in order to obtain medically relevant cells, avoiding contact with animal products, and improvement of reprogramming methods in order to increase efficiency and homogeneity and to avoid tumorigenic properties of iPSC.

Some of these limitations could be circumvented with another innovative approach, “direct reprogramming” of somatic cells from patients to specific neurons (iN). Rapid and efficient generation of patient-specific DA neurons through direct reprogramming may yield many advantages in the screening of pharmaceutical compounds as well as cellular material for analysis of molecular pathways of the disease and for transplantation studies.

## Conflict of Interests

The authors declare that they have no conflict of interests.

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## Review Article

# Skin-Derived Precursor Cells as an In Vitro Modelling Tool for the Study of Type 1 Neurofibromatosis

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The most characteristic feature of neurofibromatosis type 1 (NF1) is the development of neurofibromas. It has been suggested that these tumors are caused by somatic inactivation of the wild-type *NF1* allele, but the cell that originally suffers this mutation remains controversial. Several lines of evidence support the clonal origin of these tumors, and it has been recently suggested that skin-derived precursor cells (SKPs) could be the cell of origin of dermal neurofibromas. Nullizygous (*NF1*<sup>-/-</sup>) SKPs do give rise to neurofibromas when transplanted to heterozygous mice. Moreover, a nullizygous population of cells that is S100 $\beta$  negative is present in human neurofibromas, and *NF1*<sup>+/-</sup> multipotent progenitor cells are seemingly recruited to the tumor. This evidence supports the neurofibroma stem cell hypothesis and a putative involvement of SKPs in the aetiopathogenesis of the disease, suggesting that SKPs could become a valuable tool for the in vitro study of NF1.

## 1. Introduction

The tumor predisposition disorder von Recklinghausen's neurofibromatosis type I (NF1) is one of the most common genetic disorders of the nervous system, affecting 1 in 3500 individuals worldwide [1–4]. The disease is caused by mutation in the *NF1* gene (located on chromosome 17q11.2) that encodes the tumor suppressor protein neurofibromin, a GTPase-activating protein (GAP) [5, 6].

Neurofibromas are complex tumors that contain proliferating Schwann-like cells and other local supporting elements of the nerve fibers, as perineurial-like cells, fibroblasts, endothelial cells, pericytes, and vascular smooth muscle cells, as well as infiltration of mast cells [7]. Although several reports have studied which cell originates this tumor, the present data are somewhat contradictory. In this paper we will address the issue of the cell of origin for dermal neurofibromas to explore if the available data support the cancer stem cell hypothesis. We will discuss recent findings in the light of possible involvement of the so-called skin-derived precursor cells in the aetiopathogenesis of this complex disease.

## 2. Skin-Derived Precursors (SKPs)

Skin-derived precursors (SKPs) are a population of neural crest-derived multipotent precursor cells present in both human and mouse dermis. They can be identified in vitro as nonadherent cells isolated from the dermis that proliferate and self-renew in response to growth factors FGF-2 and EGF. Under specific differentiation conditions, they give rise to progeny of the neuronal, glial, and mesodermal lineages [8–14].

SKPs thus derive from the dermis and apparently are distinct from mesenchymal stem cells and from central nervous system neural stem cells [8, 13], although they express genes characteristic of embryonic neural crest cells, such as *Slug*, *Snail*, *Twist*, *Pax3*, and *Sox9* [8].

In vitro, SKPs can be differentiated into mesodermal lineages such as SMA+ smooth muscle cells and adipocytes, as well as into neural crest-derived tissues such as neurons and Schwann cells [8, 13]. In particular, SKPs give rise to cells with neuronal morphology that express the pan-neuronal markers  $\beta$ III tubulin and neurofilament-M and proteins characteristic of peripheral neurons such as p75NTR,



peripherin, NCAM, tyrosine hydroxylase, and dopamine  $\beta$ -hydroxylase. SKPs can also be differentiated into bipolar cells coexpressing glial fibrillary acidic protein GFAP, CNPase, S100 $\beta$ , and p75NTR, typical markers of cells with a differentiated Schwann phenotype, as well as MBP and P0 peripheral myelin protein [8, 13].

When transplanted in ovo into the chick neural crest migratory stream, SKPs mostly migrated into peripheral neural crest targets such as spinal nerve, dorsal root ganglia, and skin and expressed S100 $\beta$  [8]. In vivo, it has recently been reported that SKPs derive from Sox2+ follicle-associated dermal precursors and show characteristics of dermal stem cells. In this respect, they contribute to dermal maintenance, wound healing, and hair follicle morphogenesis [15].

### 3. Type 1 Neurofibromatosis (NF1)

The primary clinical feature of NF1 is the development of benign peripheral nerve sheath tumors, termed neurofibromas [16]. In a small percentage of NF1 patients, a particular type of neurofibromas (plexiform, see below) progress to malignant peripheral nerve sheath tumors (MPNSTs). NF1 patients are also predisposed to astrocytic brain tumors, pheochromocytoma, and juvenile myelomonocytic leukaemia [2, 17]. Noncancerous symptoms of the disease may include intellectual deficits, bone deformations, benign lesions of the iris (Lisch nodules), axillary freckling, and hyperpigmentation defects of the skin known as café-au-lait macules. Because many of the cardinal features of the disease affect neural crest-derived tissues, NF1 is considered as a neurocristopathy [16, 18–20].

NF1 is a dominantly inherited genetic disease. Half of the NF1 patients have inherited their *NF1* mutation and the other half are caused by a de novo *NF1* mutation, suggesting that the *NF1* locus may represent a mutational hotspot in the human genome [3, 4, 16, 21]. Neurofibromin, the *NF1* gene product, has a Ras GTPase activating (RasGAP) activity and negatively regulates Ras signaling [22, 23]. Neurofibromin functions as a tumor suppressor protein expressed in many cells although it is more abundant in cells from the nervous system such as neurons, Schwann cells, astrocytes, and oligodendrocytes as well as in leukocytes [6, 24, 25]. Loss or reduced neurofibromin expression leads to an increased Ras activity and it has been associated with increased mammalian target of rapamycin (mTOR) activity in astrocytes and Schwann cells [26, 27]. Moreover, neurofibromin plays a key role in the generation of cyclic AMP (cAMP) in both neurons and astrocytes [28, 29]. Loss of heterozygosity (LOH) in the inherited wild-type allele has been detected in some tumor types in NF1 patients, although it has been demonstrated that heterozygosity for *NF1* is a key element for the development of many NF1 symptoms, including neurofibroma formation [30]. An additional complexity of the disease is its variable phenotypic expression, suggesting that modifier genes and epigenetic phenomena may play an important role in disease manifestations [2].

### 4. Neurofibroma Subtypes and Cellular Components

The most common and complex feature of NF1 is the development of benign peripheral nerve sheath tumors or neurofibromas. Neurofibromas were classified by WHO into five subtypes [31]: localized cutaneous, localized intraneural, plexiform, diffuse cutaneous, and soft tissue diffuse neurofibromas (elephantiasis neuromatosa).

Cutaneous neurofibromas reside exclusively in the skin and occur in virtually all individuals with NF1. They initially appear at puberty and increase in number with age and during pregnancy, suggesting a hormonal component in disease development [32–35]. These benign tumors, ranging from 0.1 to several cm in diameter, grow as discrete lesions in the dermis. Patients sometimes develop thousands of these tumors. Depending on their location, they can be painful and disfiguring for the patient and thus affect their quality of life. In contrast, plexiform neurofibromas develop internally along the plexus of major peripheral nerves and become quite large, sometimes involving an entire limb or body region [36]. They occur in about 30% of the individuals and are thought to be congenital. While these tumors are also benign, they are debilitating and may progress to malignancy [37, 38]. The cellular make-up of these lesions is generally similar to that of dermal lesions.

In a physiological situation, a single peripheral nerve shaft is associated with myelinating or nonmyelinating Schwann cells. Several nerve fibers and associated Schwann cells are clustered into a nerve fascicle, each fascicle being surrounded by concentric layers of perineurial cells. Fibroblasts, endothelial cells, and occasional mast cells are also present in a normal nerve fascicle (Figure 1) [7, 16, 37, 38]. Neurofibromas contain all of the cell types found in normal peripheral nerve but in inappropriate numbers. Moreover, Schwann cells are found dissociated from nerves and the perineurium is often disrupted. Large amounts of intercellular collagen and ground substance are also typically present in neurofibromas [16].

### 5. How Many Mutagenic Events Are Needed for Neurofibromas to Arise?

The penetrance of NF1 is 100% by age 20, although the degree of severity is highly variable, even among family members that present the same mutation [1, 39].

Two types of congenital NF1 mutations have been found to influence neurofibroma number [40–42]. However, these two types of mutations affect only a small percentage of NF1 patients, and, moreover, patients bearing the same germline mutation can exhibit a very different number of dermal neurofibromas [43, 44], indicating that other mechanisms are implicated in neurofibroma formation.

Somatic mutations in the *NF1* gene have been found in tumors associated with NF1, leading to functional loss of both alleles of the gene [45–47]. For example, loss of heterozygosity (LOH) in chromaffin cells initiates pheochromocytomas, and LOH in melanocytes produces pigmented

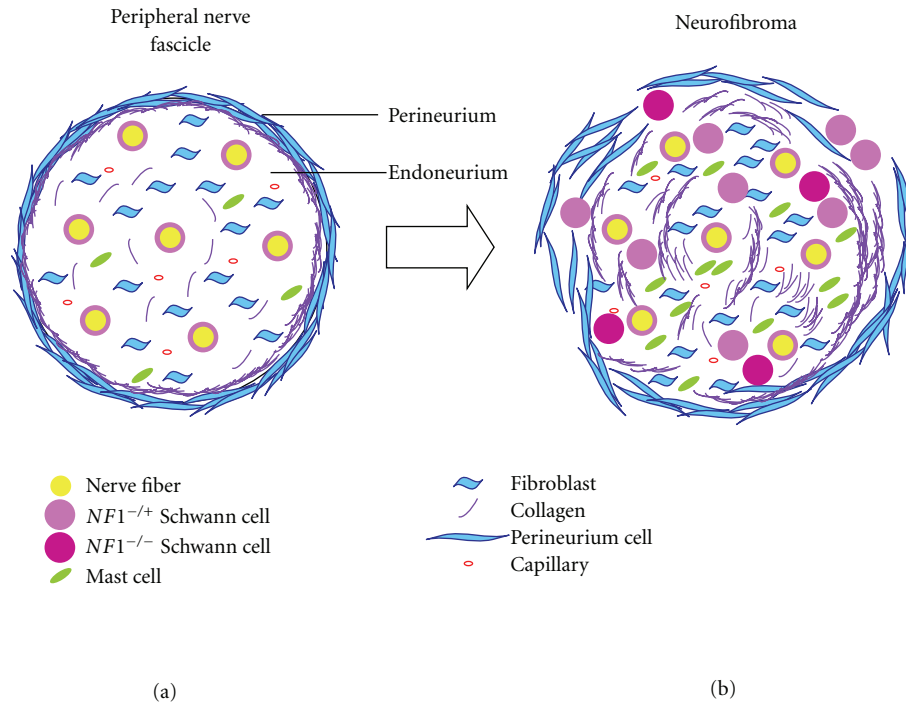


FIGURE 1: Cellular organization of a normal nerve shaft and a neurofibroma. (a) Nerve shafts are made up of axons and associated Schwann cells. Endoneurium is connective tissue composed by collagen, fibroblasts, mast cells, capillaries and extracellular matrix. Collagen fibers are tighter and more compact near the perineurium. The perineurium is composed by flattened fibroblasts, collagen and elastic fibres. (b) In a neurofibroma, the cells are the same as in a normal nerve shaft, but increased in number. There are more Schwann cells and they can be dissociated from axons. Two kinds of Schwann cells can be detected:  $NF1^{+/+}$  and  $NF1^{-/-}$ . Fibroblasts and mast cells are also increased in number but they are all heterozygous ( $NF1^{+/-}$ ). The collagen deposits are also increased and perineurium is usually disrupted.

lesions such as café-au-lait macules and Lisch nodules. LOH in myeloid cells induces myelomonocytic leukaemia, and LOH in glial cells permits astrocytoma formation [48–53].

It has also been suggested that neurofibromas are caused by somatic inactivation of the wild-type *NF1* allele, leading to complete functional abrogation of the gene [45, 54, 55]. LOH in Schwann progenitor cells permits plexiform neurofibroma formation [30, 56], and it has been suggested that LOH in skin-derived precursors leads to cutaneous neurofibroma formation [34]. Using both *NF1* intragenic polymorphisms and markers from flanking and more distal regions of chromosome 17, Colman et al. demonstrated loss of heterozygosity (LOH) of the *NF1* gene in eight neurofibromas from 22 patients and Serra et al. found LOH in 15 out of 60 dermal neurofibromas [55, 57]. Moreover, Sawada et al. identified a somatic deletion of the *NF1* gene in a dermal neurofibroma with a defined germline mutation [54]. LOH has also been detected in plexiform neurofibromas [58–60].

One possible explanation for the lack of allele loss detection in some tumors is that a more subtle somatic *NF1* mutation occurred (point mutation, small deletion, insertion, or modification through epigenetic mechanisms). These changes do not produce loss of closely linked polymorphic marker loci [61]. Alternatively, LOH may stay undetected because the presence of normal stromal or inflammatory tissue within the tumors increases sample background. Nevertheless, mechanisms that do not involve inactivation of the

normal allele cannot be excluded. In dermal neurofibromas, local trauma can be a factor in the development of the tumors [62] and it has been suggested that dermal neurofibromas could be hyperplastic instead of neoplastic lesions, due to a poorly regulated wound healing in *NF1* haploinsufficient tissues [63–65]. However most experts agree that these lesions are true neoplasms and are not hyperplastic.

## 6. Which Neurofibroma Cells Harbor Somatic *NF1* Mutations?

Being a complex genetic disease with tumors of multicellular composition, the question arises which cell type within the tumor presents the secondary somatic mutations that characterize the pathological presentation of the dermal neurofibromas. Although  $NF1^{-/-}$  fibroblasts exhibit greater proliferation capacity than their normal and heterozygous counterparts [63, 66], they are not normally found in tumors since only Schwann cells carry a double inactivation of the *NF1* gene [59, 61, 67–69].

Two different populations of S100 $\beta$ + cells (presumably terminally differentiated Schwann cells) have been demonstrated within in vitro cultures obtained from dermal neurofibromas, indicating that both *NF1* Schwann cell subtypes ( $+/-$ ) and ( $-/-$ ) coexist in these tumors [61, 68]. This fact may be explained through two alternative possibilities: (i) the

second hit mutation occurred as a secondary event within a neurofibroma that had already developed polyclonally, and thus only a subpopulation of S100 $\beta$ <sup>+</sup> cells is (–/–), or (ii) the tumors arose through a two-hit mechanism within a stem/progenitor cell that gave rise to most tumor cells, but the proliferating neoplastic clone stimulated the proliferation of infiltrating nonneoplastic cells such as heterozygous Schwann cells, mast cells, and fibroblasts.

The influence of a heterozygous environment in plexiform neurofibroma development supports the latter theory. In a conditional plexiform neurofibroma mice model (*NF1*<sup>fllox/+</sup>; Krox20cre), haploinsufficient stromal and mast cells (*NF1*<sup>+/-</sup>) are necessary and limiting for neurofibroma development [30, 70]. Accordingly, *NF1*<sup>-/-</sup> Schwann cell-derived secreted stem cell factor (SCF) causes a hyperactive recruitment of *NF1*<sup>+/-</sup> mast cells [71]. Furthermore, *NF1* mast cells secreted 2.5-fold higher TGF $\beta$  than wt mast cells, leading to a heightened fibroblast proliferation, migration, and collagen production [72]. In all, these data reinforce the idea that heterozygous fibroblast and mast cells may play a key role in the neurofibroma pathogenesis [70].

## 7. Does Neurofibroma Originate from Stem/Progenitor Cells?

Cancer is a heterogeneous disease and tumors present a significant morphological, phenotypic, genetic, kinetic, and functional diversity. Several lines of evidence suggest that this heterogeneity could be due to a hierarchical organization of tumors that resembles normal tissue development. However another possible explanation is that tumor cells are biologically equivalent and that heterogeneity derives from extrinsic or intrinsic influences that result in stochastic responses [73]. Strong evidence points to the importance of stem cells in the initiation and long-term maintenance of several cancers, as malignant germ cell cancers [74, 75], leukemias [76, 77], nervous system [78], breast [79] and colon cancers [80–83]. In these cancer types, several markers have been identified to distinguish the so called “cancer stem cells” that may form tumors when serially transplanted into immunocompromised NOD/SCID mice as compared to nontumorigenic cancer cells that do not present self-renewal capacities. Nevertheless, it is worth noting that in some cancers, most tumor cells fulfill this tumorigenic potential [84–86] and that the NOD/scid mouse transplantation assay sometimes might underestimate the frequency of human cancer cells with tumorigenic potential [85, 86].

The two-hit tumor suppressor hypothesis for NF1 predicts that all cells carry a constitutional mutation and a particular cell acquires a second mutation to initiate tumor formation [87]. Based on the two-hit model of tumorigenesis, tumor cells in neurofibromas should be of clonal origin. Nevertheless, while both alleles are inactivated in NF1-associated malignancies, the clonal nature of the neurofibromas is controversial (see below) [58, 67, 88–91].

Interestingly, there is strong evidence that an adult multipotent stem/progenitor cell could be the cell of origin for cutaneous neurofibromas. It has been demonstrated that

plexiform neurofibromas originate from embryonic neural crest-derived progenitors [30, 56, 92–96] and mice that develop plexiform tumors with 100% frequency fail to develop dermal tumors. Moreover, plexiform neurofibromas are congenital while cutaneous neurofibromas arise in puberty. The facts that dermal neurofibromas arise in the adulthood and locate in the dermis suggest the idea that dermal adult progenitor cells could be the source of these tumors [34]. Furthermore, the close relationship observed between the development of cutaneous neurofibromas and hair follicle proximity suggests that adult progenitor cells residing in the hair follicle may be the origin of these tumors. There is evidence that the neurofibromas arise in the hair follicle vicinity and even small neurofibromas can be detected histologically in close contact with the hair follicle, in otherwise apparently healthy skin areas [97, 98]. Mechanical trauma has also been suggested to play a role in the pathogenesis of neurofibromas, that is, some neurofibromas appear to arise as a dysplastic response to crush trauma [99].

Several populations of stem/progenitor cells have been described to reside in the hair follicle or surrounding areas [81, 100–114], some of them being potential candidates for an involvement in NF1 pathogenesis. Recently, it has been speculated that recruitment of Nestin<sup>+</sup> multipotent *NF1*<sup>+/-</sup> precursor cells is associated with cutaneous neurofibroma development [97]. Histologically, nestin-positive small blood vessels and spindle-shaped tumor cells can be detected in the neurofibromas. In accordance with this hypothesis, S100 $\beta$ <sup>-</sup>/*NF1*<sup>-</sup> cells are detected in high proportion (16–31%) in neurofibromas. This fact could indicate the presence of multipotent stem cells that have suffered a second-hit mutation, although a dedifferentiation from S100 $\beta$ <sup>+</sup>/*NF1*<sup>-</sup> Schwann cells, also present in the tumor, can not be excluded [91].

Finally, there is strong evidence that SKPs could be the cell of origin for dermal neurofibromas [34]. Cre-mediated recombination of *NF1*<sup>lox/+</sup> SKPs induced in vitro loss of the wt allele in these cells. When transplanted into the same *NF1*<sup>lox/+</sup> mice that originated these cells, *NF1*<sup>-/-</sup> SKPs (but not control *NF1*<sup>lox/+</sup> cells) then initiated dermal neurofibromas. However, tumor formation was only efficient in female recipients that were pregnant at the time of implantation, highlighting the hormone sensitivity observed in NF1 patients and the importance of the microenvironment during neurofibroma formation. Furthermore, deletion of *NF1* in the skin of CMV-CreERT2 *NF1*<sup>lox/+</sup> mice after topical application of tamoxifen led to local dermal neurofibroma formation, supporting the notion that the cell of origin for these tumors resides within the skin at close range of topical tamoxifen application [34, 115].

## 8. SKPs as a Tool for In Vitro Modelling of NF1 Features

Several lines of evidence now point to a stem cell origin of dermal neurofibromas. On the one hand, a number of studies have assessed the clonal origin of neurofibromas, based on

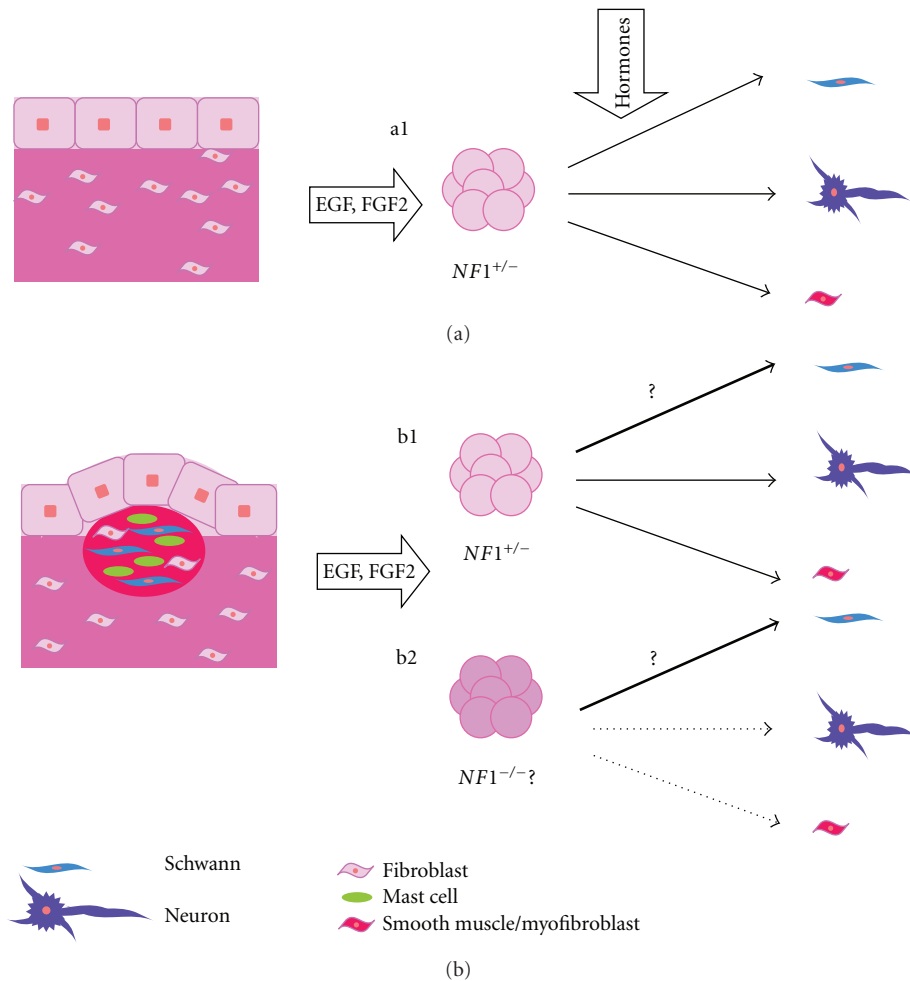


FIGURE 2: Isolation of SKPs from healthy skin and a neurofibroma of an NF1 patient. (a) Dermal multipotent stem cells form spheres in vitro, in response to EGF and FGF. In NF1 patients, SKPs from healthy skin should give rise to  $NF1^{+/-}$  SKPs in vitro (a1). SKPs can differentiate into glial, neuronal, and mesodermal lineages. If SKPs are isolated from NF1 patient neurofibromas,  $NF1^{+/-}$  SKPs (b1) are expected to form in vitro, under standard culture conditions. If  $NF1^{-/-}$  (b2) SKPs may be isolated has to be determined. If SKPs are the cell of origin of neurofibromas, they might present a predisposition to differentiate into the glial lineage.

X chromosome inactivation (XCI) clonality assay. In our view, the results are still controversial since (i) clonal cell origin may not formally be proven through XCI analyses and (ii) studies have generally been performed with low patient numbers. For instance, Skuse et al. studied eight dermal neurofibromas and concluded that all of them were of clonal origin [90]. Tucker et al. also found evidence for clonality in some of the six neurofibromas studied [91], suggesting that although other mechanisms could be at stake, at least in some neurofibromas a unique stem cell may have suffered a second-hit mutation, giving rise to a nullizygous Schwann cell progeny. On the other hand, only one kind of somatic mutation has been found in every neurofibroma analyzed and different neurofibromas of the same patient present different somatic mutations [61, 68], reinforcing the neurofibroma stem cell hypothesis. Moreover, multipotent stem cells (termed neurofibroma-derived precursor cells or NFPs) have been isolated from dermal neurofibromas. These

precursors express Nestin and show a multipotent differentiation potential, giving rise to Schwann cells, neurons, epithelial cells, and adipocytes [97]. However NFPs do not contain the somatic  $NF1$  mutation and thus their relationship with NF1 pathogenesis is currently unclear. Similarly, characterization of cells present in neurofibromas by S100 $\beta$ , a marker for the Schwann lineage, has demonstrated that a nullizygous population ( $NF1^{-/-}$ ) that is negative for S100 $\beta$  expression is present in neurofibromas. Although it cannot be discarded that they could be dedifferentiated Schwann cells, it is also possible that they could be progenitor cells that have suffered the somatic mutation and that generate the Schwann cells present in the tumor. In any case, cell characterization by a single marker is less than optimal and too many interpretations of these results are possible as to extract any meaningful conclusion.

Recently, an elegant study showed that  $NF1^{+/-}$  SKPs could form neurofibromas in a conditional mouse model,



although a key role for tumor environment was also found [34]. To date there is no data on involvement of SKPs in human neurofibroma development, although  $NF1^{+/-}$  multipotent progenitor cells are supposedly recruited to form dermal neurofibromas [97]. If SKPs were the cells of origin of dermal neurofibromas,  $NF1^{-/-}$  SKPs should be present within NF1 patient neurofibromas, although  $NF1^{+/-}$  SKPs should also be detected. If these putative  $NF1^{-/-}$  SKPs would present a predisposition to differentiate preferentially into the Schwann cell lineage should also be explored (Figure 2).

## 9. Conclusions

In summary, current evidence supports the notion that, at least in murine models, skin-derived precursor cells (SKPs) might be a cell of origin for dermal neurofibromas. It is also conceivable that human SKPs might be the cell of origin of neurofibromas, although formal proof for this is lacking. Isolation of SKPs from human neurofibromas could demonstrate if these dermal multipotent stem cells bear the somatic mutation and whether or not this mutation confers a predisposition to these precursor cells to differentiate into the Schwann cell lineage. Furthermore, isolation of SKPs from healthy skin of NF1 patients could demonstrate if there are SKPs with the somatic mutation, even in areas where the neurofibroma is histologically undetectable. In conclusion, SKPs may become a useful tool for the in vitro study of the neurofibromatosis type 1 syndrome.

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## Review Article

# The Potential of iPS Cells in Synucleinopathy Research

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$\alpha$ -synuclein is a protein involved in the pathogenesis of several so-called synucleinopathies including Parkinson's disease. A variety of models have been so far assessed. Human induced pluripotent stem cells provide a patient- and disease-specific model for *in vitro* studies, pharmacotoxicological screens, and hope for future cell-based therapies. Initial experimental procedures include the harvest of patients' material for the reprogramming process, the investigation of the patients genetic background in the cultured cells, and the evaluation of disease-relevant factors/proteins under various cell culture conditions.

## 1. $\alpha$ -Synuclein and Parkinson's Disease

$\alpha$ -synuclein is a protein that is believed to interact with presynaptic vesicles and to be involved in the regulation of dopamine transport, secretion, and reuptake [1], possibly by interacting with the SNARE complex [2]. In addition, it is believed to have additional nuclear and cytoplasmic functions. However it gained its prominence through its role in the pathology of Parkinson's disease (PD) and several other neurodegenerative diseases summarized as synucleinopathies [3].  $\alpha$ -synuclein can form fibrils and  $\alpha$ -synuclein containing aggregates, so-called Lewy bodies and Lewy dendrites which are major characteristics of PD neuropathology. Their involvement in PD occurrence and neurodegeneration has not yet been finally unraveled. The discovery of  $\alpha$ -synuclein overexpressing or point mutations in some PD patients, which have a higher tendency to form fibrils, additionally strengthened the belief that this protein has one of the central roles in PD [4]. Several *in vitro* and *in vivo* models have therefore been established to study the formation of fibrils, Lewy Bodies, and the mechanism of neurodegeneration [5]. Interestingly, the high tendency to form fibrils seems to be characteristic for the human protein. While mice overexpressing mouse  $\alpha$ -synuclein had no neural phenotype, mice overexpressing the human  $\alpha$ -synuclein suffered from neurodegeneration [6]. This showed

that although protein function and interactions as well as pathologic mechanisms could be partly analyzed in animals and cultured animal cells, all these findings have to be carefully checked in a human system which is as close to the real disease pathology as possible. In addition, the use of human cells could even reveal additional mechanistical findings that could not be mimicked in rodents.

## 2. Disease Models in Synucleopathies

Basically, disease modeling is performed in several branches. In brief, *In vivo* studies include primarily patient's clinical features associated with disease morphology and progress [7, 8]. Clinical studies for PD include the evaluation of, for example, radiological changes (organ morphology, transmitter release/uptake, signs of degeneration such as plaques or metabolic dysfunction), symptom rating, disease course, or pharmacotoxicological trials. Secondly, genetic investigations searching for disease relevant gene aberrations and familiar cosegregation are of great value for the understanding and treatment of such degenerative syndromes. Additionally, *in vivo* modeling includes several animal models starting from lower animals such as the worm *C. elegans*, the fly *D. melanogaster* or the Zebrafish *D. rerio* [9, 10]. These kinds of animal models not only provide systematic insights into genetic disease background but also help to



elucidate pathways in pathogenesis. Apart from that, they allow a relatively easy to handle model at low costs. Still, diseases like PD also involve studies using higher animals up to models mimicking the human organism, namely, monkeys and apes. Certainly, most higher animal models consist of mice models in various compositions [5]. These mice can nowadays be generated relatively easy with valuable features such as genetic knock down or even organ specific and/or in an inducible manner. These models are utilized for a variety of studies. Mouse models in general stand for investigations of, for example, the pathomechanisms, disease progression, gene function, or pharmacotoxicological evaluations. On the other hand, *in vitro* studies often make use of cellular setups. Of high interest for PD studies are cell cultures consisting of dopaminergic neurons from different sources. Until recently, most of these cell models were harvested from rodents or other animals. Investigations on these models carry the advantage of broad access to detailed cellular mechanisms. Genetic modulation of single cells additionally provide insights into cellular processes such as differentiation, migration, and function or degenerating processes such as apoptosis or necrosis. Especially, stem cells are used for studies of differentiation and maturation. In PD several different sources and types of stem cells are used. The following exemplary differences exist: (i) pluripotent embryonic stem (ES) cells are a good source for dopaminergic neurons and may be used for future cell therapeutic approaches and as platforms for pharmacotoxicological assays. Still, they inherit ethical and legal prohibitions and harbor certain dangers such as teratoma formation *in vivo*. (ii) Neural stem cells (adult stem cells) provide a source for even autologous dopaminergic neurons and can be used for patient-specific and disease-specific pathogenic investigations [11–15]. Nevertheless, these cells are extremely difficult to harvest, and this is only possible by harmful surgical intervention. Additionally, (up to now) these cells cannot be passaged over a long time and lose their potential to generate dopaminergic neurons over time. (iii) Mesenchymal stem cells from the bone marrow are thought to be amongst the most easy to harvest individual stem cell sources. These cells are also thought to be a certain source for dopaminergic neurons and provide a good hope for future cell-based therapies for a variety of neurodegenerative disorders [16]. But, the efficiency of dopaminergic differentiation is very low and research is still far away from a cell-based therapy.

### 3. Induced Pluripotent Stem Cells as a Disease Model

Studying neurodegenerative diseases in human cells is of course a difficult task. Since the affected cells cannot be propagated in culture and the supply of primary material is very limited, they cannot be widely used as a model system. As depicted in the last paragraph the use of ES cells and the subsequent differentiation into neural stem cells and neurons could partially circumvent this barrier. However their use is discussed very controversially in several countries due to ethical concerns. This issue has been resolved by the discovery of induced pluripotent stem cells (iPS cells).

iPS cells are produced from somatic cells like fibroblasts or keratinocytes and can be reprogrammed by the forced overexpression of certain transcription factors (known as the Yamanaka factors Oct4, Sox2, Klf4, c-Myc (OSKM)) into a state that strongly resembles embryonic stem cells [17]. These cells can subsequently be subjected to differentiation into virtually all cells of the organism [18] and of course to neural differentiation (as depicted in Section 4 in more detail) (Figure 1(a)), especially into dopaminergic neurons which are most affected by PD [19]. This method could prove even more valuable by the use of cells from PD patients with  $\alpha$ -synuclein mutations to evaluate and compare their iPS cell-derived neurons with healthy ones. By these means it is possible to verify findings from animal cell culture systems and other *in vitro* assays in human cells very similar or even identical to the ones which are actually affected in PD patients. Therefore it is of importance to establish a variety of iPS cell lines from different donors with  $\alpha$ -synuclein-related diseases.

### 4. Generation and Differentiation of Patient-Specific iPS Cells

The first question when reprogramming somatic cells into iPS cells is that of the cell type being reprogrammed. Traditionally, most groups used fibroblasts from punch biopsies since they are relatively easy to get and to propagate. However, when planning to generate patient-specific cell lines one has to consider that the acceptance to perform a punch biopsy is not very high since it is still an invasive and painful process. Therefore we favor the use of keratinocytes from plucked scalp hair as a starting cell source (Figure 1(b)). These cells can be obtained by noninvasive means and, in addition, have a much higher reprogramming efficiency compared to skin fibroblasts [18, 20]. Recent findings indicating that redifferentiating iPS cells favor cell types close to their origin before reprogramming reinforce the benefits of keratinocytes as starting cells since they are of ectodermal origin and closer related to neurons than fibroblasts [21].

The delivery of the four reprogramming factors Oct4, Sox2, Klf4 and c-Myc is preferably done via lentiviral transfection of a polycistronic and excisable construct. This system still harbors the highest efficiencies [22, 23]. Although there were several other methods described, including transient transfection or protein transduction [24, 25], these have very low efficiencies and are not well usable for the generation of patient-specific cell lines. The transfection of modified RNAs was described as very efficient for reprogramming but has still to be evaluated on a broader basis [26]. Lentiviral transfection of course has the negative effect of random DNA integration into the genome. This can partially be diminished by using cre-excisable lentiviral constructs. However, in order to minimize side effects caused by the integration as well as the variances between different lines it is important to evaluate a certain number of lines, preferably from different donors.

When producing patient-specific iPS cells it is important to have high reprogramming efficiencies, since the patient material is limited. Therefore the use of high-quality cultures

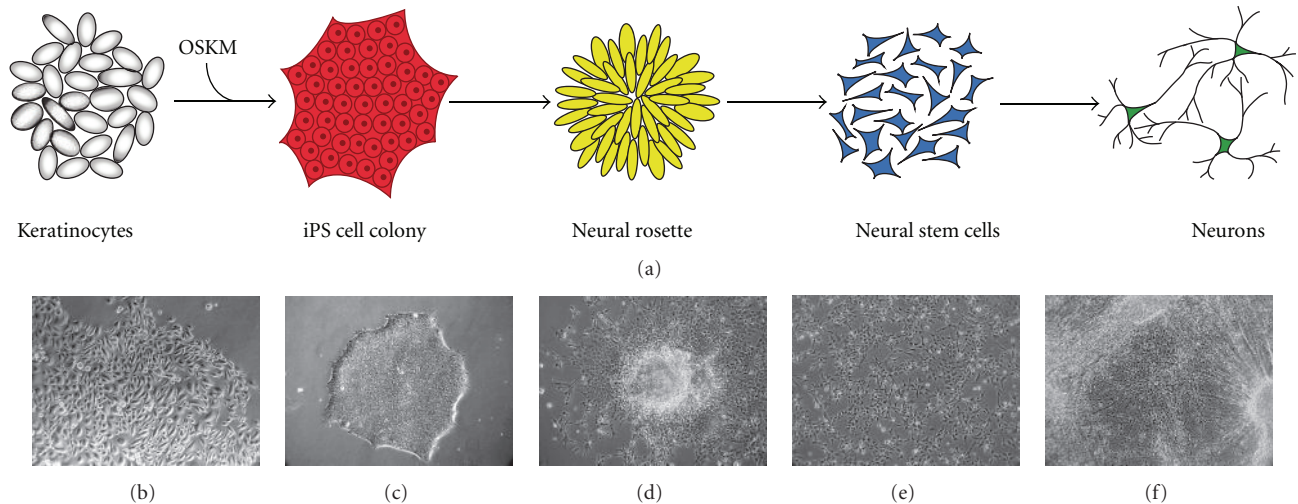


FIGURE 1: Production of iPS cell-derived neurons follows basic developmental steps. (a) Keratinocytes are reprogrammed into iPS cells by forced overexpression of the Yamanaka factors (OSKM). After differentiation into ectodermal cells neural stem cells can be isolated from the centers of the forming neural rosettes. These neural stem cells can be subsequently differentiated into neurons, (b) proliferating keratinocyte culture, (c) iPS cell colony in a feeder-free culture, (d) neural rosette shortly before dissection and isolation of neural stem cells, (e) adherent culture of neural stem cells, and (f) iPS cell-derived neurons after four weeks of culture.

of the reprogrammed cells as well as the feeder cells used in the reprogramming process is crucial. In addition several selection methods have been described to ease the isolation of true iPS cells [27, 28]. The arising iPS cells have to be thoroughly characterized to ensure their true iPS cell identity (Figure 1(c)).

The differentiation of iPS cells into neurons has already been extensively studied with ES cells [29, 30]. Available protocols, although greatly varying in detail, share some general steps. Typically, differentiation of iPS cells is started by withdrawal of FGF2. In suspension culture this is used to form embryoid bodies containing precursor cells of all lineages. The differentiation into the ectodermal and neuroectodermal lineage can, however, be highly enhanced by addition of the BMP antagonist Noggin (as well as the small molecule dorsomorphin) and even more in combination with SB431542, a TGF $\beta$  pathway inhibitor [31, 32]. Together these two substances can induce strong neural differentiation even under adherent conditions and in lines with a low neural differentiation potential. Under adherent conditions cells start to form neural rosettes (Figure 1(d)). They consist of PAX6 or Nestin-positive neural stem cells (NSCs) and mimic the development of the neural tube *in vitro*. To exclude undifferentiated cells or cells differentiating into a different fate the inner regions of the neural rosettes can be mechanically or enzymatically detached. This ensures a high purity and a similar differentiation stage of the NSCs. NSCs can be cultured under adherent conditions or in suspension as neurospheres (Figure 1(e)). However, it is not clear for how long these cells can be cultured without a reduction or change in their differentiation potential. Different culture conditions for NSCs and thereafter of the arising neurons have been reported to favor the generation of certain neuronal subtypes, like glutamatergic neurons,

dopaminergic neurons, or motor neurons (Figure 1(f)) [30, 33]. To induce final differentiation cells are treated with a mixture neurotrophic factors like the brain-derived or the glial-derived neurotrophic factor (BDNF and GDNF) as well as region-specific morphogens like Sonic hedgehog. High reproducibility in cell survival, culture quality and synapse formation has been reported for cocultures with glial cells [34]. These could be of mouse or human origin but also generated themselves from patient-specific iPS cells [35]. Of course this could be of relevance especially for diseases where glial cells cause or contribute to the pathologic effects.

## 5. Perspectives in iPS Cell-Based Synucleinopathy Research

The aim of upcoming iPS cell-based studies would be to study the morphology and electrophysiological behavior of synucleinopathy-derived neurons and compare them with healthy cells. Since  $\alpha$ -synuclein is especially involved in the synaptic compartment, alterations there would be of great interest [2]. It was already shown that iPS cell-derived human neurons express  $\alpha$ -synuclein [36]. The first synuclein-related patient iPS cell line-derived neurons with a triplication of the  $\alpha$ -synuclein (*SNCA*) gene show a higher amount of  $\alpha$ -synuclein protein compared to healthy control cells [37]. In addition to the already published relatively young neurons, we could show  $\alpha$ -synuclein in immunostainings of mature iPS cell-derived neurons with a nuclear as well as vesicular staining pattern (Figure 2(a)). They also express the gene at a higher rate compared to iPS cells or NSCs (Figure 2(b)). Interestingly, the gene *LRRK2* (leucine-rich repeat kinase 2) is also upregulated in differentiated neurons (Figure 2(c)). This PD-associated gene was described to enhance the ability of  $\alpha$ -synuclein to form aggregates [38].

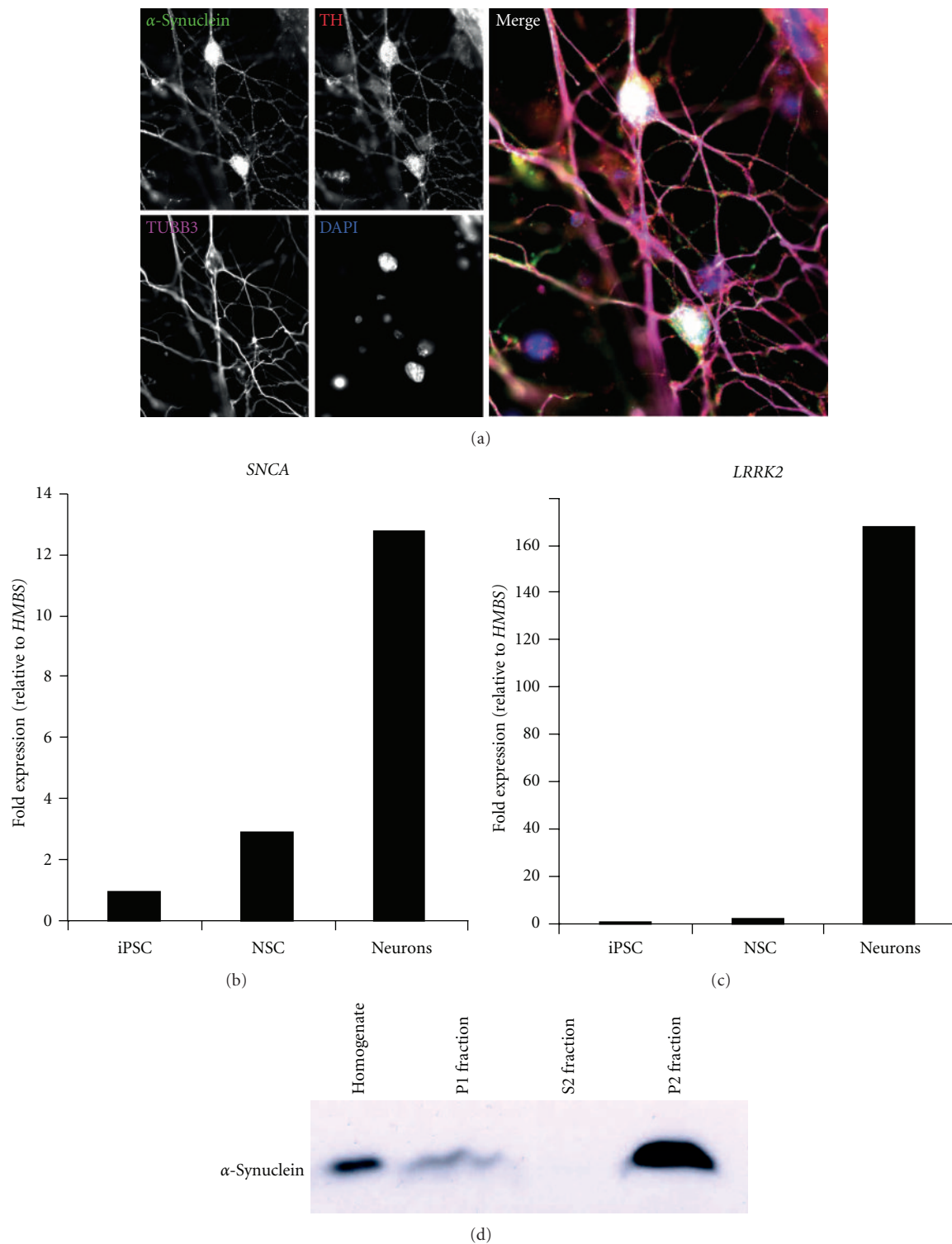


FIGURE 2: iPS cell-derived neurons express  $\alpha$ -synuclein. (a) Immunofluorescence stainings of  $\alpha$ -synuclein in TH (tyrosine hydroxylase) and TUBB3 (Tubulin beta-III) positive dopaminergic neurons after 5 months of differentiation show nuclear and vesicular localisation of  $\alpha$ -synuclein, (b) and (c) RNA expression of  $\alpha$ -synuclein (*SNCA*) and leucine-rich repeat kinase 2 (*LRRK2*) is upregulated in neurons compared to iPS cells and neural stem cells (NSCs) (normalized to the house-keeping gene *HMBS*), and (d) Subcellular fractionation of iPS cell-derived neurons shows nuclear (P1 fraction) and membrane associated, likely synaptic localization (P2 fraction) of  $\alpha$ -synuclein.

Neurons derived from patient iPS cells with a LRRK2 mutation show enhanced stress sensitivity and an elevated  $\alpha$ -synuclein levels [39]. In a subcellular fractionation  $\alpha$ -synuclein is present in the nuclear fraction (P1) but mainly in the P2 fraction containing membrane associated proteins and the synaptic compartment (Figure 2(d)). Another very intriguing study would be to evaluate ageing in these iPS cell-derived neurons. For this they have to be kept in culture for prolonged periods of time and/or additionally stressed to provoke the formation of plaque-like structures *in vitro*. This would be a very powerful tool since it then would recapitulate the neuronal changes observed in PD patients. If cultured cells can be reliably provoked to form  $\alpha$ -synuclein aggregates and plaques they also would be an ideal readout system for pharmaceutical research and evaluation of potential PD drugs. Since solely human  $\alpha$ -synuclein and its mutated forms seem to have this high tendency to form plaques the use of iPS cell-derived human neurons can be crucial to evaluate the exact pathomechanisms involved in formation of synucleinopathies. The final step would be to recapitulate the observed phenotypes of the patient-derived cells in healthy cells where the genes of interest are artificially modified. This method has already been demonstrated with  $\alpha$ -synuclein point mutations [40]. The additional use of such isogenic controls with single alterations is important to finally prove the monogenic disease potential of genes like  $\alpha$ -synuclein or LRRK2 and rule out additional but yet unknown mutations.

## Conflict of Interests

The authors declare no potential conflicts of interest.

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## Review Article

# Endothelial Progenitors as Tools to Study Vascular Disease

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Endothelial progenitor cells (EPCs) have great clinical value because they can be used as diagnostic biomarkers and as a cellular therapy for promoting vascular repair of ischaemic tissues. However, EPCs also have an additional research value in vascular disease modelling to interrogate human disease mechanisms. The term EPC is used to describe a diverse variety of cells, and we have identified a specific EPC subtype called outgrowth endothelial cell (OEC) as the best candidate for vascular disease modelling because of its high-proliferative potential and unambiguous endothelial commitment. OECs are isolated from human blood and can be exposed to pathologic conditions (forward approach) or be isolated from patients (reverse approach) in order to study vascular human disease. The use of OECs for modelling vascular disease will contribute greatly to improving our understanding of endothelial pathogenesis, which will potentially lead to the discovery of novel therapeutic strategies for vascular diseases.

## 1. Introduction

There is growing interest in endothelial progenitor cells (EPCs) because of their relevant diagnostic and therapeutic clinical applications. The association of EPCs with cardiovascular events [1] and cancer progression [2] demonstrates that EPCs have potential as both diagnostic and prognostic biomarkers. Furthermore, there are many preclinical and clinical trials that have reported benefits for a cell therapy based on delivering EPCs to ischaemic tissues such as heart [3], brain [4], retina [5], and limbs [6]. In the case of ischaemic heart disease and ischaemic limbs, despite conflicting data, meta-analysis indicated that an EPC-based cytotherapy is feasible, safe, and beneficial [7, 8]. This paper will not further discuss the diagnostic and therapeutic value of EPCs, but will focus on a lesser-known application for EPCs, that is, their potential for modelling human disease pathogenesis. Creating cellular models of human disease is an important research area where EPCs can be readily used and allows for the study of cellular and molecular mechanisms of vascular disease in a "Petri-dish". Here, we will discuss methodology for EPC isolation and different cell subtypes

and also present strategies to use EPCs as valuable tools to model vascular disease.

## 2. EPCs for Disease Modelling

Study of human disease using *in vitro*-based models usually requires large quantities of cells. This is why classically immortalised cell lines had to be established for this purpose. However, these cell lines lack a number of tumour suppressor genes or overexpress oncogenes, which is a major drawback when assessing cellular proliferation and survival. Therefore, recent interest has drifted to the usage of human embryonic stem (hES) and induced pluripotent stem (iPS) cells [9, 10], as they can theoretically be converted into any somatic cell type. We believe that EPCs, as a specific type of adult vascular stem cell [11], have great potential for modelling human disease. EPCs are easily isolated from peripheral and umbilical cord blood, they are highly proliferative, possess a stable and diploid karyotype, represent a very homogeneous cell population that is endothelial lineage-committed, and are amenable to *in vitro* manipulation and genetic modification. In addition, diseases associated with epigenetic changes to

cell function can be consistently studied through EPCs, as there is no reprogramming process required, which removes methylation or acetylation events, as is the case for iPS cells.

### 3. Isolation of EPCs

EPCs are isolated using two main methodologies: (a) cell sorting technology using different cell surface markers or (b) *in vitro* cell culture of the blood mononuclear cell fraction using specific substrates and media.

EPC cell sorting is dependent on the type and number of markers used. However, since there is no agreed consensus regarding the most appropriate combination of EPC-linked markers [12], different research teams have been sorting different cells using a diverse array of markers. Therefore, although sorted cells are all named EPCs, they actually represent distinct cell types, and this is demonstrated by the lack of consistency in reported studies using “EPCs” in various *in vitro* and animal model-based systems.

An alternative approach for isolating EPCs is cell culture. This is based on differential adhesion to specific substrates and the subsequent growth potential of isolated cells in culture. Using this methodology, two distinct types of EPCs have been identified [13, 14]. Early EPCs that appear within one week in culture are spindle-shaped cells that exhibit some endothelial properties *in vitro*, such as AcLDL uptake, Isolectin binding, and appearance of VEGFR2/CD31 on the cell surface. Despite these endothelial characteristics, these cells retain their haematopoietic nature, as demonstrated by high expression of CD14 and CD45. In fact, we have recently shown that early EPCs represent M2 alternative-activated macrophages and proposed their renaming as myeloid angiogenic cells (MACs) [15]. Other names for this cell type commonly found in the literature are circulating angiogenic cells, haematopoietic EPCs, proangiogenic monocytes, and vascular accessory cells [16].

The other EPC subtype is known as outgrowth endothelial cells (OECs) [17]. OECs appear within four weeks in culture as a cobblestone-shaped cell monolayer, exhibiting great proliferative potential and an unambiguous commitment to the endothelial lineage [18, 19]. Many studies have clearly described the OEC immunophenotype as being highly positive for the endothelial markers VE-cadherin, vWF, CD31, CD36, CD105, CD146, VEGFR2, and Tie2; negative for haematopoietic markers CD45 and CD14; and exhibit some expression of progenitor cell markers CD34, CD117, and CD133 [5, 17, 20]. OECs are also known as endothelial colony-forming cells (ECFCs), late EPCs, and non-haematopoietic EPCs. OECs are different from circulating mature endothelial cells due to the fact that they have a higher proliferative potential, shorter doubling time, and single-cell cloning capacity in contrast to mature endothelial cells that have limited proliferative potential [13, 17, 21]. Additionally, OECs retain properties of immature cells, such as greater responsiveness/sensitivity to VEGF, FGF-2, and PlGF [21], and continued expression of progenitor cell markers CD34, CD133, and CD117 [5].

OECs have been shown to possess *de novo* tubulogenic capacity *in vitro* by forming three-dimensional tubular

structures where cells interact with each other through the junction protein VE-cadherin and form a distinct vessel-like lumen [22]. This *de novo* blood vessel formation is also demonstrated *in vivo* where human OECs are transplanted subcutaneously in a collagen-fibronectin matrix into immunodeficient mice and efficiently form perfused chimeric blood vessels [18, 23, 24]. Using rhesus monkey-derived OECs in this mouse experimental system, it was recently shown that there was a decreased potential to form functional capillaries with chronological age [25]. Most importantly, it has been demonstrated that OECs directly incorporate into damaged ischaemic vasculature *in vivo* as reported using different animal models such as the murine hind limb ischaemia [13], rabbit carotid artery injury [26], the porcine myocardial infarction [27], and murine retinal ischaemia [5].

For the specific purpose of vascular disease modelling, OECs should be the preferred EPC subtype to use, as they are currently the only EPCs with both great proliferative potential and unequivocal endothelial phenotype.

### 4. Approaches for Disease Modelling with OECs

In disease modelling, the classical “reverse” and “forward” approaches used for hESCs are fully applicable to OECs (Figure 1). The “reverse” approach is based on studying OECs isolated from patients, so that “disease-specific” cells are derived and compared to “disease-free” cells. This approach is very useful as it provides a meaningful insight into physiopathology although it has two drawbacks. First, isolating OECs from certain patient groups may be problematic. For example, it is well known that diabetic patients have a lower number of circulating EPCs and when they are isolated, these cells show dysfunctional responses [28, 29]. The second drawback is that isolated OECs from patients are “already diseased”, and as the “reverse” approach is fundamentally retrospective, it may not be possible to accurately model early stages of a pathogenic process.

The “forward” approach consists of studying “disease-free” OECs that are exposed to defined disease-relevant conditions, which can be as simple as environmental changes (hypoxia, high glucose, and radiation) to more complex genetic modifications by knocking down disease-related genes. This approach is prospective and allows the study of disease pathogenesis from early stages; however, there are some technical challenges. Trying to mimic the pathologic environment can prove very complicated as the *in vivo* milieu usually comprises a diverse variety of factors combined together. Reproducing the *in vivo* environment *in vitro* requires multicell type culture systems. Another difficulty appears when the disease of interest is non-cell autonomous and therefore is directly dependent on different cell-cell interactions, and more than one cell type is needed for disease development and progression. A strategy that could easily address this latter issue of multiple cell types is the adjuvant use of iPS cell methodologies [30]. Generation of iPS cells and OECs from the same donor can provide the means to study OECs in various cell culture settings, including co-cultures with iPS cells or any other iPS cell-derived somatic cell type. This has the advantage that all

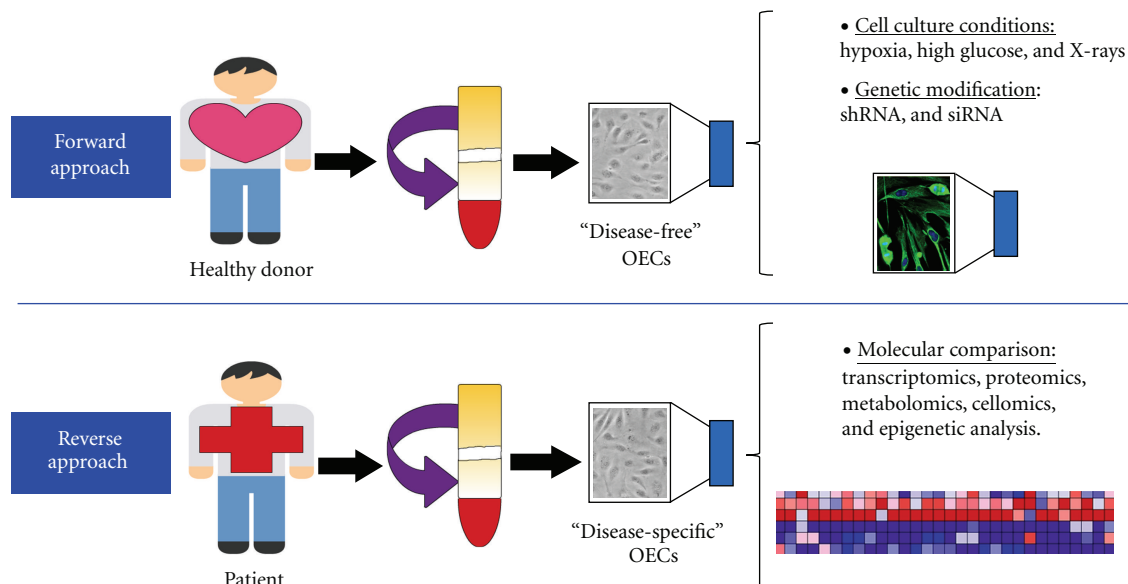


FIGURE 1: Strategies for the use of EPCs in vascular disease modelling. A specific EPC cell subtype called OEC can be isolated from human peripheral blood of both healthy donors and patients. In the forward approach, "disease-free" OECs are exposed to disease-relevant conditions or genetic modifications, while in the reverse approach "disease-specific" OECs are studied in comparison to "disease-free" OECs.

the different cell types studied alongside OECs will have the same donor which is ideal to avoid possible immunological responses arising from allogeneic transplantation.

While OECs can be studied directly, they can also indirectly facilitate the study of other supportive cells that can modulate vasculogenic activity *in vitro* or *in vivo*. As with fully differentiated endothelial cells, angiogenic activity in OECs can be directed by cytokines released from proximal myeloid cells, mesenchymal stem cells (MSCs) [24, 31], mesenchymal stromal cells, fibroblasts [23], adipose stromal cells [32], pericyte progenitors [33], astrocytes, neurons, and MACs [15, 34]. Interestingly, a mechanism involving the formation of nanotubes for the transport of organelles such as mitochondria and lysosomes has also been reported [35–37]. Delivery of miRNAs within microvesicles and exosomes represent another way cells can communicate with OECs [38, 39].

Despite these technical challenges, utilising OECs to generate cellular models of disease is an attractive methodology that is already being used and optimised. We anticipate that in the field of vascular biology, researchers will favour the use of EPCs/OECs for disease modelling.

## 5. OECs Used As Disease Cellular Models

OECs derived from patients with chronic myeloproliferative disorders (CMD) [40] indicated that this disease targets mainly the haematopoietic system, as the BCR-ABL rearrangement or JAK2-V617F mutation were not present in OECs. This finding highlighted that OECs are not the adult "haemangioblast", but represent adult stem cells fully committed to the endothelial lineage.

OECs from patients with hereditary haemorrhagic telangiectasia (HHT) [41] revealed abnormalities compatible

with vascular lesions, such as decreased endoglin expression, impaired TGF- $\beta$  signalling, disorganised cytoskeleton, and failure to form cord-like structures. These findings described a molecular mechanism to explain small-vessel fragility and frequent bleeding in these patients.

To elucidate the role of EPCs in the pathobiology of pulmonary arterial hypertension (PAH), OECs were isolated from peripheral blood of PAH patients with mutations in the gene-encoding bone morphogenetic protein receptor type II (BMPRII) and control subjects. OECs from PAH patients with BMPRII mutations were hyperproliferative when compared to controls. Furthermore, the matrigel angiogenesis assay demonstrated that *in vitro* tube formation was also significantly impaired in OEC isolated from PAH patients [42].

Von Willebrand disease (vWD) is frequently associated with angiodysplasia; therefore, the importance of vWF expression was tested in endothelial cells and animal models. vWF-deficient cells showed enhanced angiogenesis *in vitro*, and vWF-deficient mice displayed increase angiogenesis *in vivo*. These results were further confirmed by isolating OECs from patients with vWD [43] which showed increased *in vitro* angiogenesis, proliferation, and migration.

To study the role of the diabetic environment in EPC function, OECs were exposed to high glucose, and umbilical cords of diabetic mothers were used as the EPC source to isolate OECs that had previously experienced diabetic conditions *in vivo* [44]. Results demonstrated that exposure to high glucose *in vitro* or a diabetic environment *in vivo* significantly diminished OEC function such as colony formation, self-renewal capacity, and capillary-like tube formation. This study provided potential mechanistic insights into the long-term cardiovascular complications observed in newborns of diabetic pregnancies.

## 6. Concluding Thoughts

OECs are a specific EPC sub-type that is starting to be used for the study of vascular pathology. We encourage researchers in the field of vascular biology to apply their different *in vitro* and *in vivo* models of angiogenesis to OECs. Combination of forward and reverse approaches for human disease modelling with OECs is an effective system for the study of vascular disease pathogenesis. As with any new technology, we foresee some technical challenges when establishing disease models at the cellular level; nevertheless, we remain optimistic that utilising OECs for vascular disease modelling will improve our understanding of disease that subsequently leads to the development of novel therapies.

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