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Pluripotent Stem Cells for Schwann Cell Engineering

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Abstract Tissue engineering of Schwann cells (SCs) can serve a number of purposes, such as in vitro SC-related disease modeling, treatment of peripheral nerve diseases or peripheral nerve injury, and, potentially, treatment of CNS diseases. SCs can be generated from autologous stem cells in vitro by recapitulating the various stages of in vivo neural crest formation and SC differentiation. In this review, we survey the cellular and molecular mechanisms underlying these in vivo processes. We then focus on the current in vitro strategies for generating SCs from two sources of pluripotent stem cells, namely embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs). Different methods for SC engineering from ESCs and iPSCs are reviewed and suggestions are proposed for optimizing the existing protocols. Potential safety issues regarding the clinical application of iPSC-derived SCs are discussed as well. Lastly, we will address future aspects of SC engineering.

Keywords Induced pluripotent stem cells · iPSC · Schwann cell · Peripheral nerve · Myelination · Mesenchymal stem cells · Reprogramming · Differentiation · Neural crest

Introduction

Schwann cells (SCs) are the main glial cells of the peripheral nervous system (PNS). They exert multiple functions: 1) myelinating axons of the PNS, 2) providing trophic support for developing or regenerating axons, 3) regulating formation of nodes of Ranvier, and 4) regulating their own survival [1].

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Mature SCs develop from two developmental stages, namely SC precursor cells and immature SCs, which in turn develop from migrating neural crest cells (NCCs) [1]. SCs are major players in PNS disorders and in peripheral nerve injury (PNI) and the target of various treatment strategies. Therefore, engineering of SCs is of considerable importance and can serve a number of purposes:

1. In vitro *disease modeling*: Patient-derived SCs can be used for the development of in vitro models to study in detail the pathogenic processes in immune-mediated peripheral nervous system (PNS) diseases like Charcot-Marie-Tooth disease, Dejerine-Sottas disease, Guillain-Barré syndrome, chronic inflammatory demyelinating polyneuropathy, and Schwannomatosis;
2. *Gene therapy for the treatment of PNS diseases:* Knowing the role of SCs in PNS diseases may lead to approaches to modify their activity, for instance via gene therapy. It has been shown that recombinant adenoviral vectors can efficiently deliver exogenous genes to SCs in in vivo animal models [2]. These vectors might also be useful for in vitro gene targeting in diseased patient SCs before those are transplanted back into the patient [3];
3. *Treatment of PNI:* Peripheral nerve injuries are very common and can lead to considerable long-term morbidity [4, 5]. SCs play a crucial role in the intrinsic regenerative response after peripheral nerve injury. Upon nerve injury, within 24–36 h Wallerian degeneration begins. The myelin sheath degrades and is infiltrated by macrophages that, in concert with SCs, start to clear axonal debris. Although the distal axon degenerates and disappears, the connective tissue basement membrane remains. SCs proliferate and line these endoneural tubes, forming so-called bands of Büngner. In this process SCs de-differentiate into a phenotype similar to immature SCs and stimulate axonal outgrowth by the production of a variety of factors, such

as neurotrophic factors (e.g. NGF, BDNF, CNTF and NT3), ECM proteins (e.g. laminin 1 and 2, fibronectin) and adhesion molecules (e.g. L1 and N-CAM) [6–8]. Moreover, SCs regulate their own survival by means of an autocrine survival loop consisting of IGF-2, PDGF-BB, NT3, and LIF [9]. It is for these properties that in several studies nerve conduits have been coated with SCs in order to upgrade efficient neurite outgrowth, which indeed resulted in enhanced axonal regeneration [10–15]. SC transplantation has been shown to improve functional outcome in nerve injury models [16];

4. *Treatment of CNS diseases*: Remyelination of the CNS has been established in animal models using cells of the oligodendrocytic lineage [17, 18]. Since it was shown that SCs can also myelinate axons of the CNS [19], they have been considered as alternative vehicles for promoting exogenous myelination in CNS demyelinating diseases (e.g. multiple sclerosis, MS) or CNS injury [20–24]. However, SCs grafted into the CNS appear to have a limited ability to migrate and survive and, in view of their intrinsic capacity to myelinate only one axon, are inefficient for myelinating bundles of demyelinated central axons [25]. On the other hand, several authors reported the successful treatment of spinal cord injury in animal models using SCs that in vivo appeared to differentiate from implanted mesenchymal stem cells (MSCs) (see below).

In order to study or therapeutically employ large numbers of autologous (patient-derived) SCs, various stem cell populations have been considered as potential sources with, recently, focus on induced pluripotent stem cells (iPSCs). In the present review we will survey the types of stem cells available for autologous SC generation and continue with recapitulating the cellular and molecular mechanisms that play a role in in vivo neural crest (NC) formation and SC differentiation. We will outline the current in vitro strategies for generating SCs from pluripotent stem cells, both from embryonic stem cells (ESCs) and iPSCs. Subsequently, we will discuss methods to optimize SC engineering from iPSCs, and review the issues that have to be solved with regards to future clinical applications. Lastly, we will address SC engineering in relation to the newest developments in stem cell biology (e.g. direct conversion), and we will conclude with general prospects regarding future SC engineering.

Stem Cell Sources for Autologous Schwann Cells

Various types of multipotent adult stem cells may serve as an autologous source for SCs. Most adult stem cell types can be isolated with minimal invasive procedures although the yield is generally low. Given their accessibility and broad availability, multipotent mesenchymal stem cells (MSCs) are an obvious adult stem cell source of SCs for clinical applications [26–33]. Adipose tissue has been identified as a rich source for MSCs which showed in vitro capability to generate SCs, and could myelinate spinal cord axons in vivo [34–38]. However, MSCs have a limited in vitro expansibility. Another accessible source of multipotent adult stem cells with potential for in vitro SC differentiation are skin-derived (SKPs) or hair follicle neural crest stem cells (Epi-NCSCs) [39–44]. Amoh et al. reported the in vivo differentiation of human hair follicle cells into SCs after transplantation in a sciatic nerve injury mouse model, which promoted the recovery of axons [43]. These cells were also used for treatment of injured spinal cord axons in mice, giving rise to glial fibrillary acidic protein (GFAP)/CNPase-positive SCs and leading to improved remyelination and motor function [45]. Others have isolated SKP spheres, differentiated them towards SCs and subsequently applied them for induction of remyelination in injured rat spinal cord [46, 47]. Also, adult neural stem cells, the multipotent stem cells located in specific areas in the adult brain, have been shown to differentiate into S100/$p75^{NTR}$-positive SCs and were able to improve axonal regeneration in peripheral nerve injury rodent models [48, 49]. However, these cells are of limited clinical relevance for human therapies.

Recently, the focus has switched to pluripotent stem cells for generating SCs, namely embryonic stem cells (ESCs) and, in particular, induced pluripotent stem cells (iPSCs). ESCs are cell lines derived from the inner cell mass (ICM) of the pre-implantation blastocyst [50, 51]. Being pluripotent, they display self-renewal by means of symmetrical division, which can continue indefinitely in vitro under proper conditions and have the ability to give rise to cell lineages of all three germ layers, as well as to germ line cells which becomes evident when they are allowed to differentiate spontaneously in vitro into so-called embryoid bodies [52]. Undifferentiated ESCs can be recognized by the expression of specific surface markers such as stage-specific embryonic antigen (SSEA-1 in mES, SSEA-3 and SSEA-4 in hES) and tumor rejection antigens TRA-1-60, TRA-1-81, and TRA-2-54 [53]. The development of a procedure to isolate ESCs from the human blastocyst [54] has boosted the attention for ESCs as potential - though not autologous - source for cell therapy and clinical application of all types of cells, including SCs.

Takahashi and Yamanaka first reported the generation of iPSCs from mouse and human embryonic and adult fibroblasts by retroviral induction of the four transcription factors Oct4, Sox2, Klf4 and c-Myc, a combination narrowed down from a list of 24 candidate pluripotency genes [55, 56]. iPSCs are highly similar to ESCs regarding morphology, gene expression profile, epigenetic status, and in vitro differentiation potential. They express ES cell-specific markers such as SSEA and alkaline phosphatase and pluripotency

transcription factors like Oct4, Sox2, Nanog, Rex1 and UTF1. Like ESCs, iPSCs have the ability to differentiate into all of the three germ layers as evidenced by their in vitro generation of embryoid bodies and teratoma formation after implantation in vivo. Similar to ESCs, mouse iPSCs are able to give rise to adult chimeras and show competence for germline transmission [57, 58]. They form an attractive autologous alternative to ESCs for the study of degenerative diseases and for application in regenerative medicine and tissue engineering in humans. So far, the applicability of iPS has been successfully demonstrated for the treatment of Parkinson's disease and sickle cell anemia in rodent models for these diseases [59, 60].

Unlike adult stem cells, pluripotent stem cells can be considered as a potentially indefinite source of SCs. iPSCs hold a greater promise for clinical application than ESCs, as they can be patient-derived and therefore be autologous, while ESCs are non-autologous and would require immune suppression after transplantation to prevent rejection. SCs can differentiated in vitro from ESCs and iPSCs by accurately recapitulating their ontogeny, with NC formation as a crucial intermediate step. In the two next sections we will give a concise review on the molecular mechanisms underlying the developmental in vivo processes; we will highlight some of the most crucial intrinsic and extrinsic factors that will re-appear in the subsequent descriptions of the in vitro protocols for the differentiation of ESCs and iPSCs towards SCs.

Molecular Mechanisms Underlying Neural Crest Formation

Rostrocaudal specification of the developing nervous system is regulated by gradients of bone morphogenetic protein (BMP) and other morphogens, notably Wnt, fibroblast growth factor (FGF), and retinoic acid (RA), while dorsoventral specification is induced by gradients of BMP and sonic hedgehog (SHH) (reviewed in [61]). NCCs originate in the dorsal neural tube. Premigratory epithelial cells of the neuroectoderm undergo a process called epithelial-to-mesenchymal transition (EMT), giving rise to migratory NCCs. EMT is triggered by activation of signaling pathways involving Wnt, transforming growth factors (TGFs), FGFs, and BMPs, as well as transcription factors such as c-Myb, Sox9, and msh homeobox 1 (Msx-1) [62–65]. A complex interplay between these signaling pathways results in the activation of key regulators in the transcriptional network underlying EMT, leading to delamination and migration of NCCs (Fig. 1). Migration in EMT typically is characterized by the loss of the adhesion molecule E-cadherin in cell-cell contacts; E-cadherin is a hallmark of both the epithelial state and architecture [66, 67]. *E-cadherin* transcription is repressed by zinc finger transcription factor Snai1 through binding to regulatory elements in its promotor [68–72]. In an additional epigenetic mechanism, Snai1 recruits histone demethylase LSD1 to downregulate the transcriptional activity of the *E-cadherin* promotor. Moreover, phosphorylation of Snai1 Ser residues by specific kinases potentiates *E-cadherin* repression by recruiting Snai1 to the nucleus. Downregulation of E-cadherin leads to cytoplasmic activation of another class of adhesion molecules, namely integrins [73]. Integrins in turn negatively regulate expression and proper function of *E-cadherin* [74], and are required for newly migrated NCCs to interact with the ECM; their expression is regulated by transcription factors FoxD3 and SRY-box 10 (Sox10) [63]. Heterogeneity in developmental restriction within the NC cell population is thought to cause differences in responsiveness to environmental signals, thus giving rise to lineage segregation during migration and homing [75]. Migration along the rostral-caudal axis of the organism results in distinct populations of NCCs which give rise to a locally determined range of NC derivatives such as melanocytes, cranial bone structures, myofibroblasts, sensory neurons, and SCs.

Molecular Mechanisms Underlying the Differentiation of Neural Crest Cells into Schwann Cells

Migrating NCCs develop into SC precursors (SCPs) around embryonic days 12–13 (in mice); around embryonic days 15–16, SCPs transform into immature SCs, which ultimately give rise to myelinating and non-myelinating SCs (present around birth) [76, 77]. The precise molecular pathways underlying the developmental transitions in SCs are not yet fully understood. For an extensive overview of the known transcriptional and epigenetic pathways involved in maturation of SCs, we refer to the review by Pereira et al. [78]. Several transcription factors are essential during SC development, most prominently Sox10, early growth response-2 (Egr2, also known as Krox20), and POU domain transcription factor Pou3f1, also known as octamer–binding factor 6 (Oct6). SRY-related high-mobility group (HMG) domain protein Sox10 is a crucial transcription modulator expressed in both migrating NCCs and in their derivatives, such as the developing enteric nervous system (ENS), melanocytes, and SCs [79]. Targeted deletion of Sox10 leads to a failure to form SC precursors from NCCs, and hypomorphic *Sox10* mutations lead to SC defects in mice [80, 81]. Mutations in *Sox10* are associated with peripheral demyelinating neuropathy, central dysmyelinating leukodystrophy, Waardenburg syndrome, and Hirschsprung disease [82]. *Sox10* is expressed throughout SC development [83, 84] and has a direct and pivotal regulating effect on myelination, acting both on and synergistically with *Egr2/Krox20* [85, 86], as well as on other genes. *Egr2/Krox20* is the master regulator gene of myelination; mutations in this gene are associated with demyelinating neuropathies such as Charcot-Marie-Tooth and Dejerine-Sottas disease [87–89].

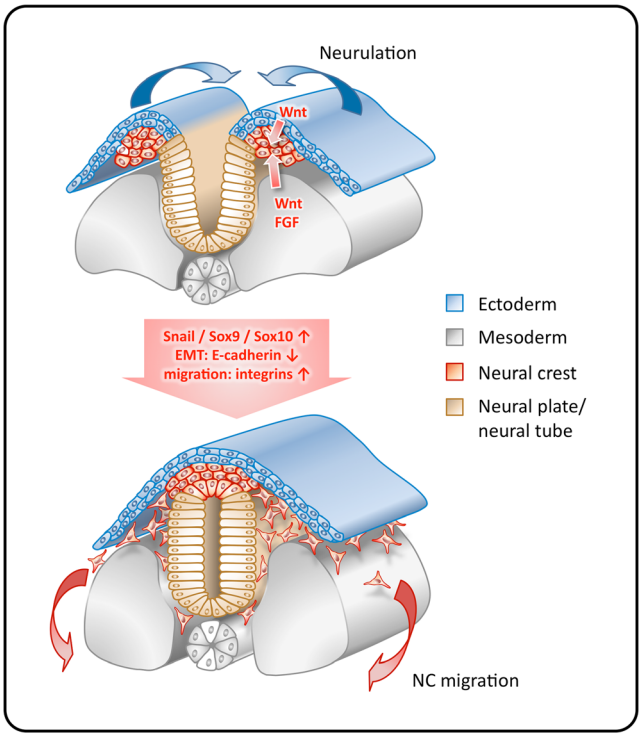

Fig. 1 NC formation is induced by Wnt and FGF signals from the neighbouring mesoderm and ectoderm. This leads to expression of early NC specifiers such as Snail, FoxD3, Sox9, and Sox10, which set the NC apart from the neuroepithelium. They also induce NC migration by downregulating E-cadherin (leading to EMT, epithelial-to-mesenchymal transition) and upregulating integrins

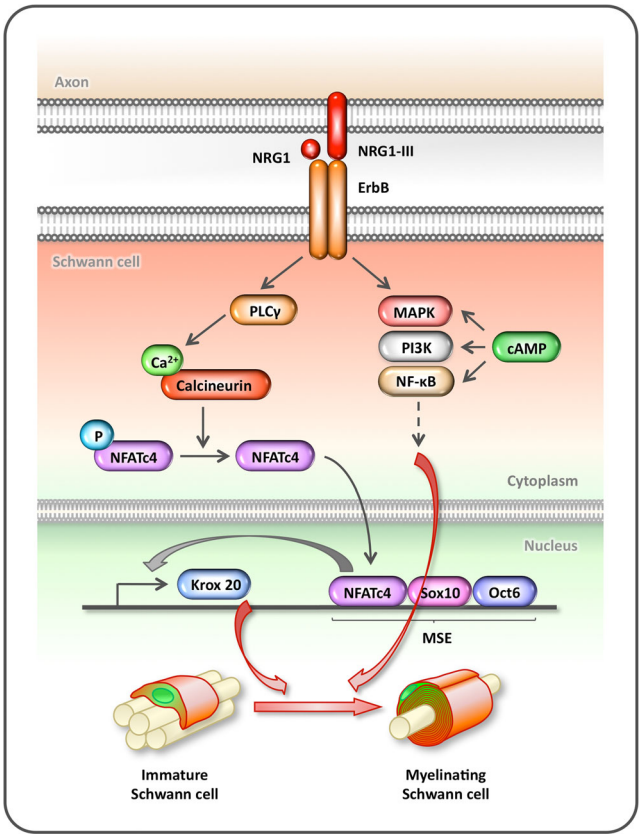

Fig. 2 Transcriptional regulation of myelination through NRG1 signaling. Soluble or axon-derived membrane-bound NRG1 activates the ErbB receptor. NRG1 stimulates myelination through signaling via ERK, PI3K, and NF-κB. cAMP synergistically activates these pathways, and also causes upregulation of ErbB receptors. In an additional pathway, calcineurin directly activates myelination genes by activating NFATc4. Sox10, together with Oct6, Brn2, and NFATc4 targets the *Krox20* gene by directly binding to its promotor, and to a downstream myelinating Schwann cell element (MSE). For additional interactions between the different transcription factors, see the main text

Ghislain et al. identified a downstream enhancer of the *Egr2/Krox20* gene, designated myelinating Schwann cell element (MSE), which is activated by direct binding of Oct6 and its related protein Brn2 [90, 91]. The same authors found that Sox10 acts synergistically with Oct6 and Brn2 to activate the MSE [86]. The *Egr2/Krox20* MSE is additionally activated by the transcription factor NFTAc4 (which also activates the *Mpz* promotor), synergistically with (again) Sox10, thus directly influencing onset of myelination [92] (Fig. 2). *NFAT4c* expression is induced by Neuregulin-1 (NRG1) [92] (see below).

Dimeric Sox10 also directly activates *Oct6* expression through binding of the *Oct6* Schwann cell enhancer (SCE) element, thus enhancing its synergistic potential with Oct6 on the *Egr2/Krox20* MSE in a feed-forward manner [93]. A second feed-forward loop regulates the expression of peripheral myelin genes as Sox10 directly as well as in combination with Egr2/Krox20 regulates the *Mpz* and *Connexin 32* promotors [81, 84, 93–97].

Differentiation of SCs from NCCs typically requires a few essential regulators that induce differentiation, proliferation, and myelination of SCs. These are: neuregulin-1 (NRG1), cyclic adenosine monophosphate (cAMP), and laminin 1. These factors are required during both in vivo and in vitro SC generation.

Axon-derived type III NRG1 is essential for in vivo SC development [98]. Experiments in vitro demonstrate that NRG1 influences cell fate decisions, growth, survival and maturation/differentiation of SC precursors, immature and mature SCs [99–101]. Shah et al. showed that NRG1 commits the differentiation of NC progenitor cells towards a glial lineage [100]. NRG1 also increases the survival and proliferation of SC precursor cells, while submitogenic levels of NRG1 promote differentiation into mature SCs [101, 102]. Acting through ErbB receptors, NRG1 stimulates SC mitogenesis and myelination through signaling via the ERK and PI3-K pathways, an effect enhanced by cAMP [103, 104]. Myelinating effects of NRG1 are also exerted through NF-κB activation [105, 106], again enhanced by cAMP [107] as well as MAPK to drive cell proliferation and PI3K/Akt to prevent apoptosis. As mentioned above, Kao described an additional pathway downstream from ErbB, in which NRG1 acts through the protein phosphatase calcineurin to directly

activate myelination genes [92] (Fig. 2). NRG1 addition to SC precursors initiates an increase in cytoplasmic Ca^{2+}, which activates calcineurin leading to activation of downstream transcription factors NFATc3 and c4. NFATc4 directly acts on the promotor of master myelination gene *Egr2/Krox20* as well as the promotor of the *Mpz* gene, which encodes an important component of peripheral myelin [92]. Besides NRG1, cAMP is a crucial inducer of SC differentiation and myelination [108]. It exerts its actions through synergistic activation of NRG1-dependent intracellular signaling pathways [104, 107], but it also increases ErbB receptor expression, thus potentiating NRG1 binding and signaling [109]. *ErbB* receptor expression is also controlled by Sox10, which through NRG1 signaling and NFATc4 creates yet another feed-forward loop [80, 92]. Interestingly, the lab of Birchmeier recently reported that NRG1/ErbB3-mediated SC maturation and myelination induction can be completely bypassed [110]. Knockdown of ErbB3 in mice resulted in almost complete absence of SCs; however, SC development and myelination could be rescued under experimental conditions by means of MAPK activation, and sustained MAPK activation even resulted in continuous myelin production [110]. These experiments show that the essential pathways for myelination are MAPK-dependent and do not necessarily require ErbB3 or NRG1 signaling.

Extracellular matrix protein laminin is essential for proper SC differentiation and myelination. SCs of the mammalian PNS constitutively express laminin 1 ($\alpha1\beta1\gamma1$) [111]. Isoforms laminin 2 ($\alpha2\beta1\gamma1$), laminin 8 ($\alpha4\beta1\gamma1$) and laminin 10 ($\alpha5\beta1\gamma1$) are specifically expressed in the peripheral nerve endoneurium [112]. Conditional disruption of *laminin γ1* (affecting laminin 1, 2 and 8), leads to impaired motor function and paralysis in mice, impaired nerve regeneration as well as the inability to produce myelin protein [113]. Yu et al. showed by means of a conditional knockout that SCs lacking laminin $\gamma1$ show impaired axon interaction, proliferation and differentiation and have reduced phosphatidylinositol3 (PI3)-kinase signaling, leading to increased apoptosis [114]. Laminin activates myelination and SC differentiation through p38 MAPK-mediated expression of transcription factors *Egr2/Krox20* and *Sox10* [115].

Altogether, properly timed expression of transcription factors in concert with signaling by extrinsic factors induces the in vivo transition from NCCs to SCs. Many of the above mentioned factors are used for induction of differentiation in in vitro studies and will be discussed in this context in the following sections.

Embryonic Stem Cells as Source of Schwann Cells

Differentiation induction of neural cells and NCCs requires the same signaling pathways. Consequently, for induction of both neural cells and NCCs, similar morphogens and model systems are used in in vitro differentiation studies [61, 116–118]. Whether the two parallel developmental processes can occur independently from each other is not entirely clear [119]. The in vitro models used for both neuroepithelial specification and NC induction include 1) neural rosette formation by 2-dimensional co-culturing of pluripotent stem cells with stromal cells (PA6, MS5), so-called stromal cell-derived inducing activity (SDIA) [118], and 2) formation of 3-dimensional aggregates called embryoid bodies (EBs) and treatment with RA [120]. EBs contain all three germ layers and express markers for EMT [121]. Both model systems are complemented by different morphogens and growth factors (Table 1), which are specifically chosen to mimic in vivo dorsalization- and caudalization-inducing effects, such as SHH, FGF8, BMP4, and FGF2. The timing and the duration of morphogen supplementation seem crucial for the functional outcome. Addition of SHH and FGF8 to differentiating pluripotent cells can be used to induce neural rosette formation [118], but is often avoided as it can suppress development of dorsal neural tissues and promote ventralization [122]. Some authors use BMP to induce NC formation [123, 124]. Although early BMP antagonism (e.g. by means of FGF2) is necessary for neural induction, late BMP4 exposure appears to suppress ventralization and to promote dorsalization, thus favoring NC formation [123]. FGF2 is also used for its caudalizing effect [62], reviewed in [119], and later on is a part of the autocrine survival loop of differentiated SCs [125]. Other factors regularly used are brain derived-neurotrophic factor (BDNF) and ascorbic acid (AA). BDNF is known to increase survival and differentiation among NCCs [126], while AA promotes neuronal differentiation from embryonic stem cells [127]; it also is an inducer of myelination [128].

A number of authors have shown the differentiation of NCCs derived from mouse and human ESCs (Table 1). These NCCs have been further differentiated into NC derivatives like sensory and sympathetic neurons [129], smooth muscle cells, melanocytes [130], and SCs [120, 123, 131]. SCs or SC-like cells expressing markers like GFAP, $p75^{NTR}$, S100β, and myelin basic protein (MBP) have been derived from human ESCs as well [118, 132, 133]. Lee et al. developed a protocol for induction of NC from human ESCs, recapitulating neuroectoderm formation using MS-5 feeder cells and subsequent NC induction, using morphogens SHH, FGF8, BDNF, and AA [118, 134]. Co-culturing of ESCs on stroma cells (SDIA) and neural rosette formation were used to differentiate and enrich $p75^{NTR}$-positive NCCs from ESCs. Final differentiation towards SCs was accomplished by culturing in medium with ciliary neurotrophic factor (CNTF), cAMP enhancer forskolin (FSK), and NRG1. Other protocols for the differentiation of ESCs in SCs have been developed using combinations of stromal

Table 1 List of studies describing differentiation of NCCs and SCs from pluripotent stem cells, and their characteristics and in vivo applications

Author	Source	Culture condition	Progenitor stage	Purification	Intermediate stage markers	Differentiation potential	SC / glia induction medium	Markers	SC Functionality	SC Markers	Tera-toma
Kawaguchi 2010	Mouse ES cells	EB formation + N2 + B27 RA + Lif + Fgf8b Forced Sox10 expression	Neural crest	Sox10	Sox9, Id2, Id3, Slug, Snail	Neurons, glia cells	FGF2/BMP 4/GDNF	GFAP			
Mizuseki 2003	Mouse/prima te ES cells	SDIA (PA6) BMP4	Neural crest		Ncx, Snail, Slug, dHand, Msx1	Neurons, smooth muscle cells					
Motohashi 2007	Mouse ES cells	ST2, dexamethason, FGF2, cholera toxin, ET3, ATRA	Neural crest	C-Kit+/ CD45-	Snail, Slug, Sox10, Pax3, Mitf-M	Melanocytes, neurons, glia cells, smooth muscle cells	BMP2-NRG1	GFAP			
Rathjen 2002	Mouse ES cells	1) Neuroectoderm culture + MEDII 2) staurosporine + FGF2	Neural crest		Sox10	Glia cells	FGF2/EGF /laminin/PD GF	GFAP			
Cui 2008	Mouse ES cells	EB formation							Nerve injury model (rat)	S100β	None
Lee 2007	Human ES cells	1) SDIA (MS-5) + SHH/FGF8/BDNF 2) FGF2/AA/BDNF	Neural crest	p75+/ HNK-1+	POU4F1, SCN3A, TFAP2B, Slug, …	Neurons, SCs, adipocytes, chondrocytes, osteocytes	CNTF/ NRG1/dbc AMP	S100β, GFAP 8%, MBP			
Pomp 2005	Human ES cells	SDIA (PA6)	Neural crest		Snail, Sox9, dHand, Msx-1, Foxd3, Ap2	Sensory neurons, sympathetic neurons					
Pomp 2008	Human ES	SDIA (PA6) Neurospheres FGF2	Neural crest		SNAI1, MSX1, SOX9	Neurons		GFAP			
Zhou 2008	Human ES cells	EB formation	Neural crest progenitors	Frizzled-3+/ cadherin-11+	Collagen type-2,GFAP, peripherin, Runx2, SMA	Neurons, glia, chondrocytes, osteoblasts, smooth muscle cells	IGF-1/NRG1	GFAP			
Jiang 2009	Human ES cells	SDIA (PA6)	Neural crest	p75	Snail, Slug, Sox 9, Sox10, Msx-1, Pax3, TRKC, p75	Neurons, glia cells, myofibroblasts		GFAP 10%, MBP			
Ziegler 2011	Human ES	SDIA (PA6) Neurospheres FGF2	Neural crest		p75, HNK-1, S100β	Neurons, SCs	NRG1/ FSK/ FGF2/AA	GFAP/ S100β 60%, p75, P0, PMP-22, BMP, PLP	DRG co-culture	GFAP	
Uemura 2012	Mouse iPS	EB formation + hormone mix (MHM) + FGF2 neurospheres							Conduit, nerve injury model (mouse)	S100β, GFAP,	None
Ikeda 2013	Mouse iPS	EB formation + hormone mix (MHM) + FGF2 neurospheres							Conduit, nerve injury model (mouse)	S100β	
Okawa 2013	Mouse iPS	SDIA (PA6) BMP4	Neural crest	p75+					Intramuscular transplantation (mouse)	S100β	None
Ma 2014 (unpublished)	Mouse iPS	EB formation + SHH/FGF8/BDNF neural crest induction: FGF2/AA/BDNF	Neural crest			SCs	NRG1/ FSK/ FGF2	CNPase/ Oct6 50%			
Wang 2011	Human iPS	EB formation, EGF + FGF2, neural rosettes, neurospheres	Neural crest	p75+	Ap2, Hnk-1, p75	Neurons, SCs, chondrocytes, osteoblasts, adipocytes, smooth muscle precursors	CNTF/ NRG1/dbc AMP	GFAP, S100β	Conduit, nerve injury model (rat)	S100β	None
Liu 2012	Human ES/iPS cells	EB formation + B27, FGF2, rock inhibitor, AA, PA6-conditioned medium	Neural crest	p75+	Msx-1, Snai1, Slug, Sox9, p75, Sox9, AP2	**Neurons, SCs, chondrocytes, osteoblasts, adipocytes, smooth muscle cells**	NRG1	GFAP 78%, S100β 85%, p75, Sox9, ErbB3, PLP1, PMP22	DRG co-culture	MBP	
Kreitzer 2013	Human iPS	SB435142, LDN-193189 matrigel, EGF + FGF2	Neural crest		P75, HNK-1, AP2, neural crest biomarkers	Neurons, SCs, smooth muscle cells		GFAP			
Menendez 2013	Human ES/iPS cells	SB435142, GSK3 inhibitor, FGF2/IGF-1/NRG1	Neural crest		p75, Hnk-1, AP2, FoxD3, Sox9, Sox10, …	Neurons, chondrocytes, osteoblasts, adipocytes, smooth muscle cells					

Grey boxes indicate non-defined conditions, or no data reported. Studies using ES and iPS of different origins are indicated with different colors. The red box indicates the studies that describe targeted SC differentiation from iPSCs. The red numbers indicate the reported differentiation efficiencies.

cells, and neurosphere formation in combination with FGF2 [129, 135]. NRG1, FSK and FGF2 were used for targeted SC induction. One group described peripheral nerve repair using ES-derived embryoid body cells without further in vitro differentiation [136].

Induced Pluripotent Stem Cells as Source of Schwann Cells

iPSCs are highly similar to ESCs regarding morphology, gene expression profile, epigenetic status, and in vitro

differentiation potential [137, 138]. They express ES cell-specific markers such as SSEA and alkaline phosphatase and pluripotency transcription factors like octamer-binding factor 4 (Oct4), SRY-box 2 (Sox2), Nanog, reduced expression 1 (Rex1), and undifferentiated embryonic cell transcription factor 1 (UTF1). Like ESCs, iPSCs have the ability to differentiate into all of the three germ layers as evidenced by their in vitro generation of EBs and teratoma formation after implantation in vivo. Similar to ESCs, mouse iPSCs are able to give rise to adult chimeras and show competence for germline transmission [57, 58]. iPSCs are a promising alternative to ESCs for mechanistic studies of disease, in vitro drug screening, evaluation of potential therapeutics, and for cell therapy and regenerative medicine - with or without gene repair (Fig. 3). In vitro differentiation of iPSCs, analogous to ES cell differentiation, can be used in strategies aimed at treatment of human disease.

To date, only few studies have shown NCC differentiation and subsequent SC differentiation from iPSCs (Table 1). Liu et al. differentiated NCC from human iPSCs using EB formation and PA6 stromal cell line-conditioned medium supplemented with FGF2, Rock inhibitor, and AA [139]. NCCs (expressing *Msx-1, SRY-box 9 [Sox9],* and *Slug*) were purified by means of FACS sorting for low affinity neurotrophin receptor $p75^{NTR}$, and SC differentiation was accomplished by culturing in medium supplemented with NRG1. iPS-derived SCs were positive for S100β, $p75^{NTR}$, Sox9, and ERBB3, as well as for myelin markers like peripheral myelin protein 22 (PMP22) and MBP, and were able to in vitro myelinate rat sensory neurons [139]. Menendez et al. published a protocol for the differentiation of NCCs from human iPSCs without the use of stromal cells, although no SC generation was shown [140]. Our group has differentiated SCs from transgenic mouse CNPase/GFP-positive iPSCs using EB formation and NC induction by means of SHH, FGF8, and BDNF, without stromal cells. After NRG1-induced targeted differentiation, we obtained efficiencies of around 50 % differentiated SCs, as identified by the expression of GFP and Oct6 (unpublished data).

iPS-derived SCs cells have been used for nerve regeneration in animal models. Wang et al. differentiated human ES and iPSCs towards neural crest stem cells (NCSCs) using both EBs and neural rosette formation, and serum-free medium containing FGF2 and EGF [141]. The NCSCs were FACS sorted for $p75^{NTR}$ expression and in vitro differentiated towards SCs expressing GFAP and S100β using CNTF, NRG1, and cAMP analogue dibutyryl cyclic-AMP (dbcAMP). However, the authors reported that these SCs were unfit for transplantation, and NCSCs were used for implantation instead. NCSCs were seeded in nerve conduits, implanted in a rat sciatic nerve injury model, and after 1 month formed S100β-expressing cells indicating in vivo glial differentiation. Okawa et al. used PA6 (SDIA) and BMP4 to differentiate GFP-positive mouse iPSCs towards NC-like cells, which were then FACS-sorted for $p75^{NTR}$ expression and implanted intramuscularly in a mouse model for diabetic neuropathy [124]. Interestingly, 4 weeks after transplantation, GFP-positive S100β-expressing SC-like cells could be detected, indicating intramuscular glial differentiation. Two research groups seeded (uncharacterized) neurospheres derived from mouse iPSCs in nerve conduits and found SC formation after implantation in a mouse sciatic nerve injury model [142, 143]. Functional recovery was higher in animals with transplanted iPS-derived neurosphere cells compared to control animals. Also, S100β expression was higher than in the control group [142, 143]. Notably, no teratoma formation was observed in any of the reported studies.

Some groups use neurosphere formation as a culture method for NC formation from both ESCs and iPSCs, with or without prior neural rosette formation [135, 141–144]. As Lee et al. pointed out, identical protocols are often used for neural and NC formation, and thus neurospheres are indeed to be expected to contain a NC subpopulation [118]. Pomp et al. indeed showed that both CNS and PNS precursors could be found within ES derived neurospheres (after SDIA) [144]. The efficiency for NC enrichment appears to depend on the stage of the cells (see below).

Considerations for Optimization of SC Engineering from iPSCs

Safety of iPSCs

Unlike ESCs, application of iPSCs is not burdened with ethical considerations. Autologous iPSCs can be generated from patient-specific cells, which is a huge clinical advantage over ESCs. The main issue regarding potential treatment with iPS-derived cells remains their safety, due to the use of genome-integrating carcinogenic retroviruses and of proto-oncogenes such as c-Myc in the reprogramming process. Expression of the reprogramming factors is only required temporarily; the transgenes are silenced by iPSCs once they have been established and endogenously express the essential pluripotency transcription factors [57]. However, unwanted retroviral transgene reactivation of c-Myc can lead to genomic instability and tumorigenesis [58, 145, 146]. Several modified protocols have been developed to address these issues: iPSCs were generated without c-Myc [145, 147–149] as well as by means of non-integrating adenoviruses [150], non-integrating plasmids [151], recombinant proteins [152, 153], mRNAs [154], small molecules [155], piggyBac transposons [156, 157], minicircle vectors [158], non-integrating episomal vectors [159, 160], and Sendai viral vectors [161]. In particular, the use of small molecules interacting at the epigenetic level may be an important step towards the efficient creation of safe

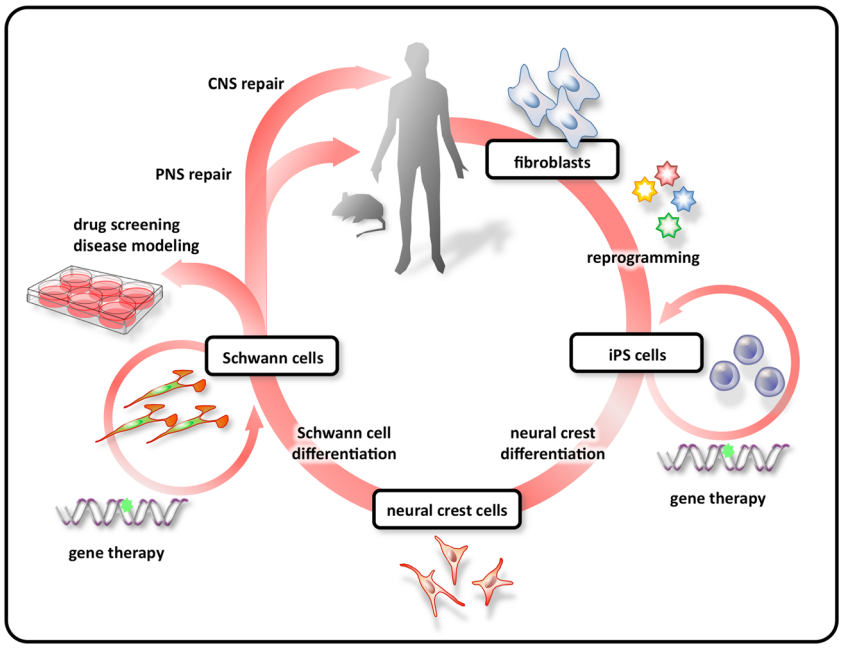

Fig. 3 Potential applications of iPSCs for SC therapy and disease modeling

iPSCs. A major issue for both ES and iPSCs is the risk of teratoma formation in vivo. Even small percentages of contaminating undifferentiated ES or iPSCs can result in teratoma formation after implantation [162, 163]. Several other issues still need to be addressed such as the relevance of epigenetic memory to iPS differentiation capacity, the reprogramming efficiency, the effect of unknown mutations in the starting cells, and the phenotypic stability of the differentiated cells. It seems likely that clinical application of iPSCs will not be possible as long as the reprogramming process for each separate clone cannot be controlled in the safest possible way. Unraveling the exact molecular mechanisms by which adult somatic cells can be reprogrammed to a pluripotent state remains therefore of great importance.

Improvement of Model Systems

SDIA treatment promotes ectodermal differentiation, while suppressing mesodermal differentiation. Although the use of SDIA is an efficient method to differentiate human pluripotent stem cells into neural cells [164], a major drawback is the risk of introducing xenogenic pathogens or antigens in a patient, if clinically applied. Generally, culture conditions with SDIA are poorly defined. Recently, a protocol was published by Menendez et al. for the differentiation of NCCs from human pluripotent stem cells without the use of SDIA, however no SC generation was shown [140]. EB formation has its own inherent drawbacks; due to differential exposure to diffusing medium components, 3-dimensional aggregates tend to show a high heterogeneity among the differentiating cell types as well as less controllability of differentiation. Also, absence of contact with extracellular matrix components may cause lack of developmental support. However, as EBs do not carry risks of xenogenic contamination, they seem clinically more relevant than the use of SDIA.

As mentiond earlier, culture of NCSCs as neurospheres has been described as a method for induction of NC [135, 141–144]. Although neurosphere formation does not seem to be mandatory for NC formation, it appears to increase efficiencies up to 10-fold [144]. Most authors refer to neurospheres as to any floating cell cluster containing CNS or PNS precursor cells, regardless of the stage of culture. Prolonged culture of undifferentiated iPSCs in medium containing FGF2 and EGF, however, will increase the amount of CNS lineage progenitors (‘true’ neural stem cells) within these spheres, at the expense of NCCs [142, 143]. Pomp et al., Ziegler et al., and Wang et al. did demonstrate the presence of NCCs in ES-derived neurospheres based on the expression of NC markers [135, 141, 144]; however, they all used SDIA, EB formation, or rosette formation prior to neurosphere culture. It seems likely that PNS progenitors can be efficiently expanded using neurosphere formation, but that induction of NC does require prior use of one of the two main model systems (SDIA, EB formation).

To become clinically relevant, the efficiency of in vitro differentiation methods should be optimized to shorten the time period needed for generation of transplantable cells, without compromising safety (e.g. increased risk for teratoma formation). Preparation of cells should be confined to a limited time period, in order to prevent missing a crucial therapeutic window. The efficiency of in vitro neural differentiation can be greatly enhanced by inhibition of BMP and TGFβ signaling through dual SMAD inhibition, which can be established with small molecules such as SB435142 and

LDN-193189 [165, 166]. These molecules not only induce neuralization but can also be used to favor NC specification, as shown in human pluripotent stem cells [165, 166]. Menendez et al. also showed that blockade of SMAD signaling enhances differentiation of human pluripotent stem cells towards multipotent NCCs [140]. NC differentiation using dual SMAD inhibition does not require stromal cells and can give up to 90 % $p75^{NTR}$–positive cells. Subsequently, Kreitzer et al. also showed spontaneous differentiation towards GFAP-positive cells. However, these cells were not yet further characterized, and, as SC generation was not the purpose of the study, no efficiency was mentioned [166].

A diagram suggesting the consecutive steps that can be taken for optimizing the tissue engineering of SCs, based on several protocols, is depicted in Fig. 4. Taking safe future clinical application into account, this protocol does not make use of SDIA, but only chemically defined morphogens after EB formation. Combination with dual SMAD inhibition might increase the efficiency of NC formation. FACS sorting for mature markers diminishes the chance of contamination with tumorigenic pluripotent cells.

Targeted differentiation of SCs requires positive regulators of myelination (see earlier). Therefore, NRG1, cAMP or cAMP substitutes such as dbcAMP or forskolin, and laminin-coated culture dishes are desired for in vitro differentiation of SCs, mimicking the in vivo micro-environment.

Proper Characterization of Tissue Engineered Schwann Cells

Most studies, except for the study by Liu [139], lack a proper proof of functionality, such as evidence of in vitro myelination and/or in vivo myelination, or proper gene expression analysis of purified iPS-derived SCs. In all in vivo studies so far, progenitor stages e.g. undifferentiated NCCs, were implanted instead of mature SCs. Furthermore, the use of markers is mandatory for proper definition of the differentiated cell phenotype. While gene expression of immature SCs (SC precursors) partially overlaps with expression in migrating NCCs, mature SCs do have a defined phenotype. Markers that are often used are GFAP, $p75^{NTR}$, S100β, and MBP. However, some markers such as S100β and GFAP are not restricted to SCs, and unless pre-differentiated NCCs are FACS sorted for NC markers such as $p75^{NTR}$ and HNK1, it cannot be ruled out that the S100β-positive or GFAP-positive cells are astrocytes, and not SCs. As S100β is mainly expressed in astrocytes, and partial neural differentiation is to be expected from iPS neurospheres, S100β expression in neurosphere-derived cells may indicate astrocytic differentiation rather than SC differentiation [143]. CNPase on the other hand is purely restricted to the two myelinating cell types, oligodendrocytes and SCs, and in combination with other markers such as S100β, GFAP, or Oct6 might show better proof of a mature SC phenotype. Expression of genuine myelination markers such as P0 and PMP-22 in an in vitro or in vivo myelination model should be the standard for proving proper functionality of iPS-derived SCs.

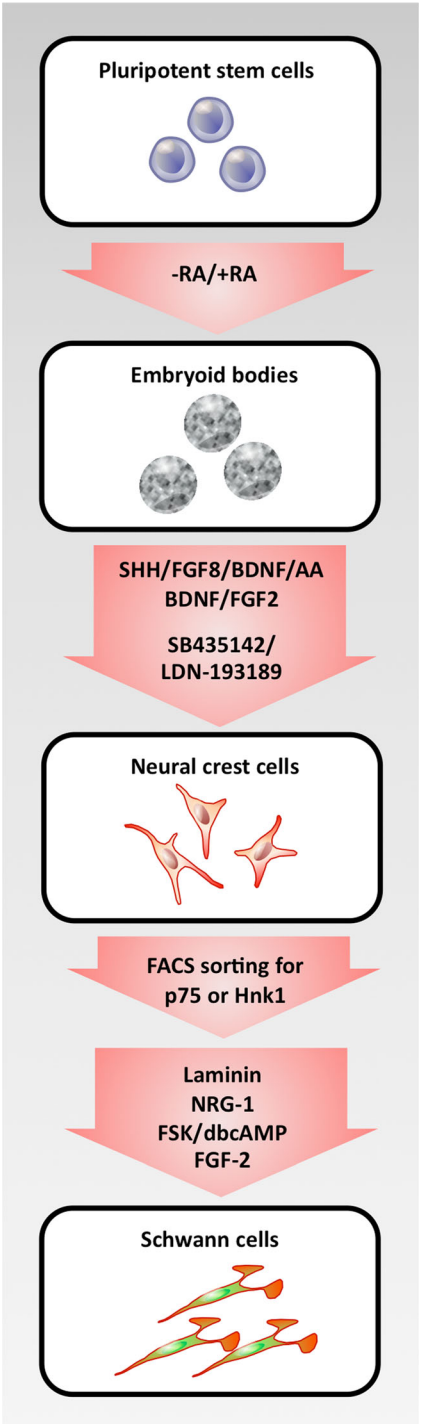

Fig. 4 A diagram schematically consecutively indicating the potential methods that can be used for optimization of in vitro differentiation of NCCs and SCs

Future Prospects: Direct Conversion?

New stem cell technologies have been developed since the iPS 'revolution'. In 2010, the group of Wernig showed that it is possible to directly convert mouse fibroblasts into functional neurons in vitro by means of lentiviral transduction of three transcription factors (*Ascl1*, *Brn2,* and *Myt1l*) [167]. These induced neurons (iNs) expressed neuronal markers, generated action potentials and formed functional synapses [167]. Recently, two studies have reported the direct conversion of mouse fibroblasts into myelinogenic oligodendrocyte precursor cells (OPCs) by transfecting the fibroblasts with a combination of either *Olig2, Sox10,* and *Zfp536*, or *Olig2, Sox10* and *Nkx6.2* [168, 169]. No direct conversion of fibroblasts towards myelinogenic SCs has yet been described. A potential gene cocktail for such a conversion might contain *Egr2/Krox20*, together with *Sox10* and *Oct6/Brn2*. *Egr2/Krox20*, master regulator gene of myelination, has been shown to upregulate myelination genes and to promote the transition of SC precursors towards non-proliferating myelinating SCs [170], while Sox10, Oct6, and Brn2 all exert their promyelinating functions through synergistic targeting of *Egr2/Krox20* (described earlier). However, a systematic search for appropriate inducing factors might still be required. Also, timing of exogenous expression will be of importance; e.g. Oct6 might require only transient expression, as misexpression may cause hypomyelination [90, 171]. The implications of the source cell type to be used are not fully clear, however the published studies indicate that direct conversion is not limited to cell types 'within' the same germ layer. A most interesting option for the application of direct conversion could be direct conversion in situ. In this approach, lost cells are replenished in vivo at the proper location near a lesion; e.g. fibroblasts might be converted into remyelinating SCs near injured peripheral nerves, bypassing time-consuming in vitro differentiation protocols.

Concluding Remarks

The groundbreaking discovery that somatic cells can be reprogrammed into iPSCs has offered an inexhaustible, autologous (patient-derived) source of any type of cell, including SCs. To induce the specific differentiation of these iPSCs into myelinogenic SCs in vitro, the conditions, pathways and processes that normally regulate the formation of SCs during embryonic and neonatal development need to be recapitulated. It is crucial to have a complete understanding of the cellular and molecular, genetic, and epigenetic mechanisms, at the different intermediate stages of in vivo SC development, in order to be able to effectively recapitulate this development in vitro. Apart from further elucidation of regulation of SC formation, it is essential that issues related to the safe clinical use of iPS-derived cells are solved. However, with the ongoing progress in the development of zero-footprint, xenogen-free production of iPSCs according to new, strict GLP regulations, clinical application of autologous iPS-derived SCs in the treatment of peripheral nerve injury or peripheral nervous system diseases, let alone CNS diseases, may not be that far.

Conflict of Interest The authors declare no potential conflict of interests

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