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Abstract

Intense research using vertebrate model organisms has gained considerable knowledge regarding the origin of peripheral neurons, such as neural crest and cranial placodes induction and diversification. However, early development in human embryos has remained largely uncharacterized, despite the roles the neural crest, cranial placodes and their derivatives play in several pathologies. The in vitro systems based on the differentiation of human pluripotent stem cells (hPSCs) strikingly recapitulate embryonic development in a dish. Extensively proved for the neurogenesis in the central nervous system (CNS) in the last 15 years, novel in vitro differentiation strategies were recently designed for the generation of peripheral nervous system (PNS)-related populations. It is the case of human neural crest, cranial placodes, cranial sensory and autonomic neurons, and enteric neurons. These novel models are equally important for enlightening the human early development and for developing new tools for the modern medicine. Better understanding of the programs for specification and maturation of the multitude of peripheral neurons is a major challenge confronting developmental and stem cell researchers in years to come.

Keywords: neural crest, cranial placodes, sensory ganglia, sympathetic ganglia, enteric ganglia, human embryo, pluripotent stem cells, peripheral neuropathies

1. Introduction

Human pluripotent stem cells (hPSCs), whether derived from the inner cell mass of an embryo at the blastocyst stage (human embryonic stem cells, hESCs) [1], or generated via reprogramming of differentiated somatic cells (human induced PSC, hiPSCs) [2], can self-renew...
indefinitely in culture while maintaining the ability to generate all the cell types in an organism.

The potential of using hPSCs for generating neural cells has been extensively exploited during the last 15 years. The derivation of central nervous system (CNS) cells was among the first differentiation protocols developed in the hESC field [3,4]; nowadays, efficient protocols are available for generating neuroepithelial cells [5], spinal cord motor neurons [6], midbrain dopaminergic neurons [7], and cortical neurons [8,9], among many others, valid from both hESCs and hiPSCs.

The effort to generate specific cell types was guided by the knowledge gained from studies on model organisms, as well as from the previous mouse embryonic stem cell differentiation studies. The cell biology of neurogenesis has been studied in most detail in CNS, but much progress has been achieved also for the peripheral nervous system (PNS) development (reviewed in [10–13]). PNS neurons are categorized into two anatomical and functional classes: sensory and autonomic neurons.

The sensory neurons are afferent neurons that relay information from a number of specific structures (including Merkel’s discs, Meissner’s, Pacini’s and Ruffini’s corpuscles, Golgi tendon organs, and muscle spindles) to the CNS. Accordingly, proprioceptive neurons provide spatial information regarding position and movement, mechanoreceptive neurons mediate touch, nociceptive neurons respond to painful stimuli or itch, and thermoceptive neurons relay information regarding temperature. These neurons form cranial ganglia (trigeminal, geniculate, petrosal, nodose, and others), and dorsal root ganglia (DRG), a metameric series of ganglia next to the spinal cord.

The autonomic neurons are largely efferent motor neurons that carry information from the CNS to the various organs of the body, providing involuntary control of the visceral organs. The autonomic PNS consists of three major components: the sympathetic (SyNS) and parasympathetic (PSNS) nervous systems, which function together to maintain body homeostasis, and the enteric nervous system (ENS), which controls gut motility. SyNS controls the involuntary activities that occur under stressful “fight or flight” conditions, while PSNS, in general, promotes the visceral activities characteristic of periods of peace and relaxation. Axons from CNS neurons situated in vegetative centers in brainstem and spinal cord project and synapse with the ganglionic cell bodies located paravertebrally, along the body axis, for SyNS, in sympathetic ganglia (SG) or inside the target organs (for PSNS) as parasympathetic ganglia, distributed all over the body. ENS is the largest component of the autonomic nervous system and comprises local neural circuits in the gastrointestinal tract consisting of sensory, inter-, and motor neurons, capable of an autonomous control, without CNS inputs. Sympathetic neurons are noradrenergic (the most) or cholinergic (few, e.g., sudomotor). Parasympathetic neurons are cholinergic (the most) or adrenergic (few). Enteric neurons, whose number surpasses that of spinal cord neurons, have remarkable cytoarchitecture and neurotransmitter diversity, including serotonin (5-hydroxytryptamine, 5-HT), GABA (γ-aminobutyric-acid-positive), and nitric-oxide-synthase (NOS)-positive neurons.
The peripheral neurons originate from neural crest and cranial placodes, two transient embryonic epithelia generated in early embryogenesis at the junction between CNS primordia and non-neural ectoderm. These ectodermal structures are notable for their ability to transform into mesenchymal-like cells, migrate extensively along highly stereotypic pathways and differentiate into numerous derivatives, according to the environmental influences encountered during their journey and at their final sites. In addition to sensory and autonomic neurons of the PNS, they form many other derivatives, including, from the neural crest, Schwann cells and satellite glia, melanocytes, endocrine cells, chondroblasts, osteoblasts, odontoblasts and smooth muscle cells, among others [10–12], and from placodes, sense organ cells and neuro-endocrine cells in the head [13].

Neural crest and placode development are multistep processes whose main features are conserved across all vertebrate groups. Section 2 summarizes, in a pan-vertebrate model, the current understanding of neural crest and placode induction, specification, migration, and diversification, pointing on the major signaling molecules and transcription factors that act in each stage.

During the past ten years, much progress has been made in elucidating the mechanisms that orchestrate the differentiation of neural crest and placode cells toward the multitude of neurons forming the PNS. It is the case of sensory neurogenesis and autonomic neurogenesis (sympathetic, parasympathetic, and enteric) in various vertebrate models, which are briefly reviewed in Section 3.

Understanding the developmental ontogeny of the diverse peripheral neural populations provides an essential framework for designing rational approaches towards hPSC directed differentiation. Recently, protocols for efficient differentiation of hPSCs toward neural crest cells (NCCs), cranial placode cells (CPCs), neural crest-derived sensory neurons, neural crest-derived enteric neurons, and placode-derived sensory neurons have been reported, and are briefly reviewed in Section 4.

The expression profiles reported from the in vitro differentiation protocols and confirmed in human embryos are combined in an in vitro model proposed in Section 5. As a lot little is known about the specification mechanisms in human embryo comparing with animal models, these in vitro approaches can gain further insights into the early human development. As the iPSC technology has opened unprecedented opportunities for medicine, current challenges and future avenues in the development of novel therapeutic strategies for PNS diseases are also discussed.

2. Peripheral nervous system development in a pan-vertebrate model

2.1. Origins: neural border, neural crest, and cranial placodes

PNS originates early in development from epiblast, in close connectivity with the CNS and epidermal development. At the beginning of gastrulation, a median domain named neural ectoderm starts to form in the anterior part of the embryo, which eventually generates the neural
plate, the neural tube, and the entire CNS; a new territory is formed between the future neural and non-neural ectodermal domains, named the neural border domain (reviewed in [11–16]).

The induction of both neural and neural border domains is established in all vertebrates by a complex interplay of signaling pathways such as fibroblast growth factors (FGFs), bone morphogenic proteins (BMPs), and Wingless and Int proteins (WNTs), although the sources of these signals and their inhibitors may vary between species. At the anterior end of the embryo, the inhibitors DKK1 (dickkopf 1, a WNT inhibitor) and noggin (a BMP inhibitor) suppress posterior signals and pattern the neural ectoderm, leading to the formation of the anterior neural plate and tube. In the lateral part of the embryo, increased activity of WNT and BMP specifies the non-neural ectoderm. The neural border originates within a zone exposed to intermediate levels of FGFs and WNTs, as well as to BMP inhibitors. The neural border cells turn on the expression of a new set of transcription factors, named the neural border specifiers, which include Tfap2, Msx, Zic, Gbx2, Pax3/7, Dlx5/6, Gata2/3, Foxi1/2, and Hairy2 (reviewed in [12–16]) (Figure 1).
At subsequent stages, the neural plate closes to form the neural tube (a process named **neurulation**). As a consequence, the border territory elevates, forming the dorsal aspect of the opposing neural folds and of the new forming neural tube. Different signaling pathways together with the neural plate border specifiers act to segregate the neural border domain medially into the neural crest and laterally into the preplacodal domain. Neural crest is induced by the presence of FGF, BMP, and WNT activity, while FGF activity and inhibition of BMP and WNT signals in this stage induce the formation of the preplacodal domain. The emergence of the neural crest is marked by the expression of a new set of genes named **neural crest specifiers**: FoxD3, Ets1 and Snail1/2, Twist and Sox8/9/10. The emergence of the preplacodal domain is marked by the expression of the **placodal specifiers** Six1, Eya1/2, and Irx1 (reviewed in [12–16]) (Figure 1).

During neurulation and somitogenesis, the anterior-posterior (A/P) patterning of the neural plate/tube and of the associated neural crest and preplacodal domains takes place. As in the neural tube, the A/P patterning of the neural crest and placode domains is regulated by the major signaling pathways: FGFs, BMPs, WNTs, sonic hedgehog (SHH), Notch, and retinoic acid (RA). Their combined action further segregates both neural crest and preplacodal domains (reviewed in [12–16], Figure 2).

Figure 2. Regionalization and specification of neural crest and preplacode domains.

Cranial neural crest anterior to somite 1 contributes to the formation of the head (including bone, cartilage, connective tissue, teeth, and cranial ganglia (the last together with cranial placodes). Posterior to somite 1, vagal, trunk, and sacral neural crests are formed. Between
somite 1–7, vagal neural crest gives rise to the ENS and cardiac outflow tracts. Between somite 7–28, trunk neural crest differentiates into DRG, SG, and norepinephrine-producing cells in the adrenal gland. Posterior to somite 28, sacral neural crest provides part of the ENS of the distal gut. Melanocytes and Schwann cells are generated from neural crest of all A/P levels (Figure 2).

Following the A/P patterning in the developing embryonic head, the preplacodal domain is divided into The anterior, posterior, and intermediate domains (Figure 2). Anterior domain comprises the adenohypophyseal, olfactory, and lens placodes. The posterior domain comprises the otic and epibranchial placodes. Between the anterior and posterior placodes develops the trigeminal placode. With the exception of the adenohypophyseal and lens placodes, all other cranial placodes give rise to sensory neurons of their associated sensory structures. The otic placode generates the sensory neurons of its associated vestibulocochlear (VIIIth) ganglion, and epibranchial placode gives rise to the sensory neurons of the geniculate, petrosal, and nodose ganglia. The trigeminal placode gives rise to the sensory neurons of the ophthalmic and maxillo-mandibular divisions of the trigeminal ganglion.

2.2. Migration: epithelial-to-mesenchymal transition and stereotypical pathways

After the neural crest and placodes are specified and patterned, their cells acquire mesenchymal properties and migratory morphology and commence migration. The epithelial-to-mesenchymal transition (EMT) was intensively studied in neural crest. Most of the work has focused on the adhesive changes that enable cells to delaminate. Cadherins are thought to be central to this process, a switch between type 1 cadherins (Ecad and Ncad) and type 2 cadherins (Cad7 and Cad11) taking place in migratory cells.

The neural crest specifiers activate the EMT effector program, which allows the neural crest cells to delaminate from the neural folds/tube and become a migratory cell type, but also to maintain them in a proliferative state. Extracellular signals such as FGF2, neuregulin, neurotrophin 3 (NT3), and epithelial growth factor (EGF) help migratory cell survival and/or proliferation and may act as instructive cues [16,17]. Migratory NCCs are complex from a regulatory viewpoint, as they are constantly exposed to different environmental signals and are also starting to differentiate into diverse derivatives.

Additionally, different signaling systems are involved in the guidance of neural crest migration and the establishment of the correct migratory pathways [17]. For example, the melanocyte precursors follow a dorsolateral migratory stream, under the epidermis, while the sensory and autonomic precursors from trunk neural crest follow a ventromedial pathway, between the neural tube and the developing somites. Sympathetic progenitors express the chemokine receptor CXCR4, which is responsible for the directed migration toward the dorsal aorta, where the chemotaxtractant SDF-1 is produced [18].

Next section briefly describes the differentiation steps of the NCCs and CPCs that are fated to become sensory neurons, as well as of the NCCs fated for autonomic neurons. However,
understanding the programs of neural crest diversification is a major challenge confronting
development biologists in years to come.

3. Peripheral neurogenesis

3.1. Sensory neurogenesis

Cranial sensory neurons are derived mainly from trigeminal and epibranchial placodes and
fewer from cranial neural crest, while the neurons forming the DRGs are derived solely from
trunk neural crest. Specification of sensory neurogenic lineages has been better documented
for the trunk neural crest (reviewed in [10, 11]). Each functional type of sensory neuron is
characterized by its own unique set of receptors and ion channels, and their differentiation
depends on unique sets of transcription factors.

A crucial molecular handle for analyses of sensory neuron development was provided by the
discovery that functionally related neuronal subtypes require specific neurotrophic factors.
Trophic factor signaling has long been recognized, particularly the neurotrophin ligand/receptor components: nerve growth factor (NGF)/TrkA, brain derived neurotrophic factor (BDNF)/TrkB, and NT-3/TrkC [19,20]. Premigratory NCCs delaminated at different time points, and sensory neurons are generated in a number of waves that derive from temporally distinct NCCs that enter in the ventromedial pathway and arrest migration adjacent to the neural tube, generating DRGs [21]. Large-diameter TrkC/TrkB+ proprio- and mechanoreceptive neurons are produced first, while small-diameter TrkA+ nociceptive neurons are subsequently generated. Later, boundary cap cells also generate a small population of TrkA+ nociceptive sensory neurons.

The first two waves of NCC differentiation are regulated by Neurogenin1 (Ngn1) and Neurogenin2 (Ngn2). Neurogenin-1 and Neurogenin-2 are potent promoters of sensory specification and can be detected in a subset of proliferative migrating cells [22]. Ngn1 appears to play a predominant role in formation of small-diameter nociceptive (TrkA+) neurons with a minor requirement in the formation of large-diameter mechanoreceptor (TrkB+) and proprioceptive (TrkC+) neurons. In contrast, Ngn2 plays a transient role in formation of large-diameter mechanoreceptor (TrkB+) and proprioceptive (TrkC+) neurons; Ngn2+ cells also contribute to a small but significant fraction of nociceptive (TrkA+) neurons.

The subsequent transition of neurogenic progenitors into post-mitotic neurons involves up-regulation of Brn3a and Islet1, as well as down-regulation of factors that maintain NCC multipotency, such as Sox10 and FoxD3 [23–25]. Brn3a and Islet1 also direct the expression of transcription factors important for sensory neuron maturation, such as the Runx family. Runx1 is critical for the continuing differentiation of nociceptive neurons, while Runx3 primarily regulates proprioceptive maturation [21,26,27].

The signaling pathways and transcriptional changes that occur during sensory neuron
specification have been well documented [28]. Many of the extrinsic and intrinsic cues that act
in the neural crest specification act also in sensory neurogenesis. WNT/β-catenin signaling acts
on sensory specification at the premigratory stage and during later stages of neuronal development [29].

During their early maturation stages, the sensory neurons begin to grow neurites that fasciculate with outgrowing spinal motor axons in the forming ventral root en route to the periphery or project centrally into the spinal cord via the dorsal root and innervate CNS targets. Somite-derived patterning cues direct the stereotyped position of early sensorimotor projections into the periphery and coordinate alignment with the developing vertebrae [30]. At further stages of maturation, peripheral innervation targets, central neurons, and associated glia produce neurotrophic factors that direct the development of receptive neuronal subtypes. Proper neurotrophic signaling is crucial also for sensory neuron survival and synapse formation [30].

3.2. Sympathetic neurogenesis

Sympathetic neurons are derived from trunk neural crest (Figure 2). Major differentiation steps and gene regulators acting on the way between neural crest progenitors and mature sympathetic neurons have been identified in several vertebrate species (reviewed in [31]).

The initial sympathetic differentiation depends on signals from the ventral neural tube and the notochord, as well as the environment surrounding the dorsal aorta. Sympathetic progenitors migrate along the ventro-medial path and reach the dorsal aorta, initially forming a continuous sympathetic chain that subsequently segregates into discrete ganglia. The ventro-medial path is shared with the DRG precursors, and the sympathetic precursors pass through the DRG primordia to reach the sites of primary sympathetic ganglia. The aorta-derived signals have been identified as members of the family of BMPs, which have an essential role in sympathetic neuron development [32]. Accordingly, migrating sympathetic progenitors express the chemokine receptor CXCR4 [18]. The cells then undergo a second migration to para-aortic sites, where secondary sympathetic ganglia are formed. Some of these cells from the primary sympathetic ganglia migrate deeper into the embryo toward the kidney, where they differentiate into predominantly neuro-endocrine cells (chromaffin cells) of the adrenal gland.

The BMP-induced differentiation involves the sequential onset of expression of Phox2b, a master gene of sympathetic neuron development, followed by Ascl1, Insm1, Hand2, Phox2a, Gata2, and Gata3. AP-2β is also important for sympathetic progenitor survival, while Hox genes for the patterning of the sympathetic chain [33]. Noradrenergic differentiation of sympathetic progenitors starts after stopping the migration in the vicinity of the dorsal aorta and is reflected by the expression of tyrosine hydroxylase (TH), followed by dopamine-beta-hydroxylase (DBH) [34] and pan-neuronal markers. However, these sympathetic neurons are immature and continue to proliferate in the sympathetic ganglia primordia under the control of a number of extrinsic signals, such as insulin-like growth factor I (IGF-1), artemin, WNTs, midkine, and BDNF (reviewed in [31]).

The vast majority of postmitotic sympathetic neurons are generated by asymmetric divisions of immature neurons, leading to a postmitotic neuron and an immature neuron that is able to
divide again [35]. Transcription factor codes that underlie the specification and sequential
generation of different sympathetic neuron classes are still not known. However, a large
proportion of transcription factors involved in the initial specification of sympathetic neurons
are expressed up to the adult stage, such as Phox2b, and even selectively expressed in adult
noradrenergic neurons, such as Hand2 [36]. These results demonstrate that several members
of the gene regulatory network that controls initial sympathetic neuron development are also
essential for the maintenance of differentiated neurons.

Survival and terminal differentiation of sympathetic neuron subtypes, as like of sensory
neurons, is controlled by target-derived neurotrophic factors. Their terminal differentiation is
controlled by specific retrograde signaling, which can result in a change of neuropeptide and
neurotransmitter phenotype. For example, sweat glands and periosteum are innervated
postnatally by noradrenergic sympathetic neurons that transdifferentiate to functional
cholinergic sympathetic neurons [37,38].

3.3. Parasympathetic neurogenesis

Different parasympathetic neurons are generated from cranial, vagal, and sacral neural crest
(Figure 2). The development of parasympathetic neurons is controlled by mechanisms that are
closely related to those acting in the sympathetic neuron lineage, but also shows some
interesting differences [31,39,40]. Signals and molecular pathways controlling the specification
and differentiation of parasympathetic neurons have been characterized in detail for the ciliary
ganglion derived from cranial neural crest and for the enteric neurons arriving from the vagal
neural crest.

For the cranial neural crest, BMP5 and BMP7 are expressed at the site of autonomic ganglion
formation and are shown to be essential and sufficient for ciliary neuron development. The
ciliary ganglion and other parasympathetic ganglia, like the sympathetic ganglia, depend on
Ascl1 and Phox2b expression [41,42]. Unlike in the sympathetic lineage, parasympathetic
neuron precursors do not express Gata3, AP-2β and Hox genes. A transient expression of
noradrenergic characteristics (like TH and DBH) is observed, which is maintained in a very
small subpopulation of cells. The majority of cells acquire a cholinergic phenotype, character‐
ized by the expression of choline acetyltransferase (ChAT) and vesicular acetylcholine
transporter (VACHT). For the cranial parasympathetic ganglia, as for the DRGs and in contrast
to sympathetic ganglia, neuron differentiation starts after withdrawal from the cell cycle
[31,41]. However, some postmitotic neurons in the ciliary ganglion do not start to differentiate
after cell cycle exit but rather remain as quiescent postmitotic neuron progenitors that later
differentiate to mature neurons [42].

Recent exciting work has shown that Schwann cell progenitors in the developing cranial nerve
can generate parasympathetic neurons [43,44]. Thus, the developing cranial nerve appears to
serve as both a guide and source of progenitors for the parasympathetic ganglia it will
eventually innervate.

Detailed analysis revealed that both parasympathetic ganglia arise from cells that accompany
the cranial nerve fibers as they grow towards the site of parasympathetic ganglion formation.
These migratory cells express SOX10, which indicates their crest origin, and additionally is a marker for Schwann cells [45–47]. During their migration, however, they turn on the expression of Phox2b, characteristic for autonomic neurons. These be-fated precursor cells are not restricted to cranial nerves but are also found in developing limb nerves where they generate small ganglia composed of Phox2b neurons. It is yet unclear which signals control the fate switch in the migration bi-fated precursor cells and how progenitors become restricted to a parasympathetic fate rather than to a sympathetic neuronal fate.

3.4. Enteric neurogenesis

The ENS develops from both the vagal and sacral neural crest. Vagal neural crest generates most of the ENS and migrates caudally to colonize the entire length of the bowel. Vagal neural crest acquires the ENS formation ability after further programming during migration to the foregut. This involves signaling by Hedgehog and retinoic acid systems which upregulate Ret expression of vagal neural crest. Enteric neuron precursors express Phox2a and TRKC. The mechanisms generating the large diversity of neurotransmitter phenotypes, including serotonin (5-hydroxytryptamine, 5-HT), GABA (γ-aminobutyric-acid-positive) and nitric-oxide-synthase (NOS)-positive neurons, are not yet known [39, 40].

4. Peripheral neurogenesis

4.1. Neural crest and placode induction protocols

During the last 15 years, many protocols for generating different neural progenitors and neurons from hESCs were developed. The early methods to differentiate human PSCs towards neural lineages were focused on producing neural stem cells and some CNS derivatives, whereby neural crest cells were observed as a by-product of neural differentiation. Some of these protocols involved dense self-organizing cell conglomerates, such as embryoid bodies, neural rosettes, or confluent cultures, that produce undefined signaling [48,49], while other protocols relied on the use of stromal feeder co-cultures [50,51]; however, these undefined protocols were not able to generate more than 10% of cells with neural crest identity.

A step forward in the increasing efficiency of neural induction in hPSCs was the introduction of the “dual SMAD inhibition” protocol, which generated high yields of neuroectodermal cells and CNS fates. This protocol uses the concomitant inhibition of BMP pathway with Noggin and of TGFβ/activin/Nodal signaling pathway with SB431542 (SB) [5]. Based on this protocol, different strategies were developed to direct the neuroectoderm cell towards neural crest cells, using dual SMAD inhibition and WNT signaling [52–54]. Most of them used the small molecule CHIR99021 (CHIR) to enhance WNT/β-catenin signaling by inhibiting GSK3β. Other protocols were able to generate more neural crest cells by using the WNT agonist CHIR and only the TGFβ/activin/Nodal receptor inhibitor, but not BMP inhibitors [55, 56]. The efficiency of these protocols was variable, and additional separation methods were implemented to increase the neural crest yield, relying on surface markers such as HNK1 (human natural killer-1) and p75NTR (NGFR) [52–56].
A very recent protocol [57] dramatically improved the yield of neural crest cells in defined serum free-media under WNT activation by simply eliminating both the BMP inhibitor noggin and the TGFβ/activin/Nodal receptor inhibitor SB. This study established a rapid (5 days) and efficient protocol for generating a high yield of neural crest cells. This approach significantly shortens the total length of time required for the neural crest induction. At day 5, the differentiating cells expressed in high proportion (more than 80%) the neural crest markers SOX10, PAX7, and TFAP2A. Additionally, it was clearly demonstrated with this protocol that blocking the FGF and BMP pathways or increasing FGF and BMP activity dramatically decreased the neural crest induction (below 5% of the total cell population).

Another recent report [58] demonstrated that de-repression of endogenous BMP signaling during dual SMAD inhibition is sufficient for the selective induction of human cranial placode cells. Gene expression analysis at day 11 revealed, upon withdrawal of Noggin at day 2 or 3 of differentiation, a robust induction of the preplacodal markers SIX1, EYA1, and DLX3, and more than 70% of cells adopting a SIX1+ cranial placode fate.

Leung et al. [57] also investigated the cranial placode induction in the same defined culture conditions as for neural crest induction, but without CHIR. They found that E-cadherin, and preplacodal markers such as EYA2, FOXC1 and ISL1 were highly induced in day 5 cultures. Upon CHIR administration, however, these surface ectoderm and preplacodal transcripts were repressed.

The capacity of human neural crest and placode-like cells generated with different protocols to contribute to terminal derivatives associated with neural crest and placode development was tested in different approaches. Most of the neural crest-like cells were tested for their multipotency and were subjected to terminal differentiation into different derivatives, including sensory neurons, sympathetic neurons, enteric neurons, Schwann cells, melanocytes, chondrocytes, and osteoblasts. The placode-like cells were tested for their competence to generate sensory neurons, lens cells, and hormone-producing anterior pituitary cells [58].

Early protocols were based mainly on spontaneous differentiation, identifying different proportion of specific derivatives such as sensory neurons (Brn3a+/periferin+), sympathetic progenitors (TH+/periferin+), glial precursors (S100β+ GFAP+), and melanoblasts (MITF+ SOX10+). Some recent protocols were designed for the generation of sensory and enteric neurons, which are briefly presented below.

4.2. Sensory neuronal differentiation protocols

Human NCCs generated peripheral sensory neurons as well as other neural crest derivatives applying different protocols. For peripheral neuronal differentiation, NCCs were plated on a special coated surface in order to facilitate neuronal growth (such as Matrigel or Geltrex-coated plates, laminin/fibronectin or collagen), followed by a treatment with a cocktail of neurotrophic molecules to sustain sensory neuronal differentiation and maturation: brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and nerve growth factor (NGF). Markers associated with peripheral sensory neurons (PRPH BRN3a, and ISL1) and glial progenitors (S100β) were detected after 7–14 days [48–53, 56–61].
Chambers et al. [59] applied another protocol which resulted in the highly efficient derivation of human nociceptive neurons from PSCs within a short period of time. Specification into nociceptive neurons was achieved by adding an inhibitor of tyrosine kinase signaling (SU5402), a blocker of the NOTCH signaling (such as M N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester, DAPT, which blocks γ-secretase) and a GSK-3β inhibitor (CHIR), which promotes WNT/β-catenin signaling [59–61].

The differentiation of neural crest cells toward a sensory phenotype was marked by the expression of the pro-neural transcription factors NGN2 and NGN1. Also consistent with early sensory neurogenesis, the expression levels of PRPH, BRN3A, ISL1 were found to be highly up-regulated. The majority of neurons derived from all iPSC lines co-expressed BRN3A and PRPH, a combination that had been shown to be specific for peripheral sensory neurons in vivo. Most of the PRPH positive cells co-expressed ISL1, another recognized markers of sensory specification [23–25].

The increasing expression of the glutamatergic markers VGLUT1 and VGLUT2 as well as very low expression levels of markers for non-sensory neuronal phenotypes (such as MASH1, HB9, DBH, GAD, and VACHT) additionally supported an efficient sensory specification. The increasing expression of the synaptic marker SYNAPTOPHYSIN (SYP) indicated that the neurons were maturing to become functional. These newly differentiated neurons up-regulated markers of proprioceptive (TRK C, RUNX3), nociceptive (TRK A, RUNX1), or mechanoeceptive (TRK B) subtypes.

Optimization of long-term differentiation will be necessary in order to sustain the maturation and subtype specification in vitro.

4.3. Autonomic neuronal differentiation protocols

A very recent study [62] describes an efficient strategy to derive enteric neurons with vagal origin and cranial parasympathetic neurons from human ESCs, based on CHIR and the dual SMAD neural crest induction protocol. Enteric neuron specification involves additional treatment with retinoic acid from day 6 to day 11. The differentiated cells were sorted for CD49D at day 11. ENC cells from the 11 day induction protocol were aggregated into 3D spheroids and cultured in defined medium containing CHIR and FGF2. After 4 days of suspension culture, the spheroids were plated on poly-ornithine/laminin/fibronectin-coated dishes in medium containing GDNF. The enteric neuron precursors migrated out of the plated spheroids and differentiated into neurons in 1–2 weeks, further maturing for up to 60 days.

Purified CD49D⁺ precursors, derived in the presence of retinoic acid, expressed HOXB2–HOXB5 indicative of vagal identity, but not more caudal HOX transcripts such as HOXB9. In further agreement with vagal identity, CD49D⁺, retinoic-acid-treated neural crest precursors expressed markers of early enteric lineages including PAX3, EDNRB and RET. Replating 3D spheroids under differentiation conditions yielded immature neurons expressing TUJ1 and the enteric precursor marker PHOX2A and ASCL1 (day 20). Most PHOX2A⁺ cells were positive for TRKC a surface marker expressed in enteric neuron precursors. Temporal expression analyses showed maintenance of ENC neuronal precursor marker expression by day 40 of
differentiation, followed by an increase in the percentage of mature neurons by day 60. In agreement with enteric neuron identity, a broad range of neurotransmitter phenotypes was observed, including serotonin-positive (5-hydroxytryptamine+, 5-HT+), GABA+ (γ-aminobutyric-acid-positive), and nitric-oxide-synthase-positive (NOS+) neurons.

The CD49D+ crest progenitors not treated with RA were HOX-negative, expressing a cranial autonomic phenotype. CNC-derived precursors differentiated into tyrosine-hydroxylase-expressing neurons and gave rise to TRKB-positive rather than TRKC-positive precursors, and no 5-HT+ neurons were observed [62].

5. From in vitro differentiation of PSCs to human PNS development and diseases

5.1. An in vitro neuronal differentiation model

Based on the recent reports presented above, a model for neuronal differentiation is proposed here (Figure 3). HESCs resemble epiblast cells in terms of signaling requirements and gene regulatory network for self-renewal and maintenance. Combining various chemically defined conditions, cell plating strategies, and activation of signaling pathways, such as activin, Nodal, FGF, and WNT, can direct the differentiation of hESCs into a number of cell...
types, including the ones generating neural plate, neural crest and placodes. As in development, different peripheral neurons can be generated from regionally patterned neural plate, neural crest, and cranial placode-like cells.

Through the blocking of BMP, activin, and Nodal (via dual SMAD inhibition), hPSCs loose stemness/pluripotency markers and acquire characteristics of neuroepithelial cells (NECs) and further neural progenitor cells (NPCs) and CNS neuronal types. Through the WNT activation (via GSK3 inhibition with small molecules such as CHIR), hPSCs acquire characteristics of non-neural ectodermal cells, neural border cells and neural crest cells. In the absence of WNT activity, markers associated with non-neural ectoderm, placodal and epidermal tissues arise. All these steps are characterized by batteries of transcription factors which are in accordance with the pan-vertebral model. This model relies on milestone-markers which have been identified in a specific fashion in embryonic human NC [63].

In the specification and differentiation steps, including further treatment with CHIR, the NCCs acquire a cranial phenotype, generating mainly cranial parasympathetic neurons. Additional patterning of NCCs with CHIR and RA allows the generation of enteric neurons and DRG sensory neurons. For sensory differentiation, FGF signaling should be blocked (with the inhibitor SU). Placode precursors generate mainly sensory neurons with a trigeminal phenotype. For all the neuronal differentiation steps, blocking NOTCH signals with DAPT dramatically increases the neuronal yield.

5.2. Modeling human early development

For obvious reasons, neural crest and placode development, as well as the sensory and autonomic neurogenesis, are difficult to be studied in human embryos. Access to gastrula stage embryos is extremely limited, and their experimental manipulation almost impossible. Nevertheless, neural crest marker expression patterns have been studied at various developmental stages [63]. However, no study in human embryos related the earliest events occurring during gastrulation and at the onset of neurulation.

The newly devised protocol using hPSC differentiation will certainly facilitate future studies on human PNS development. It is already the case in the same very recent study [57], demonstrating that in early human development, the earliest NC populations arise independently of neural and mesodermal tissues, from precursors that precede the standard neural plate border characteristics. By examining the expression dynamics of a number of neural-related transcripts, no correlation for the induction of PAX6+ NECs and SOX10+ NCCs was found. Furthermore, knockdown assays also suggested that reduced PAX6 function did not negatively interfere with NC induction. These recent data substantiate the notion that human NC forms independently of PAX6+ neuroectoderm. An early origin of human NCCs independent of neural progenitors has been suspected, in accordance with the recent embryological evidence has also suggested the capacity of non-neural ectoderm to form NC without acquiring a neural character (see section 2).

Neural induction in hESCs requires inhibition of either BMP or activin, or both [5]. The fact that administration of noggin blocked expression of NC and/or neural border markers suggests
that human NC induction requires BMP activation, which is distinct from neural induction. It is also worth noting that transcripts for BMP ligands can be readily detected under CHIR treatment, supporting the suggested contribution of this pathway during later events in NC development, such as sensory and autonomic differentiation.

5.3. Human peripheral neurons and their application in medicine

The availability of hPSCs can provide not only a source for investigating the early human development, but also a source for generating human neurons for in vitro studies. Obviously, human neurons are difficult or impossible to be obtained from adult tissue, in primary culture, as it is the case for many other cells (e.g., blood cells, fibroblasts, skin cells). Many functional approaches can be performed with hESC-derived peripheral neurons, such as the electrophysiological investigation of different human neurons in vitro [64]. Many compounds can be tested for their neurotoxicity using assays based on human peripheral neurons, together with other central neurons [60].

In particular, specific neuronal subtypes generated from the patient iPSCs have become a source for studying disease mechanisms underlying different neurological disorders from which the affected neurons were not possible to be available before. The patient-derived in vitro models can recapitulate the disease in a dish, for determining its etiology and progression and could develop into a key drug discovery platform. During recent years, the generation of iPSC lines from human material has become routine. Even more, high-quality iPSCs obtained with non-integrative methods and the complete epigenetic resetting were reported [65]. This brings the iPSCs more close to the clinics, increasing their potential for use in regenerative medicine.

Protocols for sub-types of neurons with different degrees of heterogeneity are currently being used for different peripheral neuropathies (PNPs). Recent studies proved the utility of iPSC-derived neural crest cells and peripheral neurons for neurocristopathies (NCPs) and neurodegenerative diseases (NDDs).

NCPs are a major group of human congenital disorders caused by neural crest developmental deficiencies. To better understand the etiology of NCPs and perhaps identify targets for therapeutic intervention, it is critical to understand the detailed mechanisms of neural crest specification, migration, and differentiation in human embryos. The most common NCPs in which the peripheral neurons are affected are familial dysautonomia (FD) and Hirschsprung disease (HiD).

A first example is provided in a study using hiPSCs derived from FD patients, a pathology characterized by the degeneration of sensory and autonomic neurons [66]. Most FD patients carry a point mutation in the IKBKAP gene (I-k-B kinase complex associated protein), leading to a tissue-specific splicing defect, resulting in reduced levels of normal IKBKAP protein. Neural crest precursors obtained in vitro from FD hiPSCs show low levels of normal IKBKAP transcript, defective neuronal migration, and differentiation. These characteristics were used to monitor the effects of various drugs on NC-induced hiPSCs and have validated a
compound restoring the IKBKAP splicing defect in these cells. These compounds rescue IKAP protein expression and the disease-specific loss of autonomic neuron marker expression [66].

Another example is HiD, in which the enteric neuron progenitors fail to colonize the full length of the bowel during early development. To investigate possible therapies for the HiD, a very recent study used hPSC in vitro under conditions that encouraged them to differentiate into cells resembling ENS precursors [62]. These progenitors were further transplanted into the colon of mice with a genetic mutation that causes a Hi-like disease. The transplant prevented premature death in these mice, although how the cells achieved this condition is not clear. Finally, hiPSC-derived ENS precursors enable the identification of candidate therapeutic targets [62]. In this case, the cell-based therapy can transform the future of patients with severe phenotype such as total intestinal aganglionosis.

In another class of PNP, specific peripheral neurons are affected by a neurodegenerative process. It is the case of Friedrich Ataxia (FRDA), an autosomal-recessive disease caused by a GAA triplet expansion in the first intron of the Frataxin (FXN) gene [67]. Major neuropathologic findings comprise a degeneration of DRGs, with loss of large sensory neurons, followed by cerebellar and cardiomyocytes degeneration. FRDA-iPSC lines have been established and were successfully differentiated into sensory neurons [67], as well as cardiomyocytes [68]. So far, no overt phenotype was observed in iPSC-derived neurons in FRDA in contrast to the reported mitochondrial phenotype in FRDA-iPSC-derived cardiomyocytes [67,68]. However, during the differentiation process of FRDA iPSCs to peripheral neurons via generation of neural crest cells, a differential expression of the frataxin protein was observed between control and FRDA iPSCs, with the FRDA sensory neurons lacking the up regulation found in control neurons. One limitation of this model is related to the maturity of neurons derived from iPSCs. Most iPSC-derived neurons seem to more closely resemble embryonic neurons than mature and aged neurons, and long-time cultures are required to model non-congenital disorders; additionally, it may be necessary to devise protocols that favor aging and degeneration-associated features [69].

To fully tap into the potential of the iPSC technology and to progress toward a fundamental understanding of the causes of disease selectivity in the loss of neuron subtypes, it is necessary to establish reproducible and tailored protocols for differentiation of iPSCs specifically into these neuronal subtypes in vitro.

Better identification of a dysfunctional pathway in patients suffering from complex PNPs is the primary requirement for rational therapeutic drug development. The human iPSC-derived models could impact positively the screening of compound libraries and the drug safety screens and in the same time reduce the animal dependency of the current drug development pipeline. iPSC technology is seen as an important driver of personalized medicine. Prior to treatment, patient-derived iPSCs or differentiated progenies can be used to tailor a particular drug type and dose according to the genetic and cellular profile. Based on all these facts, the hPSC-based PNS platform will allow the development of cell- and drug-based strategies for the treatment of different PNS diseases.
The most promising aspect of patient-derived cellular models is the idea of curing genetic diseases in vitro. Hence, patient donor cells (like fibroblasts) could be genetically corrected, reprogrammed into iPSCs and further differentiated into the desired progenitor cell. Another possibility is to generate isogenic iPSC lines by genetic modifications through target specific CRISPR/Cas9 technologies [70].

6. Conclusions

hPSCs have provided a revolutionary impact on modeling human PNS development, pathophysiology of different PNPs as well as on novel therapies for them. In the last few years, major efforts have been made to use hPSC to bypass the difficulties linked to working on the human embryo. The knowledge accumulated by embryologists working on various vertebrate model organisms has allowed the development of several strategies to manipulate cell fate choices from stem cells grown in vitro. These new in vitro cellular models have provided insights into the steps underlying determination and differentiation of diverse neural crest and placode cell lineages. Additionally, recent studies showed that it is now possible to differentiate patient-derived iPSCs into disease-susceptible neuronal phenotypes. The experiments described here proved the presence of disease-inherent phenotypes in iPSCs and the iPSC-derived affected neurons and even revealed new pathophysiological insights and promising candidate substance testing in some PNPs.

However, a lot of work is still needed to optimize and integrate the specific differentiation protocols in the disease-related iPSC models. More importantly, these need to be translated in vivo for all the PNPs, and a better appreciation of the precise signals and switches that dictate the survival, proliferation, and differentiation of neural crest cells into their distinct derivatives will facilitate their application in therapeutic and regenerative medicine. Nevertheless, patient iPSC-derived neurons provide a unique opportunity to gain insights into the pathophysiology of PNPs, as well as for drug screening, cell therapy, and personalized medicine.

As presented above, the in vitro derivation of PNS lineages from human PSCs did not achieve the same tremendous progress as of CNS lineages, despite the great medical needs also for peripheral neurons. However, the very recent progress in deriving human NCCs, placode cells, and enteric neurons marked a new stage in the human PNS development and diseases. However, understanding the programs of peripheral neurogenesis, especially in the later developmental stages, is a major challenge confronting development biologists in years to come.

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