Signals and Switches in Mammalian Neural Crest Cell Differentiation

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SUMMARY

Neural crest cells (NCCs) comprise a multipotent, migratory cell population that generates a diverse array of cell and tissue types during vertebrate development. These include cartilage and bone, tendons, and connective tissue, as well as neurons, glia, melanocytes, and endocrine and adipose cells; this remarkable lineage potential persists into adult life. Taken together with a limited capacity for self-renewal, neural crest cells bear the hallmarks of stem and progenitor cells and are considered to be synonymous with vertebrate evolution. The neural crest has provided a system for exploring the mechanisms that govern developmental processes such as morphogenetic induction, cell migration, and fate determination. Today, much of the focus on neural crest cells revolves around their stem cell-like characteristics and potential for use in regenerative medicine. A thorough understanding of the signals and switches that govern mammalian neural crest patterning is central to potential therapeutic application of these cells and better appreciation of the role that neural crest cells play in vertebrate evolution, development, and disease.

Outline

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1 INTRODUCTION

At the end of gastrulation, after generation of the three primary germ layers is complete, the ectoderm is subdivided into two distinct domains: the non-neural or surface ectoderm and the neural ectoderm. The surface ectoderm will eventually form placodes, skin, and dermis, whereas the neural ectoderm will ultimately give rise to the central nervous system. The neural ectoderm (also known as the neuroepithelium or neural plate) extends almost the entire length of the vertebrate axis, and during neurulation, the left and right halves elevate and fuse to form a neural tube. It is during this neurulation process that neural crest cells (NCCs) are formed within the dorsal-most part of the neuroepithelium at the junction with the surface ectoderm, a region termed the "neural plate border." Explants of neural plate cultured in vitro do not endogenously generate neural crest cells. Thus, neural crest cell induction has been viewed as a multistep process, requiring an inducer (i.e., the ectoderm or paraxial mesoderm) and a competent receiving tissue (i.e., the neural plate). Furthermore, these interactions between non-neural and neural tissues are contact-mediated, suggesting that inductive signals pass to the neuroepithelium to induce neural crest cell formation (Selleck and Bronner-Fraser 1995).

Initially, neural crest cells are integrated within the neuroepithelium and are morphologically indistinguishable from the other neuroepithelial cells. However, in response to contact-mediated inductive signals from the surface ectoderm and underlying mesoderm, neural crest cells are born and undergo an epithelial-to-mesenchymal transition, after which they delaminate from the neuroepithelium. Some neural crest cells may also be derived from the surface ectoderm. Neural crest cells then migrate extensively to several different locations in the embryo (Fig. 1). Although the bone morphogenetic protein (BMP), fibroblast growth factor (FGF), and Wnt signaling families have each been identified as key signaling regulators of neural crest cell formation in diverse species such as avians, fish, and amphibians, there is no conclusive evidence that supports an absolute role for these same factors in mammalian neural crest cell induction (Crane and Trainor 2006). These signaling pathways appear to be more important for specifying cell-type differentiation within the mammalian neural crest cell lineage. Therefore, the signals and switches governing mammalian neural crest cell formation remain to be identified.

The delamination of neural crest cells from the neural tube requires significant cytoarchitectural and cell-adhesive changes and is typically recognized by the activity of members of the *Snail* transcription factor gene family. *Snail1*, for example, demarcates neural crest cells in mouse embryos (Sefton et al. 1998). Snail1 and Snail2 can directly repress the cell adhesion molecule E-cadherin by binding to its promoter, which is thought to facilitate cell migration (Cano et al. 2000; Bolos et al. 2003). However, in contrast
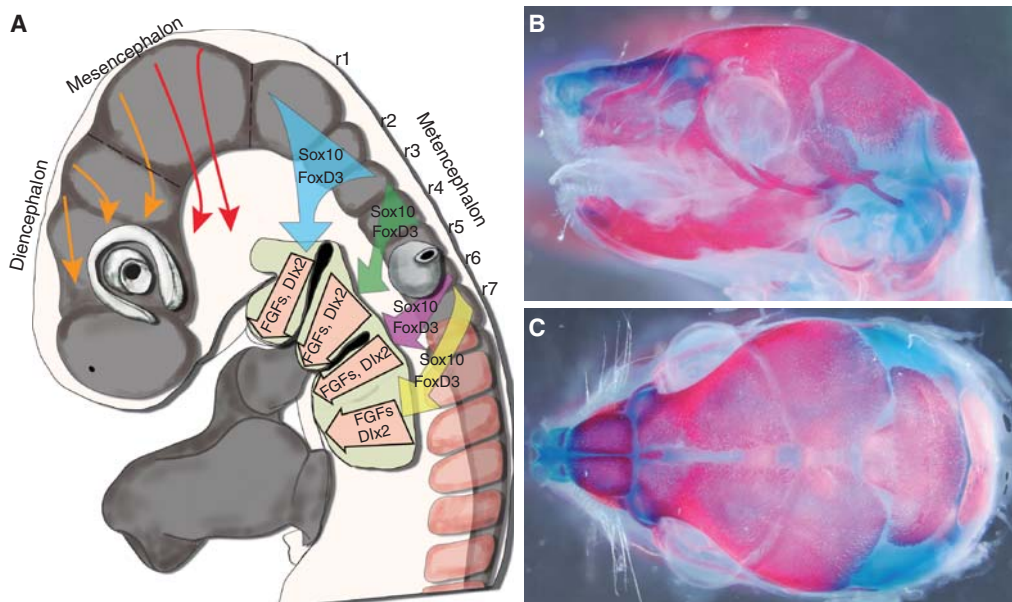
Figure 1. Cranial neural crest cell migration and differentiation. (*A*) Schematic representation of the pathways of mammalian cranial neural crest cell migration and the respective expression and interaction of *Sox10*, *FoxD3*, FGF, and *Dlx* signals and switches that govern neural crest cell differentiation. (*B*) Lateral, and (*C*) dorsal views of Alizarin Red- and Alcian Blue-stained bone and cartilage, respectively, in an embryonic day 18.5 (E18.5) mouse embryo.

to avians, fish, and amphibians, a requirement for the *Snail* genes in mammalian neural crest cell induction is conspicuously absent. Conditional loss-of-function analyses of *Snail1* and *Snail2* either individually or in combination do not inhibit neural crest cell induction and delamination in mice (Jiang et al. 1998; Murray and Gridley 2006). To date, only mutations in *Zfhx1b*, which is also known as *Smad-interacting protein 1* (*SIP1*), have been shown to affect neural crest cell formation and delamination in mammalian embryos (Van de Putte et al. 2003). *Zfhx1b* knockout mice do not develop post-otic vagal neural crest cells, and the delamination of cranial neural crest cells is perturbed. This is due to the persistent expression of E-cadherin throughout the epidermis and neural tube. Hence, appropriate regulation of cell adhesion is critical for formation, epithelial-to-mesenchymal transition (EMT), and subsequent delamination and migration of mammalian neural crest cells.

During normal mammalian embryogenesis, neural crest cell induction and delamination begin at the level of the midbrain and continue as a wave that extends progressively caudal toward the tail. Thus, neural crest cells are born along nearly the entire length of the neuraxis and, based on their axial level of origin, can be classified into distinct axial groups: cranial, cardiac, vagal, trunk, and sacral, each of which shows specific migration pathways and differentiation capacities. The cranial neural crest gives rise to the majority of the bone and cartilage of the head and face, as well as to nerve ganglia, smooth muscle, connective tissue, and pigment cells (Fig. 1A). The cardiac neural crest contributes to heart development by forming the aorticopulmonary septum and conotruncal cushions, whereas the vagal and sacral neural crest gives rise to enteric ganglia of the gut. Finally, the trunk neural crest give rise to neurons and glia that contribute to the peripheral nervous system, to secretory cells of the endocrine system, and to pigment cells of the skin. The remarkable capacity of neuroectoderm-derived neural crest cells to differentiate into both neuronal and mesenchymal derivatives has led to the neural crest being described as the fourth germ layer (Hall 1999). There are a couple of possible mechanisms that can account for the ability of neural crest cells to differentiate into such diverse cell types and tissues. Neural crest cells could comprise a heterogeneous mixture of progenitor cells, with each progenitor giving rise to a distinct cell type within the body. This would require some degree of neural crest cell specification before their emigration from neural tube and be largely dependent on intrinsic signals regulating their development. Alternatively, neural crest cells could be multipotent, with their differentiation into multiple distinct cell types being dependent on extrinsic signals emanating from the tissues with which they contact

during their migration. The question of extrinsic versus intrinsic specification of neural crest cells and the appropriateness of their classification as true stem cells or progenitor cells has been addressed extensively elsewhere (Trainor and Krumlauf 2001; Trainor 2003; Trainor et al. 2003; Crane and Trainor 2006). Suffice it to say that differing opinions are attributable to semantic arguments and the context-dependent nature of specific experiments. Neural crest cells show some of the key hallmarks of stem and progenitor cells, and their development is governed by a balance between intrinsic and extrinsic signals; however, neural crest cells are only generated transiently during embryogenesis. Neural crest cell differentiation has thus proven to be a significant model for understanding cell signaling and remains relevant because of the importance of neural crest cells in vertebrate development, evolution, and disease. Therefore, in this article, we discuss the signals and switches that regulate mammalian neural crest cell differentiation with a particular emphasis on skeletogenic and neuronal specification, the primary derivatives of the head and trunk, respectively.

2 CRANIAL NEURAL CREST CELLS

The craniofacial complex is anatomically the most sophisticated structure of the vertebrate body. Composed of a neurocranium (brain case) and viscerocranium (components derived from the pharyngeal arches), the cranioskeletal complex houses and protects the brain as well as the majority of the sense organs (Fig. 1B,C). It is important to note that the craniofacial skeleton across all craniates is of dual origin, being derived from both neural crest cells and mesodermal cells; however, the majority of the bone, cartilage, and connective tissue is derived from the neural crest (Jiang et al. 2002; Yoshida et al. 2008). In the mammalian neurocranium, for example, the meninges and frontal bones are derived from neural crest cells as is the suture mesenchyme, whereas the parietal bone is mesoderm-derived (Jiang et al. 2002). Two types of bone formation occur in the head. Intramembranous bone formation occurs by the direct differentiation of mesenchymal condensations into osteoblasts that lay down a mineralized matrix. In contrast, in endochondral bone formation, chondrocytes derived from mesenchymal condensations produce a cartilaginous framework that subsequently becomes hypertrophic and is replaced by osteoblasts and bone matrix.

2.1 *Hox*-Positive versus Non-*Hox*-Negative Activity in Cranial Neural Crest Cells

Cranial neural crest cells in mouse embryos migrate in stereotypical patterns that are highly conserved across

vertebrates (Kulesa et al. 2004) and use a complex array of intrinsic and extrinsic signaling cues (Trainor 2005). For craniofacial skeletogenesis, following colonization of the facial prominences and branchial arches, neural crest cells aggregate, condense, and differentiate from a common osteochondral progenitor toward more specific chondrogenic or osteogenic cell fates in response to signals from the surrounding epithelia, which include the neuroepithelium, endoderm, ectoderm, and mesoderm (Hall 1999; Trainor and Krumlauf 2001).

Neural crest cells have unique transcriptional identities correlating with their anterior–posterior axial origin within the neural plate. Most importantly, the cranial neural crest cells are subdivided into *Hox*-gene-negative- versus *Hox*-gene-positive-expressing cells. The first pharyngeal arch and more rostral populations of neural crest cells do not express *Hox* genes. *Hox* gene expression is associated with second and more caudal pharyngeal arch populations of neural crest cells. In mice, neural crest cells that colonize the first arch form skeletal tissue such as Meckel's cartilage, the maxillae, and the dentary bones, whereas neural crest cells of the second arch form Reichert's cartilage. The proximal region of Meckel's cartilage develops into two of the middle ear bones, the malleus, and the incus, whereas Reichert's cartilage forms the stapes (third bone of the middle ear), the styloid process of the temporal bone, the lesser horn, and part of the hyoid bone. Both endochondral and intramembranous ossification occurs during first pharyngeal arch differentiation, in contrast to primarily endochondral ossification in the second pharyngeal arch.

In mice, targeted inactivation of *Hoxa2* results in lethality at birth and homeotic transformations of second arch neural crest-derived elements into proximal first arch derivatives, including a partial duplication of Meckel's cartilage and ossification centers of the middle ear bones (Rijli et al. 1993). In these mutants, ectopic intramembranous ossification takes place in the second arch, resulting in duplicated jaw structures. Therefore, *Hoxa2* is essential for proper patterning of neural crest cell differentiation and, in fact, functions as an inhibitor of intramembranous and endochondral ossification (Rijli et al. 1993; Kanzler et al. 1998). Consistent with this, overexpression of *Hoxa2* in the first branchial arch of avian (Grammatopoulos et al. 2000) and frog (Pasqualetti et al. 2000) embryos suppresses jaw formation. Inroads have been made into the mechanisms by which *Hoxa2* specifically influences cranial neural crest cell differentiation (Kanzler et al. 1998). During normal development, *Hoxa2* is widely expressed in the second arch mesenchyme but is excluded from the chondrogenic condensations in the core of the arches. In the absence of *Hoxa2*, ectopic chondrogenesis coincides with an expansion of *Sox9* expression into the normal *Hoxa2* expression

domain, where it is not normally expressed. *Sox9* is a direct regulator of the cartilage-specific gene *Col2a1* (Bell et al. 1997; Ng et al. 1997), and, using a mouse transgenic approach, it has been shown that changes in *Sox9* expression are indeed responsible for the ectopic elements found in the second arch of *Hoxa2* mutants. This is also supported by misexpression of *Sox9* in the second arch, which produces a phenotype resembling that of the *Hoxa2* mutants. Therefore, *Hoxa2* acts very early in the chondrogenic pathway upstream of *Sox9* during neural crest cell differentiation. In addition, *Runx2* is up-regulated in the second branchial arch of *Hoxa2* mutant embryos. *Runx2* is required for bone formation, suggesting that the inhibition of *Runx2* activity might mediate *Hoxa2* suppression of intramembranous and endochondral bone formation.

Hox gene expression in cranial neural crest cells is considered to be inhibitory to skeletogenic differentiation and, in particular, incompatible with jaw formation (Trainor and Krumlauf 2001). Thus, the lack of expression of *Hox* genes in ectomesenchymal cells is imperative for proper patterning and skeletal development of the vertebrate face (Couly et al. 2002). Furthermore, the simultaneous inactivation of all *Hoxa* cluster genes leads to multiple jaw and first arch structures, partially replacing second, third, and fourth arch derivatives (Minoux et al. 2009). This pattern of *Hox* and non-*Hox* gene expression in cranial neural crest cells is conserved across vertebrate gnathostomes, and, interestingly, the vertebrate agnathan *Lampetra fluviatilis* shows expression of *Hox6* in the first branchial arch (Cohn 2002), which may help to explain the absence of jaw formation in lampreys despite the presence of neural crest cells. Thus, jaw evolution may have coincided with suppression of *Hox* gene expression in the first branchial arch (Trainor 2003; Trainor et al. 2003). However, the presence or absence of *Hox* gene expression in the first branchial arch is likely too simplistic a model for jaw skeletal potency because another species of lamprey, *Lethenteron japonicum*, appears to maintain a *Hox*-expression-free mandible (Takio et al. 2004).

2.2 Signals and Switches in Ectomesenchymal Fate

Ectomesenchymal differentiation of neural crest cells has been shown to vary temporally and spatially. Lineage tracing of neural crest cells in the zebrafish has revealed that there is a spatial correspondence between the time of emigration and ultimate fate of individual cells (Schilling and Kimmel 1994). Early-migrating neural crest cells typically become the skeletal and connective tissue of the face and pharyngeal region, whereas later-migrating neural crest cells take on primarily a neural fate. In mice and avian species, clonal neural crest cell differentiation assays and

lineage tracing studies have shown the existence of neural crest cell progenitor cells with both ectomesenchymal and neural potential from the same cranial region, strongly supporting the multipotentiality of cranial neural crest cells that become fate-restricted as a product of the environmental signals encountered through migration into their target sites (Calloni et al. 2009). Consistent with this, upon delamination and migration, neural crest cells express glial determination factors such as *Sox10* (also a migrating neural crest cell marker) (Britsch et al. 2001) and *FoxD3* (Dottori et al. 2001) in accordance with their general glial potential (Fig. 2). However, after reaching the branchial arches, *FoxD3* and *Sox10* expression diminishes, and, instead, neural crest cells express ectomesenchymal markers such as *Dlx2* and *Dlx5* (Depew et al. 1999, 2005; Blentic et al. 2008), which are conserved in both mouse and chick. Recent work in avians has also shown that the expression of *Dlx2* and *Dlx5* may be responsible for initiating the formation or condensation of mesenchyme into cartilage

and bone, a process common to all vertebrates (Gordon et al. 2010). Moreover, *Dlx5* acts as a mediator downstream from *Bmp2* signaling in the promotion of *Runx2* expression (Lee et al. 2003, 2005). Furthermore, single and compound *Dlx* mouse mutants have revealed roles for this gene family in collectively regulating regional identity along the proximodistal axis of the pharyngeal arches and specifying their specific cranioskeletal derivatives (Robledo et al. 2002; Depew et al. 2005). Yet even at this stage, ectodermal versus neuroglial fate has not been irrevocably determined. Neural crest cells isolated from the branchial arches of avian embryos and transplanted back into the neural tube of younger embryos are still capable of colonizing the cranial ganglia and contributing to neuroglial derivatives. However, this plasticity in neural crest cell fate persists for only ~72 h postcranial NCC migration; after this time point, pharyngeal arch NCCs transplanted back into younger embryos avoid the cranial ganglia territories (McKeown et al. 2003).
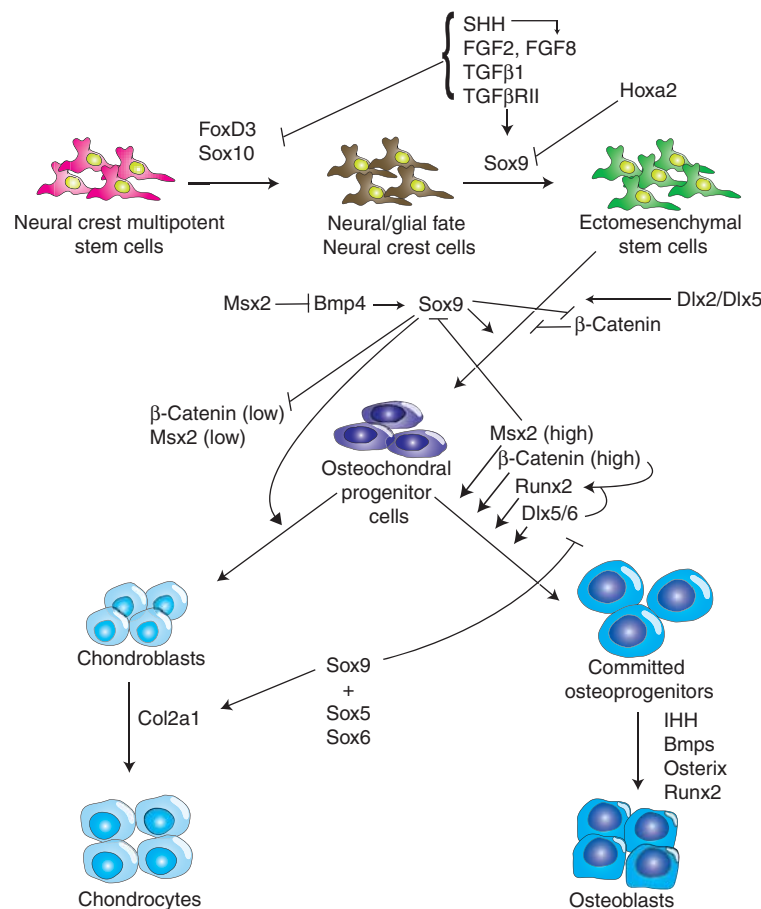
Figure 2. Signals and switches regulating cranial neural crest cell differentiation. Schematic representation of the signals and switches that govern neural crest cell segregation from a stem or progenitor cell into neuroglial or ectomesenchymal cells. This is then followed by differentiation of ectomesenchymal cells into an osteochondral progenitor cell and then bifurcation of potential into chondroblasts/chondrocytes or osteoprogenitors/osteoblasts.

2.2.1 Fibroblast Growth Factors

FGF signaling is known to play a role in promoting the fate of neural crest cells toward a skeletogenic type. In fact, FGF2 increases proliferation and skeletal fate (Li et al. 2010) of cranial neural crest cells in vitro (Sarkar et al. 2001; Abzhanov et al. 2003; Ido and Ito 2006) as well as in vivo (Abzhanov and Tabin 2004; Vitelli et al. 2006) in mouse and avian models. Interestingly, *Fgf8* is expressed in the pharyngeal ectoderm, and its downstream signaling is mediated through the *Fgfr1* receptor, which is expressed in neural crest cells entering the pharyngeal arches (Wada et al. 1997; Wilke et al. 1997; Walshe and Mason 2000; Blentic et al. 2008). *Fgf8* can induce *Sox9* expression and cartilage differentiation in vitro (John et al. 2011), and consistent with this, loss of FGF signaling leads to a failure of pharyngeal arch cartilage development (Sarkar et al. 2001; David et al. 2002; Walshe and Mason 2003; Ido and Ito 2006). Furthermore, *Fgfr1* hypomorphic mice display craniofacial skeletal and other body defects (Partanen et al. 1998), whereas *Fgfr1*-nulls are embryonic-lethal (Deng et al. 1994). Conditional knockouts of *Fgfr1* in NCCs display skeletal defects such as cleft palate and smaller skeletal elements (Trokovic et al. 2003), which may be a result of lower NCC proliferation or delay in differentiation. Electroporation of a dominant-negative *Fgfr1* into chick embryonic neural crest cells results in neural crest cells that are entering the branchial arches failing to down-regulate *Sox10*. Thus, the failure to down-regulate glial markers such as *Sox10* owing to the inability to respond to local FGF signaling cues supports a role for FGF signaling in the pharyngeal arch in promoting ectomesenchymal neural crest cell fate switching (Figs. 1A and 2). Consistent with this, FGFR2 is a well-known regulator of bone formation during embryonic development (Veistinen et al. 2009). Both gain- and loss-of-function studies in mice have shown that FGFR2 maintains a critical balance between the proliferation and differentiation of osteoprogenitor cells. Recently, de novo *FGFR2* mutations were identified in a sporadically occurring perinatal lethal skeletal disorder known as Bent Bone dysplasia, which is characterized by poor mineralization of the calvarium, craniosynostosis, dysmorphic facial features, osteopenia, and bent long bones (Merrill et al. 2012).

2.2.2 Transforming Growth Factors

Transforming growth factors (TGFs) have recently been shown to function as important switches mediating ectomesenchymal versus neural fate, both in vitro and in vivo (Fig. 2). Cranial and trunk neural crest cells exposed to TGFβ1 down-regulate *Sox10* expression and differentiation into ectomesenchymal fates in mouse stem cell lines (John et al. 2011). Similar to FGF signaling, *Tgfβ1* concomitantly induces *Sox9* expression along with the formation of osteoblasts and chondrocytes as well as smooth muscle cells (McGonnell and Graham 2002). Consistent with Tgfβ1 signaling acting as a crucial switch in inducing ectomesenchymal fates, murine conditional inactivation of *TgfβrII* in neural crest cells in vivo led to a reduction of *Sox9* expression in the pharyngeal arches and delayed ectomesenchymal fate acquisition (Mori-Akiyama et al. 2003). Furthermore, the skeletal elements that did develop were grossly malformed. In addition, the neural crest cells also failed to contribute to smooth muscle (Shah et al. 1996; Wurdak et al. 2005). Concomitant with the loss of *Sox9*, *Sox10* expression was maintained in *TgfβrII*-null neural crest cells. Interestingly, overexpression of *Sox10* in mouse neural crest stem cells (NCSCs) serves to maintain neurogenic potential at the expense of ectomesenchymal differentiation. Thus, *Sox10* can counter the effects of *Tgfβ1* (Kim et al. 2003). *Sox10*, however, is also important for smooth muscle differentiation, because inhibition of *Sox10* leads to a failure of neural crest cell differentiation into smooth muscle in vivo and in vitro (Britsch et al. 2001; Dutton et al. 2001; Paratore et al. 2001, 2002). Collectively, this reveals TGFβ signaling as a fate switch selector for *SoxE* family members that govern divergent cranial neural crest cell differentiation fates (Fig. 2).

2.2.3 Hedgehog Signaling

Sonic Hedgehog (Shh), a morphogen with broad roles in organogenesis, has also been shown to play an important role in neural crest cell fate specification (Fig. 2). In vitro clonal culture of avian cranial neural crest cells in the presence of recombinant Shh not only induced ectomesenchymal fates but also was able to produce nodules of chondrogenic differentiation in cultures of both cranial and trunk neural crest cells (Dupin et al. 2010). The most common cell type obtained in this study (at 18.5%) was a multipotent cell with ectomesenchymal and neural fate, known as the GNMFC (glial, neuronal, melanoblastic, myofibroblastic, chondrocytic). Because of its wide differentiation capacity, the GNFMC progenitor is thought to comprise the apex of a hierarchy of neural crest cell progenitors that differentiate progressively. Sustained Shh treatment of neural crest progenitors in culture biases cell fates toward skeletal differentiation via the formation of chondrocytes and osteoblasts (Dupin et al. 2010). Consistent with this, *Gli3* mutant mice show ectopic ossification in the interfrontal suture together with craniosynostosis, which phenocopies the severe craniofacial malformations characteristic of Grieg cephalopolysyndactyly syndrome (Veistinen et al. 2012). Furthermore, the conditional dis-

ruption of HH signaling by removing the Smoothened receptor in neural crest cells results in decreased proliferation and increased apoptosis, revealing the importance of Hedgehog signaling in mouse cranial NCC survival (Jeong et al. 2004).

The foregut endoderm, facial ectoderm, and neuroepithelium are all sources of Shh signaling. Endodermal Shh signaling has been shown to influence the differentiation of ectomesenchyme toward a chondrogenic fate in the pharyngeal skeleton through both mouse and avian extirpation experiments (Epperlein 1974; Hall 1980; Couly et al. 2002; Aoto et al. 2009) as well as in mouse, chick, and zebrafish genetic studies (Piotrowski and Nusslein-Volhard 2000; Brito et al. 2008). Furthermore, extirpation of specific axial domains of endoderm during the neurula stage of avian embryogenesis disrupts specific cranial structures (Couly et al. 2002). Rotations and heterotopic transplantations of axial domains of endoderm affect the orientation of skeletogenic elements and induce ectopic skeletogenic elements, respectively. *Shh*-null mutant mice have shown the importance of this signal from the endoderm through its role in actively maintaining the expression of key differentiation genes such as *Fgf8* and *Sox9* in the first pharyngeal arch (Yamagishi et al. 2006).

The facial ectoderm also expresses *Shh*, particularly in a region termed the frontonasal ectodermal zone (FEZ) (Hu et al. 2003), a site where *Shh* expression abuts an *Fgf8* domain and induces proliferation and outgrowth of the underlying NCC-derived mesenchyme as well as providing dorsoventral polarity. Ectopic grafting of the FEZ leads to formation of an ectopic upper beak (Hu and Marcucio 2009) in the upper jaw and ectopic lower jaws when transplanted to the developing mandible (Hu et al. 2003). These same patterning signals and structures are present in mice and humans, whose subtle morphological differences underlie changes in interactions between other tissues, such as the neural epithelium, neural crest mesenchyme, ectoderm, and endoderm, forming complex species-specific feedback signaling and phenotypes (Schneider and Helms 2003; Marcucio et al. 2005, 2011; Hu and Marcucio 2009, 2012). Miscommunication between these midline signaling centers leads to midline facial defects such as holoprosencephaly and hypertelorism as seen in humans and many other vertebrate species. In the frontonasal region, the anterior neural fold ectoderm has also been found to signal to the underlying ectomesenchyme to pattern the ectethmoid (dorsal) portion of the nasal capsule (Gitton et al. 2011) in avians. In contrast, the underlying mesethmoid cartilage of the avian nasal capsule is induced by endodermal Hedgehog signaling from the foregut (Benouaiche et al. 2008).

Indian hedgehog (Ihh) activity had previously been considered inhibitory to osteogenesis (Abzhanov et al.

2007); however, recent analyses show a strong pro-osteogenic role for IHH in calvarial development, because its loss leads to a reduction of osteogenic markers such as *Bmp2/4*, as well as a reduction in bone size and delay in matrix mineralization work (Lenton et al. 2011). Thus, similar to FGF and TGFβ, HH signaling also plays an important role in determining neural crest cell fate, particularly with respect to ectomesenchymal derivatives (Fig. 2).

2.3 Skeletal Connective Tissue Fate Determination

2.3.1 Wnt, Sox9, and Runx2 Signaling: Regulators of Skeletogenic Fate

As described above, *Sox9* is a transcription factor that directly targets the activity of collagen type II (*Col2a1*) in chondrocytes (Bell et al. 1997) and plays a critical role in skeletogenesis, particularly endochondral bone formation (Mori-Akiyama et al. 2003). Misexpression of *Sox9* in mouse pharyngeal arches results in ectopic cartilage formation (Kanzler et al. 1998; Healy et al. 1999), such as in the case of *Hoxa2*$^{-/-}$ mice. *Sox9* activity is thus sufficient for and indicative of cell fate commitment toward the chondrogenic pathway. Consistent with this, inhibiting *Sox9* expression in neural crest cells before mesenchymal condensation alters *Col2a1*, *Runx2*, and *Osterix* activity, which prevents cartilage differentiation and thus endochondral ossification (Akiyama et al. 2002; Mori-Akiyama et al. 2003). This is consistent with *Sox9* mutations in humans, which cause campomelic dysplasia, a skeletal syndrome characterized by skeletal malformation of the endochondral bones (Wagner et al. 1994). Thus, in mammalian embryos, *Sox9* plays an important role in cranial neural crest cell fate determination. However, *Sox9* alone is insufficient to induce ectomesenchymal fates. *Sox9* is coexpressed with *Runx2* in osteochondral progenitor cells (Akiyama et al. 2005), and *Runx2* is considered a master regulator of the intramembranous mode of ossification (de Crombrugghe et al. 2001; Karsenty 2008). However, complete commitment toward an osteoblastic fate requires *Osterix* expression downstream from *Runx2* (Nakashima et al. 2002).

Wnt signaling has emerged as a key regulatory pathway crucial to the fate choice between a chondrocytic or osteoblastic fate in mammalian studies (Hartmann 2006). The expression of Wnt signaling in the perichondria and calvaria where osteoblastogenesis is activated highlights a conserved role for this pathway in both endochondral and intramembranous ossification. Canonical Wnt signaling encompasses ligand binding to the receptor Frizzled and coreceptors Lrp5 and Lrp6, which inhibits Gsk-3β phosphorylation of β-catenin and leads to its stabilization. Stabilized β-catenin translocates to the nucleus, where it binds to the LEF/TCF transcription factors and leads to targeted

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gene expression (for review, see MacDonald et al. 2009). Conditional deletion of β-*catenin* in neural crest cells via *Wnt1-Cre* recombinase in mice leads to a loss of cranial bones (Brault et al. 2001). Conversely, enhanced activity of *Lrp5* results in an increase in bone mass (Boyden et al. 2002), along with dental malformations and colon cancer (Logan and Nusse 2004). Although canonical Wnt signaling has been shown to promote bone formation, it simultaneously inhibits chondrogenesis (Boyden et al. 2002; Guo et al. 2004). Conditional inactivation of β-*catenin* in both dermis and chondrocytes (using *Dermo1*-Cre and *Col2a1*-Cre, respectively) suppresses osteoblast differentiation but concomitantly increases the generation of chondrocytes. In these conditional loss-of-β-*catenin* experiments, early osteoblastic markers such as *Runx2*, *Collagen1a1*, and alkaline phosphatase continued to be expressed in perichondria and periostia (Day et al. 2005; Glass et al. 2005; Hill et al. 2005); however, *Osterix*, which is involved in the definitive commitment to an osteoblastic lineage, failed to be expressed. Regardless of neural crest or mesodermal origin, mesenchyme with a conditional deletion of β-*catenin* generated cartilage at the expense of osteoblasts. Thus, canonical Wnt signaling is a key promoter of osteogenesis (Fig. 2) but, at the same time, is an important antagonist of the chondrogenic pathway (Kolpakova and Olsen 2005; Hartmann 2006). Interestingly, the conditional deletion of Wnt/β-catenin in the dermis gives rise to skeletogenic differentiation in mice (Day et al. 2005; Hill et al. 2005; Tran et al. 2010) and may thus provide some insight into the wide array of dermal armor and skeletal elements found across various extant and fossil vertebrates (Vickaryous and Sire 2009; Fraser et al. 2010), which may potentially be of neural crest origin.

How canonical Wnt signaling regulates both endochondral and intramembranous ossification has begun to be deciphered and appears to depend on a balance between *Sox9* and *Runx2*. As mesenchyme begins to condense, *Sox9* and *Runx2* are coexpressed in a common osteochondral progenitor cell (Day et al. 2005). To proceed along the endochondral path, the ratio of *Sox9* must initially be higher relative to *Runx2*. However, subsequently during hypertrophy, Wnt signaling and *Runx2* activity become elevated at the expense of *Sox9*, which collectively promote osteoblastogenesis. Interestingly, in vitro evidence suggests that Sox9 binds *Runx2* directly, suppressing its activity (Zhou et al. 2006). During intramembranous ossification, osteochondral mesenchymal condensation begins with high levels of Wnt activity, which promotes elevated *Runx2* to give rise to the intramembranous skeleton of the skull. Interestingly, SOX9 can bind to the TCF/LEF-binding region of β-catenin and influence its degradation, which blocks downstream canonical signaling (Akiyama et al. 2002).

This shows the reciprocal antagonism that exists between the chondrogenic and osteogenic pathways and the importance of the precise regulation of Wnt/β-catenin levels that is required in order to control cell fates. Canonical Wnt signaling, like *Runx2* and *Osterix*, is therefore a key regulator of osteogenesis, while also serving as an inhibitor of chondrogenesis.

Chondrocyte differentiation through *Sox9* is therefore achieved by the inhibition of osteoblast-promoting genes such as β-*catenin* (Day et al. 2005). Indeed, expression of a stable form of β-*catenin* in mice inhibits chondrogenesis, mimicking the loss of *Sox9*. Consistent with this, the conditional deletion of β-*catenin* in chondrocytes mimics overexpression of *Sox9* (Akiyama et al. 2004). Thus, an antagonistic relationship exists between *Sox9* and β-*catenin* in the regulation of cartilage and bone development (Mori-Akiyama et al. 2003). Similar to *Sox9*, β-catenin signaling plays multiple roles during NCC differentiation by influencing chondrogenesis as well as sensory neurogenesis. Collectively, this illustrates the reiteration of the same signaling pathways during multiple stages of NCC development, and this is a common theme during embryogenesis.

2.3.2 Muscle Segment Homeobox Genes

Muscle segment homeobox transcription factors *Msx1* and *Msx2* also play a role in fate determination for controlling cranial neural crest cells, specifically during craniofacial development (Satokata and Maas 1994; Bei and Maas 1998; Satokata et al. 2000; Ishii et al. 2005). *Msx1* and *Msx2* are strongly expressed in migrating neural crest cells, whose expression continues during their colonization of the facial prominences and branchial arches (Hill et al. 1989; MacKenzie et al. 1991; Catron et al. 1996). Mouse *Msx1* loss of function results in multiple craniofacial abnormalities, including frontal bone development defects (Satokata and Maas 1994). Similarly, *Msx2*-null mutant mice show defective skull ossification and persistent calvarial foramen, suggesting that *Msx2* plays a critical role in regulating calvarial morphogenesis (Ishii et al. 2003). *Msx2* loss of function therefore causes pleiotropic defects in bone growth (Satokata et al. 2000) that result from osteoprogenitor anomalies.

Interestingly, *Msx2* and *Sox9* are coexpressed in a subpopulation of cranial neural crest cells within the branchial arches (Semba et al. 2000). Because *Sox9* is a transactivator of chondrogenesis and *Msx* genes can act as transcriptional repressors, it was hypothesized that *Sox9* expression was indicative of cranial neural crest-derived chondrogenic cell lineages and that *Msx2* represses chondrogenic differentiation until cranial neural crest cell migration was completed. In mice, *Msx2* diminishes after the completion

of migration, at which point *Sox9*-mediated chondrogenic differentiation proceeds. Consistent with this, inhibition of *Msx2* in migrating neural crest cells accelerates the rate and extent of chondrogenesis, as indicated by increased expression of *Col2a1*, aggrecan, and Alcian Blue staining. This suggests that *Msx2* is a repressor of chondrogenic differentiation and that an important early event in craniofacial morphogenesis is transient coexpression of *Sox9* and *Msx2* during cranial neural crest cell migration, which is followed by restricted expression of *Sox9* within cranial neural crest cell-derived chondroprogenitor cells (Fig. 2).

Msx genes are critical for osteogenic lineage differentiation, and they control osteogenesis by regulating *Runx2* (Satokata et al. 2000). Recent work has shown a synergistic interaction between *Msx1* and *Dlx5* in the osteogenesis of the mouse frontal bone (Chung et al. 2010). In agreement with this, mutations in *MSX1* in humans have been associated with tooth agenesis and orofacial clefting conditions on their own or as part of syndromes such as Witkop syndrome (Jumlongras et al. 2001). *MSX2* mutations underlie craniosynostosis type 2 (Jabs et al. 1993), parietal foramina (Wilkie et al. 2000), and cleidocranial dysplasia (Garcia-Minaur et al. 2003) syndromes. Significantly, the entire ossified calvaria is missing in $Msx1^{-/-};Msx2^{-/-}$ double mutants, indicating that *Msx1* and *Msx2* function together to regulate osteogenesis during calvarial development (Ishii et al. 2005; Han et al. 2007), where neural crest cell migration was found to be normal with delayed migration; mispatterning along the neural tube and mixing of crest cells between streams were also noted. Thus, *Msx* genes control multiple steps in neural crest cell patterning and differentiation into skeletogenic tissues.

2.4 Ectomesenchymal Fate—Differences between Cranial and Trunk Neural Crest

An important feature that distinguishes cranial neural crest from trunk neural crest is their ability to differentiate into mesenchymal tissues. Mammalian cranial NCCs contribute to cartilage and bone (Fig. 1B,C), the articulating disk of the temporomandibular joint, and odontoblasts and mesenchyme that support tooth development (Chai et al. 2000). In contrast, mammalian trunk NCCs do not appear to generate bone and cartilage endogenously. However, this property may have been lost during evolution, because avian trunk neural crest progenitors cultured in media that promotes bone differentiation will, in fact, form both bone and cartilage cells, albeit at low frequency (McGonnell and Graham 2002). Furthermore, if these same trunk NCCs are transplanted into the developing avian head, experiments not yet amenable within mammalian embryos, they will also contribute appropriately to cranioskeletal components

such as the scleral cartilages of the orbit and Meckel's cartilage of the lower jaw.

This implies that NCCs from all axial levels can perhaps generate the full repertoire of neural crest derivatives under appropriate conditions. Consistent with this, quail cranial neural plate transplanted in place of a chick trunk neural plate generates NCCs that contribute to all typical trunk neural crest cell derivatives, and in addition produce ectopic cartilage (Le Douarin and Teillet 1974; Le Lievre and Le Douarin 1975). Similarly, mouse neural crest cells transplanted *in ovo* into avian hosts maintain their species-specific molecular identity and character (Trainor and Krumlauf 2000; Fontaine-Perus and Cheraud 2005; Tokita and Schneider 2009). Donor murine NCCs migrate normally within their avian hosts; however, their long-term differentiation has not yet been explored with respect to bone and cartilage. Nonetheless, it is known that mouse NCC transplants can support tooth development in avian hosts (Mitsiadis et al. 2003). Interestingly, the turtle plastron, whose nine bones have been homologized to the exoskeletal components of the clavicles, interclavicular bone, and gastralia, has recently been shown to be derived from late-migrating trunk NCCs (Cebra-Thomas et al. 2007; Gilbert et al. 2007). The skeletogenic potential of trunk neural crest cells is of considerable evolutionary significance, because early vertebrates, many of which have been identified in the Burgess Shale in Canada, together with fossilized fish had extensive postcranial exoskeletal coverings, and this external armor is likely to have been trunk neural crest–derived.

3 TRUNK NEURAL CREST CELLS

Cranial NCCs differentiate primarily into bone, cartilage, and connective tissues, but they also generate neurons and glia. In contrast, trunk NCCs give rise primarily to sensory neurons and glia that are central to formation of the peripheral nervous system (Fig. 3A). The primary function of the peripheral nervous system (PNS) is to sense and react to stimuli from the viscera, smooth muscles, skin, and the exocrine glands of the body. Accordingly, the PNS is loosely categorized into two anatomical and functional classifications, the sensory and autonomic nervous systems.

The sensory nervous system (SNS) comprises afferent sensory neurons that sense stimuli and convey information to the central nervous system (CNS; brain and spinal cord) and in the trunk consists of a metameric series of ganglia called dorsal root ganglia (DRG). The DRGs are composed of a variety of sensory neuron cell bodies whose axons enable the sensation of touch (low-threshold mechanoreceptor neurons), temperature (thermal sensory neurons), pain (nociceptive neurons), movement, and spatial

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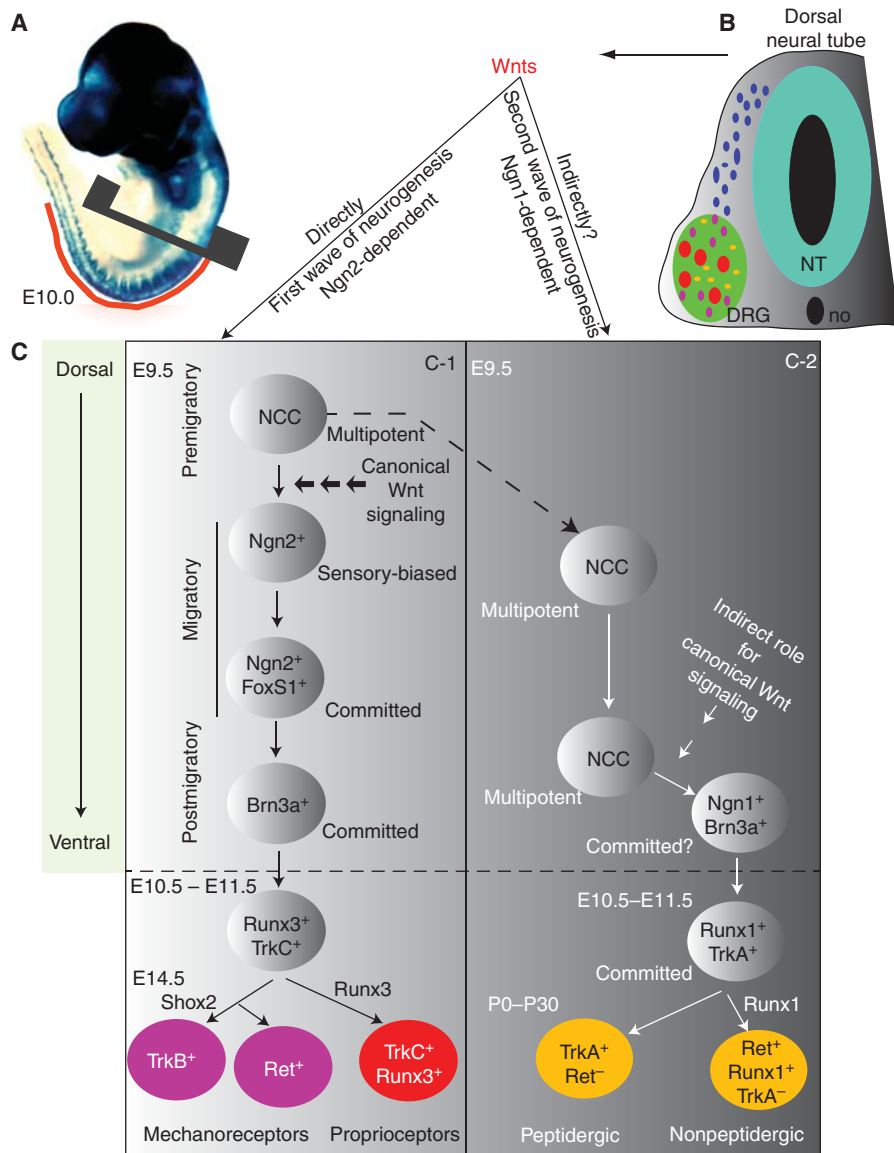
Figure 3. Neural crest differentiation into trunk sensory neurons. (*A*) *Wnt1Cre;R26R^{lacZ}* labels all of the migrating neural crest cells (NCCs) at E10.0. (*B*) A cross section through the trunk reveals the ventromedial path of NCC migration that populates the dorsal root ganglia (DRG). Differentiation of NCC during and postmigration leads to formation of three morphologically distinct types of sensory neurons: small-diameter nocipeptive (peptidergic and nonpeptidergic) (shown in yellow) and large-diameter mechanoreceptors (red) and proprioceptors shown in red and (pink), respectively. (*C*) Trunk sensory neurogenesis occurs in two waves, both of which are directly or indirectly dependent on canonical Wnt signals emanating from the dorsal neural tube. (*C-1*) During the first wave of sensory neurogenesis, Wnt signals act on migratory NC cells to induce the expression of *Neurogenin2* (*Ngn2*). *Ngn2* expression biases the neural crest fate toward sensory neurogenesis. Postmigratory cells of the first wave express *Brn3a* and differentiate into large-diameter proprioceptor (*TrkC^+*, shown in red) and mechanoreceptor (*TrkB^+* or *Ret^+*, shown in purple) neurons. *Runx3* plays an important role in formation of *TrkC^+* proprioceptors, whereas *Shox2* has an important role in regulating expression of *TrkB* in mechanoreceptors. (*C-2*) The second wave of neurogenesis occurs subsequently in embryogenesis and gives rise to nociceptor neurons. *Neurogenin1* and *Brn3a* expression in the postmigratory second-wave cells marks them for sensory differentiation. *Brn3a* directly activates *TrkA* in these cells, which then, based on the differential expression of *Runx1*, differentiate into nonpeptidergic (*Runx1^+;Ret^+*) and peptidergic (*TrkA^+*) nociceptor neurons (peptidergic cells are shown in yellow). E, Embryonic day; P, postnatal day; NT, neural tube; no, notochord; DRG, dorsal root ganglion; NCC, neural crest cell. (Figure adapted and modified based on Marmigere and Ernfors 2007.)

Table 1. DRG neurons are identified with the expression of specific neurotrophic receptors

Sense	Sensory neuronal subtype	Diameter	Receptor(s) expressed	Neurotrophic requirement	References
Temperature	Thermal	Small	*TrkA Ret*	NGF BDNF	Snider and Wright 1996; Chen et al. 2006;
Pain	Nociceptive	Small	*TrkA Ret*	NGF BDNF	Luo et al. 2007
Touch	Low-threshold mechanoreceptor	Large	*TrkB*	BDNF	Gonzalez-Martinez et al. 2004; Shimizu et al. 2007; Perez-Pinera et al. 2008
Movement and spatial position	Proprioceptive	Large	*TrkC*	NT-3	Snider and Wright 1996; Hasegawa and Wang 2008

position (proprioceptive neurons), all of which are derived from trunk NCCs. 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 (Table 1).

The autonomic nervous system (ANS) largely comprises efferent motor neurons that carry information from the CNS to the various organs of the body and is loosely defined as the motor component of PNS. It provides a way for the CNS to send commands to the rest of the body and provides involuntary control of the visceral organs. The ANS 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. Axons from CNS neurons (brainstem and spinal cord) project and synapse with the ganglionic cell bodies located paravertebrally (for SyNS) or inside the target organs (for PSNS). In the gut, the noradrenergic and cholinergic ganglia form the enteric nervous system (ENS), which exclusively innervates the gastrointestinal (GI) tract. Even though usually grouped as a subdivision of the ANS, the ENS comprises local neural circuits consisting of sensory, inter-, and motor neurons, capable of autonomous control without CNS input. Consistent with this, it is also called the second brain of the body (Gershon 1998). Neural crest cells contribute to all of the neurons and glia of the PNS, except for some cranial sensory neurons. The signals that regulate the individual contributions of NCCs to each of the different components and sublineages of the PNS are described below.

3.1 Sensory Neuron Differentiation

3.1.1 Neurogenins Regulate Trunk Sensory Neuron Differentiation

Trunk sensory neurogenesis occurs in waves (Fig. 3). The first wave is characterized by migrating NCCs forming large-diameter touch and movement neurons, whereas the second wave occurs after the DRGs coalesce, producing small pain as well as large touch and movement neurons. A third wave

of sensory neurogenesis has also been described that generates primarily nociceptive ($TrkA^+$) neurons from neural crest-derived boundary cap cells (Maro et al. 2004). The first two waves of NCC differentiation are regulated by *Neurogenin1* (*Ngn1*) (Ma et al. 1996) and *Neurogenin2* (*Ngn2*) (Fode et al. 1998), which are vertebrate homologs of the *Drosophila* bHLH (helix−loop−helix) proneural gene *atonal*. Analyses of individual *Ngn1* and *Ngn2* mouse mutant embryos suggest that *Ngn2*, which is expressed in early-migratory neural crest, regulates the first wave of sensory neurogenesis, whereas *Ngn1*, which is expressed in the coalesced DRG, regulates the second wave (Fig. 3B) (Ma et al. 1999; Marmigere and Ernfors 2007). *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 (Fig. 3B,C). 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 (Zirlinger et al. 2002).

Ngn1 and *Ngn2* control expression of other bHLH transcription factors such as *NeuroD* that function as neural differentiation factors. *Ngn1;Ngn2* double-mutant embryos show a complete lack of *NeuroD* expression and a concomitant absence of DRGs. Because both *Ngn1* and *Ngn2* play a role in the formation of $TrkA^+$, $TrkB^+$, and $TrkC^+$ neurons, there is no doubt that neurogenins promote sensory lineage determination of NCCs (Fig. 3C). However, it is not yet clear whether individual neurogenins specify the subtypes of sensory neurons. The neurogenins expressed in sensory progenitors turn on expression of *Delta1* cell-autonomously. Consequently, the $Ngn^+;Delta1^+$ cell acquires a neuronal fate, and through lateral inhibition, Delta−Notch signaling prohibits neurogenesis in the surrounding cells. These surrounding cells subsequently form the glia of the sensory ganglion (Wakamatsu et al. 2000). Consistent with this, mutations in the Notch signaling antagonist *Numb* cause a defect in trunk sensory neuron differentiation despite normal migration of neural cells into the DRG anlage (Zilian et al. 2001).

3.1.2 Wnt Signaling Regulates Neurogenin Expression and Activation in Sensory Neuron Progenitors

Avian in vitro studies have revealed that although neural tube cultures consisting of premigratory NCCs can generate sensory neurons, cultures consisting exclusively of migratory neural crest cells lose this potential (Le Douarin and Kalcheim 1999; Bronner-Fraser 2004). Consistent with this, two reports have defined a necessary and sufficient role for dorsal neural tube-derived canonical Wnt signaling in differentiation of trunk sensory neurons (Fig. 3B) (Hari et al. 2002; Lee et al. 2004). Genetic ablation of β-*catenin* specifically in premigratory neural crest cells results in the complete absence of DRGs and melanocytes, and inhibition of β-catenin function leads to loss of both the early and late waves of sensory neurogenesis, observed by the absence of *Ngn2* and *Ngn1* expression (Hari et al. 2002). However, the lack of active Wnt signaling in postmigratory NCCs in DRG and sympathetic ganglia (SG) at E12.5 and subsequently, suggests that Wnt signaling is not directly involved in the induction of the second wave of DRG neurogenesis (Kleber et al. 2005). Whether Wnt signaling has an indirect role in regulation of late sensory neurogenesis in the DRG, via an intermediary factor, remains to be determined.

Interestingly, constitutive activation of canonical Wnt signaling in neural crest progenitor cells in the dorsal neural tube in vivo promotes sensory neuron differentiation at the expense of other NCC progeny. Sustained β-catenin activation leads to ectopic sensory lineage differentiation, marked by presence of $Ngn2^+$ cells, even at sites of sympathetic neurons and glia formation, as well as the anterior region of the embryo, which is usually devoid of NCC-derived sensory neurons. In vitro clonal analysis proved that this effect of canonical Wnt signaling in sensory cell differentiation is not caused by Wnt-mediated "sensory progenitor" cell proliferation or expansion but, rather, represents true determination of cell fate specification (Lee et al. 2004). Therefore, the differentiation or lack of sensory neurons following constitutive activation or inhibition of canonical Wnt signaling, respectively, is mediated via *Neurogenins*. Wnt signaling also has a well-documented role in melanocyte differentiation; however, whether a neural crest cell differentiates into sensory neuron or melanocyte is dependent on the stage of NCC development at which it is exposed to active Wnt signaling.

3.1.3 Transcription Factors Determine the Sensory Neuronal Subtype

The expression of various receptors and ion channels specific to each subtype of sensory neuron requires a sequential expression of cohort of transcription factors. The POU-domain transcription factor *Brn3a* (aka *Pou4f1*) is expressed in terminally differentiating sensory neurons of DRGs and is important for the correct development and/or survival of a subpopulation of proprioceptive, nociceptive, and mechanoceptive sensory neurons (McEvilly et al. 1996). *Brn3a* binds directly upstream of NGF receptor *TrkA* (Ma et al. 2003), which is expressed in all nociceptors during embryonic development (Fig. 3C). The expression of *TrkA* in the nociceptive sensory neurons of the DRG also requires the transcription factor Klf7 (Lei et al. 2005). Postnatally, however, some nociceptors down-regulate *TrkA* expression and concomitantly activate the GDNF receptor *Ret*, thus segregating the nociceptors into inflammation-sensing $TrkA^+$ (aka peptidergic neurons) and neuropathic pain-sensing Ret^+ neurons (nonpeptidergic neurons) (Molliver and Snider 1997; Snider and McMahon 1998; Huang and Reichardt 2001). These two neuron types express a distinct set of ion channels and receptors (Bradbury et al. 1998; Dong et al. 2001; Potrebic et al. 2003; Zylka et al. 2003), and the *Runt*-related transcription factor *Runx1* is important for this segregation (Chen et al. 2006; Kramer et al. 2006; Marmigere and Ernfors 2007; Inoue et al. 2008; Abdel Samad et al. 2010). The specification of subsets of *TrkB*-expressing mechanoreceptor neurons requires *Shox2* (*Short statured homeobox 2*) (Scott et al. 2011), whereas the maintenance of $TrkC^+$ proprioceptive neurons, which are required for movement, depends on *Runx3* (Levanon et al. 2002). *Runx3* loss of function leads to a lack of motor coordination (Inoue et al. 2002; Levanon et al. 2002).

3.2 Autonomic Neuron Differentiation

3.2.1 Sympathetic Neuron Differentiation

The sympathetic nervous system (SyNS) is made up of a ventrally located metameric series of ganglia aligned parallel to the vertebrae on both sides of the embryo. Although the signals that regulate NCC differentiation into sympathetic neurons are site-specific, the trunk neural tube and the path of trunk neural crest migration confer competency to the trunk NCCs for sympathetic differentiation. The long-standing perception of sympathetic differentiation suggests the presence of a common sympatho-adrenal (SA) progenitor cell within the trunk neural crest that, based on the local environmental cues, can differentiate into three related cell types: sympathetic neurons, neuroendocrine chromaffin cells, and small intensely fluorescent (SIF) cells, all of which show catecholaminergic (ability to produce adrenaline and noradrenaline) traits (Unsicker 1973; Unsicker et al. 1978a; Vogel and Weston 1990). Sympathetic progenitor NCCs migrate along the ventro-medial

path and reach the dorsal aorta, initially forming a continuous sympathetic chain that subsequently segregates into discrete ganglia. 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 neuroendocrine cells (chromaffin cells) of the adrenal gland (Fig. 4A,B) (for review, see Le Douarin and Kalcheim 1999). The ventro-medial path taken by sympathetic precursor neural crest cells is shared with the trunk DRG precursor crest, and the sympathetic precursors pass through the DRG to reach the sites of primary sympathetic ganglia. Mouse knockout studies have shown that the growth factor *Neuregulin-1* and its receptor tyrosine kinase heterodimer *ErbB2/ErbB3* are important positive regulators of neural
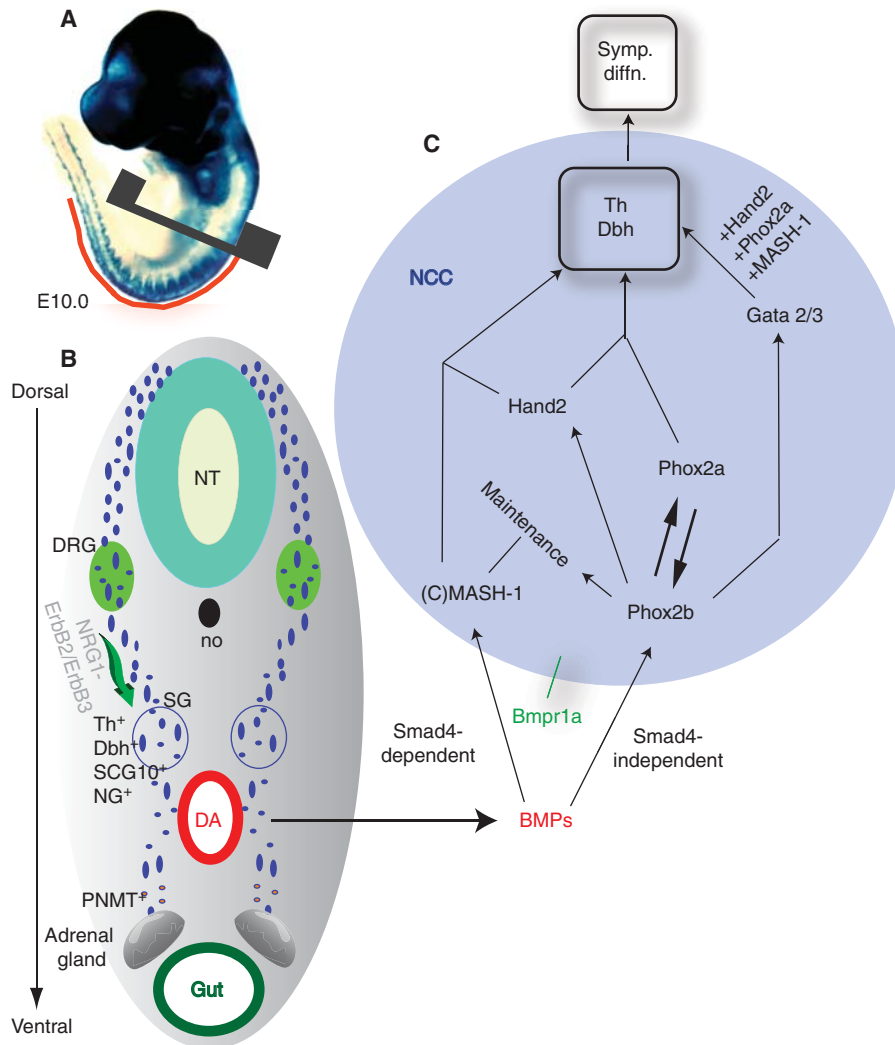
Figure 4. Neural crest differentiation into sympathetic neurons. (A) $Wnt1Cr^e;R26R^{lacZ}$ labels all the migrating neural crest cells (NCCs) at E10.0. (B) A cross section through the trunk reveals the ventro-medial path of NCC migration. NCCs migrate beyond the dorsal root ganglia (DRG) to coalesce at the dorso-lateral side of the dorsal aorta (DA) to form sympathetic ganglia (SG). Neuregulin1–ErbB2/ErbB3 signaling has a role in regulating the ventral migration of NCCs beyond DRG. A subpopulation of cells from the SG migrates further ventrally to the adrenal gland to differentiate into the neuro-endocrine chromaffin cells expressing PNMT (phenylethanolamine N-methyltransferase). (C) BMP signaling from the dorsal aorta plays a very important role in differentiation of the crest cells coalescing at the sites of SG formation. BMP ligands through Smad4-dependent and -independent mechanisms activate expression of *Mash1* and *Phox2b*, respectively. Phox2b regulates the expression of *Hand2*, *Phox2a*, and *Gata* factors and maintains *Mash1* expression within the differentiating crest cells. The combinatorial activity of all of these factors leads to activation of two enzymes—Th (tyrosine hydroxylase) and Dbh (dopamine β-hydroxylase)—crucial for noradrenaline synthesis. Symp. diffn., sympathetic differentiation; NT, neural tube; no, notochord.

crest cell migration beyond the developing DRG (Britsch et al. 1998).

3.2.2 BMPs Regulate Induction of Sympathetic Neuron Differentiation

Avian studies defined the requirement for embryonic tissues like the notochord, neural tube, and somitic mesoderm for priming the competency of migrating NCCs to differentiate into sympathetic neurons (Cohen 1972; Norr 1973; Teillet and Le Douarin 1983; Howard and Bronner-Fraser 1986). Furthermore, in vitro and in vivo work in avian models revealed BMPs to be sufficient to induce adrenergic differentiation (Varley et al. 1995; Reissmann et al. 1996; Shah et al. 1996; Varley and Maxwell 1996). Overexpression of *Bmp4* and *Bmp7* in migrating avian neural crest increases the size of sympathetic ganglia and forms ectopic ganglia, whereas blocking BMP signaling prevents sympathetic neuron generation. Consistent with this, NCC-specific inactivation of the type I BMP receptor (*Bmpr1a*) *Alk3* in mice does not affect neural crest cell migration or recognition of the SyNS site, but leads to failure of NCCs to aggregate and express sympathetic neuron markers. The influence of BMP signaling is on differentiation (but not migration) and the initial coalescence of the sympathetic progenitor crest (Schneider et al. 1999). The expression of *Bmp2* and *4* in mouse dorsal aorta, the site of primary (SG) chain formation, supports an endogenous role for BMP signaling in sympathetic neuron differentiation (Fig. 4B) (Shah et al. 1996). BMPs belong to the transforming growth factor β (TGFβ) family of secreted ligands and signal via activation of cytoplasmic coreceptor proteins SMADs (R-SMADs, SMAD 1,5,8 for BMPs), which, along with co-Smad and SMAD4, regulate gene expression. Although *Smad4* is not involved in colonization or survival of sympathetic ganglia (Buchmann-Moller et al. 2009; Morikawa et al. 2009), NCC-specific inactivation of *Smad4* leads to defects in murine SyNS development (Nie et al. 2008). Treatment of these mutant embryos with noradrenaline extends the life span of the mutants, showing a requirement for *Smad4* in sustaining the proliferation of NCC-derived sympathetic neurons (Morikawa et al. 2009).

3.2.3 Sympathetic Neuron Differentiation Involves a Linear Hierarchal Genetic Cascade

Sympathetic differentiation is unique such that there is no single master regulator of sympathetic fate. Rather, multiple players such as *Mash1*, *Phox2a/2b*, *Hand2*, and *Gata3*, which are all capable of cross-regulating each other, comprise a network that collectively governs sympathetic differentiation (Fig. 4C). Combinatorial expression of each of

these factors defines the different sympathetic neuronal subtypes. Most of these factors depend on BMP signaling for their activity (Shah et al. 1996; Lo et al. 1997, 1998; Schneider et al. 1999; Howard et al. 2000; Tsarovina et al. 2004). *Phox2* (*Phox2a*, *Phox2b*) genes are expressed in sympathetic, parasympathetic, and enteric ganglia of the developing PNS and are necessary and sufficient for specification of sympathetic neurons from trunk neural crest cells (Pattyn et al. 1999; Stanke et al. 1999). Mice lacking *Phox2b* die before birth because of the failure of autonomic ganglia formation leading to lack of noradrenaline in the body. The initial expressions of *Phox2b* and *Mash1* are independent of each other (Schneider et al. 1999); however, *Phox2b* is required for the maintenance of *Mash1* expression, because *Mash1* expression is lost in the ganglia of *Phox2b* mutant mice (Pattyn et al. 1999, 2000; Brunet and Pattyn 2002). *Phox2a* and *Phox2b* can induce transcription of each other and independently induce transcription of the noradrenaline synthesis enzymes *Th* (tyrosine hydroxylase) and *Dbh* (dopamine β-hydroxylase) (Zellmer et al. 1995; Kim et al. 1998; Yang et al. 1998; Lo et al. 1999). Adding to this complexity, MASH-1 has been shown to regulate the expression of *Phox2a* (Hirsch et al. 1998; Lo et al. 1998) and *Hand1* (Ma et al. 1997), and interactions between Hand2 and Phox2a are capable of inducing *Dbh* transcription (Fig. 4C) (Rychlik et al. 2003; Xu et al. 2003). During the normal course of sympathetic ganglia development, *Hand2* expression follows that of *Phox2b* and *Mash1*. *Hand2* expression has not been analyzed in *Mash1* mutants; hence, it remains unclear if *Mash1* and/or *Phox2b* have any role in regulating *Hand2*. *Phox2b*, however, regulates expression of *Gata3* (Tsarovina et al. 2004). *Gata3*-null mouse embryos display drastic reductions in *Th* and *Dbh* expression in the developing sympathetic ganglia (Lim et al. 2000) and die at midgestation (Pandolfi et al. 1995) of drastic noradrenergic differentiation defects.

3.2.4 Sympatho-Adrenal Progenitor Differentiation into Distinct Cell Types Is Based on Environmental Cues

Trunk neural crest cells generate both noradrenergic and cholinergic neurons (fewer in number compared with the noradrenergic). Neurotropins NGF and NT3 play a general role in survival and differentiation of sympathetic neuroblasts, and both are capable of inducing *Th* and *Dbh* expression in vivo. However, analyses of superior cervical ganglion (SCG) neurons of the SyNS in the NGF and NT3 loss-of-function mouse embryos indicate a differential requirement for NGF and NT3 in cholinergic and adrenergic differentiation. NGF seems to have a specific role in expression of acetylcholine receptor, whereas NT3 is important

for the expression of *Th* and *Dbh* enzymes (Andres et al. 2008). This differential in vivo requirement for NGF and NT3 neurotrophins might be due to differences in spatial availability of the factors to the progenitor neural crest and be an added way to regulate cholinergic versus adrenergic differentiation.

Chromaffin cells are neuro-endocrine cells present in the medullary region of the adrenal gland. Derived from the NCCs, these cells are postsynaptic sympathetic neurons modified such that upon electrical sympathetic input they secrete catecholamine neurotransmitters (predominantly adrenaline and in limited amounts noradrenaline) into the blood circulatory system. For differentiation into chromaffin cells, sympathetic precursors turn off *Th* expression while concomitantly activating the enzyme phenylethanolamine *N*-methyltransferase (PNMT) (Fig. 4B). PNMT catalyzes the conversion of noradrenaline to adrenaline. Sympathetic progenitors can be differentiated in vitro into sympathetic neurons or chromaffin cells depending on whether they are exposed to NGF or glucocorticoids, respectively (Anderson 1993; Francis and Landis 1999), and treatment of postnatal chromaffin cells with NGF causes them to *trans*-differentiate into neurons (Unsicker et al. 1978b; Doupe et al. 1985). Glucocorticoid receptor (GR) mutant mice ($GR\alpha^{-/-}$) lack the adrenaline-synthesizing enzyme PNMT, however this fails to affect development of adrenal chromaffin cells, and no transformation into a neuronal phenotype is observed (Finotto et al. 1999). This suggests that sympatho-adrenal progenitors can differentiate into distinct cell types in response to environmental molecular cues.

4 CONCLUSIONS

Neural crest cells show a remarkable capacity for differentiation, and in this article, we highlighted the complexity of signals and switches, which govern cranial neural crest cell differentiation into bone and cartilage and the differentiation of trunk NCCs into primarily neuronal derivatives. NCCs continue to fascinate scientists, and, although clearly important to embryonic development and disease pathogenesis, much of the current focus resides in their evolution and their stem cell-like characteristics and application to regenerative medicine.

Neural crest cells are synonymous with vertebrate evolution, and there is tremendous interest in identifying their specific origin and the acquisition of their incredible properties. There is no doubt that the properties of NCCs were acquired gradually over time. Understanding the signals and switches that govern NCC differentiation may eventually reveal how and when the NCCs and their distinctive properties came to be.

The discovery that NCCs show multipotency and self-renewal capacity was pioneering for the field of stem cell biology. The stem and progenitor cell properties of NCCs, combined with their remarkable diversity of cell type and tissue differentiation, have made NCCs an attractive proposition in regenerative medicine. Even though NCC progenitor cells are generated transiently in the embryo, many NCCs appear to retain their capacity throughout embryogenesis and even into adulthood. Consequently, there have been concerted efforts to isolate embryonic and adult NCCs as well as derive NCCs from human and mouse embryonic stem cell (ESCs) and induced pluripotent stem cells (iPSCs). More research, however, is needed to further our knowledge regarding the capacity of NCC progenitors to differentiate into cells of several different lineages. More importantly, this needs to be performed in vivo, 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.

Currently, however, very little is known regarding the key factors or signaling cascades and switches that are essential for mammalian NCC induction. The Wnt, BMP, and FGF signaling pathways that show well-established and characterized roles in NCC formation in avians, fish, and amphibians appear to be required primarily for lineage specification of NCCs in mammalian embryos. It is important to note that as neural stem cells differentiate into NCCs, there is a clear switch in Sox expression state with Sox2 being inactivated in concert with the activation of Sox9 and then Sox10 in progenitors and migrating NCCs, respectively (Remboutsika et al. 2011). These parameters may present avenues for ultimately identifying the key signals required for mammalian NCC formation, which will provide insights into the evolution of neural crest cells and their properties as well as facilitate ways to generate neural crest cells from hESCs and iPS cells, both of which will be enormously beneficial in the application of NCCs to tissue engineering and regenerative medicine.

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