

REVIEW

Incremental evolution of the neural crest, neural crest cells and neural crest-derived skeletal tissues

Brian K. Hall¹ and J. Andrew Gillis^{2,*}¹Department of Biology, Dalhousie University, Halifax, NS, Canada²Department of Physiology, Development and Neuroscience, University of Cambridge, Cambridge, UK

Abstract

Urochordates (ascidians) have recently supplanted cephalochordates (amphioxus) as the extant sister taxon of vertebrates. Given that urochordates possess migratory cells that have been classified as 'neural crest-like' – and that cephalochordates lack such cells – this phylogenetic hypothesis may have significant implications with respect to the origin of the neural crest and neural crest-derived skeletal tissues in vertebrates. We present an overview of the genes and gene regulatory network associated with specification of the neural crest in vertebrates. We then use these molecular data – alongside cell behaviour, cell fate and embryonic context – to assess putative antecedents (latent homologues) of the neural crest or neural crest cells in ascidians and cephalochordates. Ascidian migratory mesenchymal cells – non-pigment-forming trunk lateral line cells and pigment-forming 'neural crest-like cells' (NCLC) – are unlikely latent neural crest cell homologues. Rather, *Snail*-expressing cells at the neural plate of border of urochordates and cephalochordates likely represent the extent of neural crest elaboration in non-vertebrate chordates. We also review evidence for the evolutionary origin of two neural crest-derived skeletal tissues – cartilage and dentine. Dentine is a *bona fide* vertebrate novelty, and dentine-secreting odontoblasts represent a cell type that is exclusively derived from the neural crest. Cartilage, on the other hand, likely has a much deeper origin within the Metazoa. The mesodermally derived cellular cartilages of some protostome invertebrates are much more similar to vertebrate cartilage than is the acellular 'cartilage-like' tissue in cephalochordate pharyngeal arches. Cartilage, therefore, is not a vertebrate novelty, and a well-developed chondrogenic program was most likely co-opted from mesoderm to the neural crest along the vertebrate stem. We conclude that the neural crest is a vertebrate novelty, but that neural crest cells and their derivatives evolved and diversified in a step-wise fashion – first by elaboration of neural plate border cells, then by the innovation or co-option of new or ancient metazoan cell fates.

Key words: ascidians; cartilage; cephalochordates; chondrogenesis; dentine; developmental origins; evolutionary origins; gene co-option; gene regulatory networks; innovation; neural crest; neural crest cells; odontogenesis; skeleton; vertebrates.

Introduction

Chordates

All chordates – cephalochordates, urochordates and vertebrates – share a dorsal notochord and a dorsal hollow nerve

cord that defines the midline of the dorso-ventral axis. The three subphyla have been regarded as sharing a common chordate ancestor ever since Kowalevsky discovered that cephalochordates and larval ascidians possess a notochord (Kowalevsky, 1866, 1867, 1871, 1877; Bowler, 1996; Holland, 2000; Holland & Chen, 2001; Mikhailov & Gilbert, 2002; Hall, 2005a). Although a monophyletic clade, three divergent body plans exist among extant urochordates. These body plans have long been recognized as three classes – benthic tunicates (ascidians, sea squirts),¹ pelagic appendicularians (larvaceans) and pelagic salps (thaliaceans). Only ascidians

Correspondence

Brian K. Hall, Department of Biology, Dalhousie University, Halifax, Nova Scotia, Canada B3H 4R2. T: + 1 9024943522; F: + 1 9024943736; E: bkh@dal.ca

*Present address: Department of Biology, Dalhousie University, Halifax, NS, Canada.

Accepted for publication 15 February 2012
Article published online 14 March 2012

¹The terms tunicate, ascidian and sea squirt are used interchangeably in this paper.

have been studied in any depth, including fate-mapping cell lineages, with most data coming from two genera, *Ciona* (mostly *C. intestinalis*) and *Halocynthia roretzi*. The embryology of much of tunicate diversity remains unstudied, as does the embryology of larvaceans and salps. Extant cephalochordates, on the other hand, are represented by a single body plan found in 14 species of amphioxus in two genera, *Branchiostoma* and *Asymmetron*. Much developmental work has been done on this group, but no cell-lineage fate map is available (see Holland & Holland, 2007).

The fossil record of chordates and early vertebrates shows that ascidians, hemichordates, cephalochordates and jawless vertebrates had already arisen by the Early Cambrian. *Haikouella lanceolata* and *H. jianshanensis* are hemichordates. *Shankouclava shankouense* is an ascidian. *Cathaymyrus diadexus* and *C. haikoensis* are cephalochordates with a notochord and segmented body musculature, while *Myllokunmingia fengjiao* and *Haikouichthys ercaicunensis* are jawless vertebrates with notochord, myomeres, a dorsal fin, cartilaginous gills clefts, and cartilaginous olfactory and optic capsules (Shu et al. 1996; Mallatt & Chen, 2003; Fedonkin et al. 2007; Janvier, 2007; Hall, 2008b, 2009). The homologous cartilaginous skeletal elements in extant vertebrates are neural crest derivatives (Hall, 1999, 2009; Le Douarin & Kalcheim, 1999; Long et al. 2011).

The neural crest and neural crest cells

The 'neural crest' is a morphological term for the dorsal folds of the neural tube, seen as the neural plate rolls up to form the hollow dorsal neural tube during neurulation. Possession of a neural crest derived from dorsal neural ectoderm is a shared-derived (synapomorphic) characteristic of vertebrates. The origin of the neural crest and neural crest cells may therefore be viewed as the derivation in vertebrates of a distinct cell population within the broadly evolutionarily conserved boundary between chordate neural and non-neural ectoderm.

'Neural crest cells' are mesenchymal cells derived from the neural crest epithelium. Two key features of neural crest cells are migratory ability and multipotency (Donoghue et al. 2008). Neural crest cells delaminate from ectoderm, undergo an epithelial-to-mesenchymal transformation, and migrate away from the future brain and spinal cord, giving rise to a plethora of derivatives in the head and trunk. Neural crest-derived mesenchymal cells differentiate into at least 21 different cell types, including neurons (sensory, adrenergic and cholinergic); satellite, Schwann glial and chromaffin cells; melanocytes; connective tissue and skeletal cells (fibro-, chondro-, osteo- and odontoblasts); myoblasts (cardiac, striated and smooth); adipocytes and angioblasts (Le Douarin & Kalcheim, 1999; Le Douarin et al. 2004; Vickaryous & Hall, 2006; Hall, 2009). Some of these cell types also arise from mesoderm – fibroblasts, chondroblasts and osteoblasts being prime examples. Neural crest-derived cells

contribute to (or form in their entirety) spinal and enteric ganglia, the parasympathetic, peripheral and sympathetic nervous systems, the thyroid and adrenal glands, the craniofacial and viscerocranial skeletons, teeth, connective and adipose tissues, smooth and striated muscles of the heart, the eye, blood vessels and endothelia. Each of these cell types, tissues and organs has its own characteristic features, the elaboration of which is beyond the scope of the review (but see the references above).

When assessed on the basis of number of differentiated cell types, the neural crest forms a greater number of cell types than does mesoderm (Vickaryous & Hall, 2006). Based on this developmental potential and diversity of cell fates, the neural crest can be classified as the fourth germ layer, and the only germ layer found exclusively in vertebrates: the embryos of diploblastic animals possess ectoderm and endoderm; the embryos of triploblastic invertebrate embryos possess ecto-, endo- and mesoderm; vertebrate embryos possess these three germ layers plus the neural crest (Hall, 1997, 2000a,b, 2008a,c, 2009; Le Douarin & Kalcheim, 1999).

Evolutionary origin(s) of the neural crest

Classically, the neural crest was thought to have originated along the vertebrate stem, coincident with the evolution of a brain, a muscular pharynx and paired sensory organs. In their ground-breaking 'new head hypothesis,' Gans & Northcutt (1983) linked the shift from passive filter feeding to active predation with the evolutionary diversification of the neural crest and ectodermal placodes (see also Gans & Northcutt, 1985; Maisey, 1986; Gans, 1987, 1989, 1993; Maderson, 1987; Hanken & Hall, 1993; Donoghue, 2002; Mallatt & Chen, 2003; Hall, 2005c, 2008a,b,c, 2009; Schlosser, 2006, 2010; Donoghue et al. 2008).² Gans & Northcutt (1983) and Northcutt & Gans (1983) proposed that an epidermal nerve plexus or nerve net primitively controlled ciliary function during movement and filter feeding, and that as the central nervous system took over the neural control of locomotion, other derivatives of this primitive nerve net – namely, neural crest cells and ectodermal placodes – would have been freed to diversify and evolve other functions (*ibid*; Bowler, 1996; Northcutt, 1996; Baker & Bronner-Fraser, 1997).

Since the publication of the 'new head hypothesis', however, it has been recognized that ascidians possess: first, ectodermal 'placodes' (i.e. focal regions of neurogenic ectoderm, which may bear some relationship to vertebrate neurogenic placodes; Wada et al. 1998; Manni et al. 2004;

²Historical note: the 'senior' professor of Zoology referred to in the first paragraph of Donoghue et al. (2008) who claimed that 'the only interesting thing about vertebrates was [is] the neural crest', was [is] the first author of the present paper.

Schlosser, 2006, 2010; Hong & Saint-Jeannet, 2007); and second, migratory NCLC (see below); cephalochordates possess neither placodes nor NCLC. Furthermore, recent detailed phylogenomic analyses place urochordates and not cephalochordates as the immediate sister group to vertebrates (Fig. 1; Boulrat et al. 2006; Delsuc et al. 2006; Dunn et al. 2010; Philippe et al. 2011). If ascidian NCLC can be homologized with vertebrate neural crest cells, then the neural crest may, in fact, have a deeper (Precambrian) origin in chordate phylogeny as a synapomorphy of Olfactores (a clade consisting of urochordates + vertebrates, *ibid*) rather than of vertebrates. So, are ascidian NCLC homologous with, or evolutionary precursors of, vertebrate neural crest cells? And is there any evidence for neural crest or NCLC in cephalochordates?

We begin this review by briefly outlining studies that have identified genetic markers and defined a 'gene regulatory network' (GRN) underlying the development of the neural crest, from the early specification of neural crest precursors, to the ultimate differentiation of neural crest-derived cells. We then use this GRN, alongside cell behaviour, cell fate and embryonic context, as a basis for

assessing putative latent neural crest homologues – evolutionary developmental precursors of the neural crest that may, in effect, be represented as a series of progressively more plesiomorphic 'neural crest character states' on a cladogram (Hall, 1994; Stone & Hall, 2004) – in cephalochordates and urochordates. Finally, we discuss the evolution of neural crest skeletal fates. With respect to the acquisition of neural crest cell fates, it is now evident that the diversification of neural crest cell fates occurred, in some instances, by the evolution of new cell types and, in other instances, by the co-option of developmental programs for cell types from other tissues. We discuss how neural crest cells have acquired skeletogenic fates, using odontoblasts (a 'new' – i.e. neural crest-specific – cell type) and chondrocytes (primitively a mesodermally derived invertebrate cell type) as examples.

Neural crest 'markers', and the molecular specification of the neural crest and neural crest cells

A combination of genomic analyses and bioinformatics enabled Martinez-Morales et al. (2007) to identify 615 'neural crest genes' – defined as genes that are associated with the neural crest or neural crest cells. Of these, 91% are found in metazoans that lack neural crest cells. Therefore, the origination of the neural crest was not directly associated with major rounds of evolution of new gene families, and the notion of 'neural crest genes' is not valid. Interestingly, Martinez-Morales et al. (2007) found that half their 'neural crest genes' code for extracellular ligands that are expressed by other cell types. Based on this observation, they suggest that much of neural crest evolution may have been driven by the evolution of ligand regulation (with ligand-mediated signalling, in turn, functioning in the commitment of neural crest cells to individual cell lineages).

The HNK-1 carbohydrate epitope is often defined and used as a neural crest 'marker'. HNK-1 is present on the surface of migrating avian, frog and some teleost neural crest cells, and is often used as an indicator of neural crest cell induction in experimental studies. However, HNK-1 is far from a neural crest marker; HNK-1 is expressed in a number of non-neural crest-derived vertebrate cell types and is not expressed in the neural crest cells of several vertebrate taxa (including mouse, shark and zebrafish – Holley & Yu, 1987; Kuratani & Horigome, 2000). In fact, as effectively all genes expressed in the developing neural crest or neural crest cells are also expressed in different ectodermal and/or mesodermal development contexts, one is hard-pressed to identify a single neural crest 'marker'.

Many genes that play important inductive or regulatory roles during the development of the neural crest and neural crest cells have now been identified and characterized, including genes expressed in gastrula-stage embryos that indicate the early specification of the neural crest cell, genes

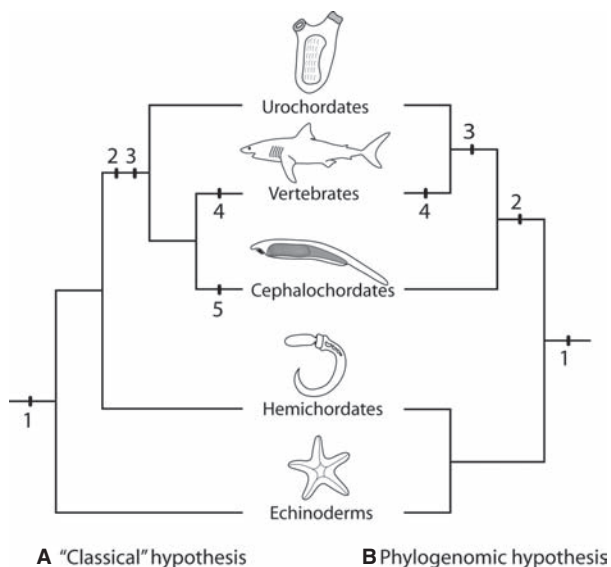


Fig. 1 (A) The classical hypothesis of chordate interrelationships (based primarily on morphology) placed cephalochordates as the extant sister group to vertebrates. (B) The currently accepted phylogenetic hypothesis (based on extensive phylogenomic and EST sequence analysis) resolves urochordates as the sister group of vertebrates. Numbers on the phylogeny indicate key steps in the evolution of the vertebrate neural crest. 1. Dorsoventrally patterned neuroectoderm is a primitive feature of deuterostomes. 2. The origin of *Snail*-expressing cells at the border of the neural plate. 3. The origin of migratory 'NCLC', as seen in ascidians (if these are deemed latent homologues of vertebrate neural crest cells – see text). 4. The origin of the neural crest. Note that if ascidian 'NCLC' are latent homologues of neural crest cells, then the classical hypothesis would require loss of 'NCLC' in cephalochordates (5).

expressed at the neural crest border in neurula-stage embryos, and genes expressed in pre-migratory, delaminating, migratory and differentiating neural crest cells. The interactions of these genes and their products have been sufficiently well characterized – both functionally and across phylogenetically informative taxa (e.g. chick, mouse, frog and lamprey) – to allow the construction of a putative vertebrate neural crest GRN (Meulemans & Bronner-Fraser, 2004; Sauka-Spengler et al. 2007; Sauka-Spengler & Bronner-Fraser, 2008a,b,c; Betancur et al. 2010). Among the genes in the neural crest GRN are members of the *Fgf*, *Wnt* and *Bmp* gene families, which are involved in induction of the neural plate; *Pax3/7*, *Zic* genes, *Msx1/2* and *Dlx3/5*, which are involved in establishing the neural plate–epidermal boundary ('neural plate border specifiers'); and *Snail1/2*, *FoxD3*, *Tfap2a*, *Sox8/9/10* and *Twist*, which are involved in the specification of neural crest cells and distinct neural crest cell sublineages ('neural crest specifiers'). While an extensive review of the molecular interactions governing neural crest specification is beyond that scope of this paper – and given that these data have been eloquently reviewed elsewhere (*ibid*) – we simply present selected downstream effectors of early ectodermal Bmp signalling (Fig. 2) as one example of how genes at different levels of the neural crest GRN interact to regulate neural crest development.

Bmp-signalling in the ectoderm is involved in the segregation of neural from non-neural (e.g. epidermal) domains, with high levels of Bmp activating the epidermal program, and low levels of Bmp (a consequence of dorsal expression of the secreted Bmp antagonist Noggin) activating the neural program (Liem et al. 1995; Marchant et al. 1998). A high level of Bmp upregulates the expression of factors such as *Msx1* (which is a direct positive transcriptional regulator of keratin), while low levels of Bmp signalling upregulate the expression of *Sox2* and *Zicr1* (which, in turn, activate expression of the neural genes *N-cam* and *N-tubulin*; Suzuki et al. 1997; Mizuseki et al. 1998). Attenuated levels of Bmp signalling at the neural–epidermal ectoderm boundary activate expression of neural plate border specifiers, such as the *Zic* genes and *Pax3/7* (Nakata et al. 1997, 2000; Aruga et al. 2002). These border specifiers, in conjunction with Bmp, Fgf and Wnt signalling, in turn, regulate the expression of the neural crest specifiers *Snail1*, *Snail2* (*Slug*), *FoxD3* and *Twist* (all of which are expressed in pre-migratory neural crest cells; Nakata et al. 1997, 2000; Brewster et al. 1998; Mizuseki et al. 1998; Sasai et al. 2001).

Within the neural crest GRN, there is ample evidence for cross-regulation among neural crest specifiers. For example, in *Xenopus*, overexpression of *FoxD3* results in the upregulation of *Slug* and *Twist* (Sasai et al. 2001), overexpression of *Sox10* results in the upregulation of *Slug* (Aoki et al. 2003), overexpression of *Snail* induces expression of *Slug*, *FoxD3* and *Twist* (Aybar et al. 2003), and loss of *Sox10* function results in the inhibition of *Slug* and *FoxD3* expression

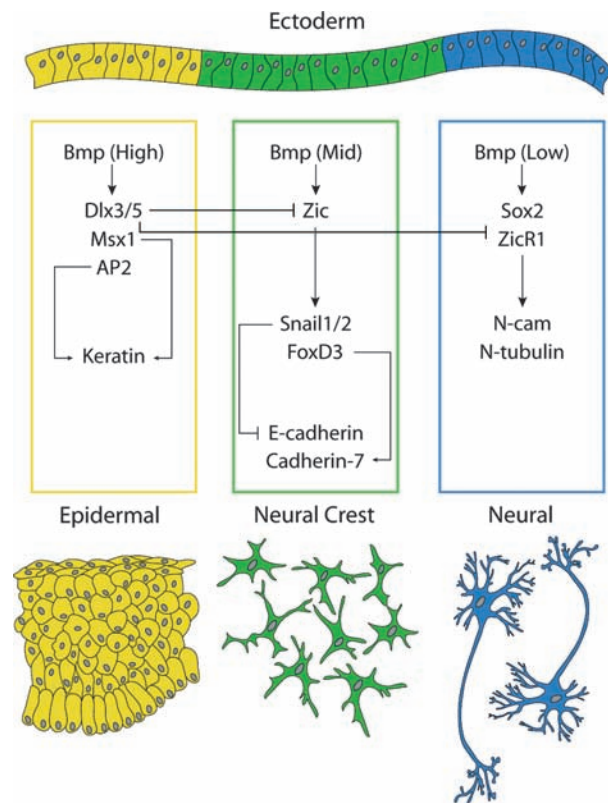


Fig. 2 A simplified schematic illustrating how selected factors downstream of Bmp signalling interact to specify epidermal, neural and neural crest cell fates in vertebrate embryos. Molecular interactions are not necessarily direct, and are based on the putative vertebrate neural crest gene regulatory network (after Meulemans & Bronner-Fraser, 2004; Sauka-Spengler et al. 2007; Sauka-Spengler & Bronner-Fraser, 2008a,b). For details, see text.

(Honore et al. 2003). Using a genetic approach in zebrafish, Arduini et al. (2009) have shown that the simultaneous loss of *FoxD3* and *Tfap2a* function results in a loss of neural crest *Sox9* and *Sox10* expression, and a corresponding absence of all neural crest-derived structures (including neural crest-derived chromatophores, pharyngeal cartilages and neural crest-derived peripheral neurons). More recently, Wang et al. (2011) demonstrated that *FoxD3/Tfap2* double mutant zebrafish also exhibit an early perturbation of *Wnt* and *Bmp* signalling during gastrulation, and suggest that, in addition to their well-established roles in neural crest cell sublineage specification, *FoxD3* and *Tfap2a* may also play earlier roles in neural crest induction. Importantly, however, Arduini et al. (2009) observed that neural crest induction occurred normally in the absence of *FoxD3* and *Tfap2* function, and so the precise roles of *FoxD3* and *Tfap2a* in neural crest induction remain unclear.

A number of 'neural crest effector' genes have also been identified and characterized. These genes are downstream of the neural crest specification genes, and play integral roles in the delamination, migration and differentiation of

neural crest cells. Expression of the neural crest specifiers *Snail* and *FoxD3* are negative and positive transcriptional regulators of *E-cadherin* and *cadherin-7*, respectively, and the downregulation and upregulation of *E-cadherin* and *cadherin-7*, respectively, are required for the delamination of neural crest cells during epithelial-to-mesenchymal transition (Nakagawa & Takeichi, 1998; Cano et al. 2000). Another example of a neural crest effector gene is *Col2a*, which encodes type II collagen – the major collagenous component of vertebrate cartilage extracellular matrix. Expression of *Col2a* in differentiating chondrocytes is directly regulated by the neural crest specifier *Sox9* (Ng et al. 1997), though note that *Sox9* also regulates the expression of *Col2a* in mesodermally derived cartilage (reinforcing the fact that many neural crest specifiers function similarly in non-neural crest developmental contexts).

Given the extensive molecular characterization of the neural crest and neural crest cells in vertebrates, we may now use the vertebrate neural crest GRN – along with cell behaviour, cell fate and embryonic context – to assess putative evolutionary antecedents of the neural crest and neural crest cells in non-vertebrate chordates.

Putative cellular and molecular precursors of the neural crest and neural crest cells in non-vertebrate chordates

Cephalochordates

Due to their 'simplified' vertebrate-like adult body plan, cephalochordates have long been regarded as the sister group of the vertebrates, and have been used as a 'proxy' for the last common ancestor of chordates (Holland & Holland, 2001). Such an approach is fraught with problems, however, as no single extant taxon can truly represent a collection of primitive character states (such an 'ancestor' is necessarily hypothetical, and a product of careful character optimization on a phylogenetic tree). Cephalochordates generally lack derivatives known in vertebrates to arise, exclusively or in part, from neural crest cells, such as peripheral pigment cells, bone-, cartilage- or dentine-forming cells. Amphioxus dorsal root nerves are ensheathed by glial cells, and these have been compared with the Schwann cells of the vertebrate peripheral nervous system (Bone, 1960; Peters, 1963), though peripheral glial cells are also present in a number of marine invertebrate taxa that unequivocally lack a neural crest (Coles & Abbott, 1996).

Studies examining the expression of selected Amphioxus orthologues of candidates in the vertebrate neural crest GRN – referred to with the prefix *Amphi* and the orthologous vertebrate gene – have revealed that the 'neural plate border specifiers' *AmphiPax3/7*, *AmphiMsx* and *AmphiZic* are all expressed at the lateral edge of the neural plate, and that *AmphiDlx3/5* is expressed in ectoderm adjacent to the neural plate (Holland et al. 1999; Sharman et al. 1999; Gostling &

Shimeld, 2003). As in vertebrates, these cells give rise to the dorsal neural tube, and so these gene expression patterns likely reflect a conserved mechanism for patterning the dorsal–ventral axis of the nervous system in chordates (indeed, for bilaterians; Holland & Graham, 1995; Meinertzhagen & Okamura, 2001). However, only a single 'neural crest specifier,' *AmphiSnail*, is expressed in the neural plate border in amphioxus (Langeland et al. 1998). Ectodermal cells at the lateral border of the neural plate in amphioxus embryos do not appear to delaminate and migrate, and so the function/fate of *AmphiSnail*-expressing cells is unclear. It would be interesting to examine whether *AmphiSnail* functions as a transcriptional regulator of cell-adhesion molecules in amphioxus, as *Snail* does in vertebrates. Additionally, while the early fate map of amphioxus is reasonably well understood (Holland & Holland, 2007), there are no data (e.g. vital dye-labelling data) available on the fate of *AmphiSnail*-expressing cells. Thus, while evidence for cephalochordate neural crest cell antecedents and neural crest derivatives is currently scant, further molecular characterization and *in vivo* fate mapping of *AmphiSnail*-expressing neural plate border cells in amphioxus may yet reveal a latent neural crest homologue in this group. Functional analyses of regulatory interactions among factors in the putative neural crest GRN in amphioxus will be facilitated by the availability of a cephalochordate genome sequence (Putnam et al. 2008).

Ascidians

Urochordates (Urochordata) include the ascidians (tunicates, sea squirts), larvaceans (appendicularians) and thaliaceans (salps). Larvaceans and thaliaceans are virtually unstudied with respect to the presence of a neural crest or neural crest cells. Conversely, ascidians – particularly the two species *Ciona intestinalis* and *Halocynthia roretzi*, which are found in northern European and in Japanese, Chinese and Korean waters, respectively – have been the focus of intense study in the field of developmental biology (Whittaker, 1987; Satoh, 1994, 2007, 2008, 2009; Jeffery, 1997, 2006, 2007; Meinertzhagen & Okamura, 2001; Jeffery et al. 2004, 2008; Kumano & Nishida, 2007; Bishop et al. 2010). Although studied since the early 19th century (Kowalevsky, 1871), the recent description by Jeffery et al. (2004) of NCLC in ascidian embryos, the sequencing of the *Ciona* genome (Dehal et al. 2002) and phylogenetic analyses that place urochordates as the sister group to vertebrates (Bourlat et al. 2006; Delsuc et al. 2006) have brought ascidians to centre stage in the neural crest evolutionary play.

The colonial mangrove ascidian *Ecteinascidia turbinata* possesses peripheral yellow-orange pigment cells in the tunic, and these pigment cells are reminiscent of vertebrate erythrophores (a subtype of neural crest-derived pigment cell, or chromatophore). Vital dye-labelling experiments in the giant tadpole larvae of *E. turbinata* have shown that cells giving rise to pigment arise near (though not

unequivocally from) the neural tube and developing central nervous system, and migrate either through the mesoderm, or between the mesoderm and epidermis, to populate the body wall and siphon (Jeffery et al. 2004). These migrating cells are mesenchymal in organization, though they migrate as single cells rather than in streams. These cells also express the carbohydrate epitope HNK-1, which is expressed in migrating avian neural crest cells, and they express the zinc finger transcription factor *Zic3*, which is expressed in the neural plate border of vertebrate embryos (though not in migrating neural crest cells). Such HNK-1-positive mesenchymal cells – termed NCLC – have been observed in 12 other species of ascidians with disparate adult organizations, developmental modes and larval sizes, and often appear to co-express tyrosinase, the rate-limiting enzyme in melanogenesis (Jeffery, 2006). If, as recent molecular phylogenies suggest, ascidians are the sister group to vertebrates, and if the NCLC reported in ascidians are forerunners of neural crest cells ('proto-neural crest cells'), then it has been speculated that the primitive fate of these neural crest precursors may have been pigment (Jeffery, 2007).

More recently, analysis of cytochalasin-treated 'cleavage-arrested' *Ciona intestinalis* embryos has suggested that the HNK-1-positive NCLC described by Jeffery et al. (2004) and Jeffery (2006) derive from the A7.6 blastomere pair. This blastomere pair gives rise to a previously characterized migratory cell population known as trunk lateral cells. Similar expression patterns of HNK-1 and the trunk lateral cell-specific marker *Tlc2* (Takahashi & Satoh, 2001) led Jeffery et al. (2008) to assert that the NCLC of *C. intestinalis* (and other ascidians) are these trunk lateral cells. Jeffery et al. (2008) demonstrated that the *C. intestinalis* A7.6/trunk lateral cell lineage expresses orthologues of seven of 16 genes from the vertebrate neural crest GRN, including four patterning genes involved in neural plate induction (*Bmp*, *Wnt*, *Notch*, *Fgf*) and five 'neural crest specifier' genes (*Twist*, *FoxDb*, *Ap2-1*, *Myc*). Based on these expression data, Jeffery et al. (2008) speculate that ascidian NCLC/trunk lateral cells and vertebrate neural crest cells are homologous cell types,

Given these observations, and the sister group relationship of ascidians and vertebrates, are NCLC/trunk lateral cells latent neural crest cell homologues? There are a number of problems with this conjecture.

(1) Firstly, ascidian NCLC have not been definitively shown to arise from the neural tube. An examination of Dil injection sites relative to the position of the neural tube in *E. turbinata* (Jeffery et al. 2004) reveals that a large population of cells adjacent to the neural tube was also labelled in these lineage-tracing experiments. While this is certainly understandable, given the size of the animal in question, caution must nevertheless be exercised when assessing the putative embryonic origin of *E. turbinata* NCLC.

(2) There is also no evidence from lineage-tracing studies in other ascidians that A7.6-derived cells contribute to

the border of the neural plate, or even to ectoderm more generally (Satoh, 1999). In terms of molecular marker expression, HNK-1 is not an exclusive marker for neural crest cells, and while the A7.6/trunk lateral cell lineage of *C. intestinalis* does express a subset of 'neural crest specifiers' (*Twist*, *FoxDb*, *Ap2-1*, *Myc*), as discussed above, these genes are not exclusive to the developing neural crest, and are also expressed in developing vertebrate mesodermal derivatives.

(3) It is important to note that, in the ascidians *Halocynthia roretzi* and *Ciona intestinalis*, the A7.6 lineage of trunk lateral cells gives rise to a variety of mesendodermal fates, including blood cells, longitudinal mantle muscles, oral siphon muscles and pharyngeal epithelium (Hirano & Nishida, 1997; Tokuoka et al. 2004), and so it is difficult to disentangle roles for 'neural crest specifiers' in A7.6-derived NCLC vs. other mesendodermal derivatives without further functional studies.

(4) Finally, echinoderm larvae possess pigment cells that derive from migratory mesenchymal cells of mesodermal origin (Gibson & Burke, 1985). It is therefore possible that pigment cells primitively derived from the mesodermal lineage in deuterostomes (echinoderms, hemichordates and chordates), with a subsequent co-option of this developmental program to the neural crest along the vertebrate stem.

There are, nevertheless, cells at the neural plate border of ascidians that may well represent true latent neural crest homologues. Presumptive dorsal epidermal cells adjacent to the neural plate in *Ciona intestinalis* express orthologues of the vertebrate 'neural plate border specifiers' *Dlx3/5* and *Msx*, while *Pax3/7* is expressed in both presumptive dorsal epidermis and dorsal neural tube (Wada et al. 1996; Mazet et al. 2003; Imai et al. 2004). Furthermore, as in amphioxus and vertebrates, the neural crest specifier *Snail* is expressed in neural plate border ectoderm in the ascidians *Halocynthia roretzi* and *C. intestinalis* (Corbo et al. 1997; Erives et al. 1998; Wada et al. 1998). The ascidian caudal neural tube consists of one dorsal, one ventral and two lateral rows of ependymal cells (Nicol & Meinertzhagen, 1991). In *H. roretzi* 76-cell embryos and *C. intestinalis* 110-cell embryos, *Snail* is expressed in the A8.15 blastomeres and the A8.15/A8.16 blastomeres, respectively. These cells give rise to the lateral ependymal cells of the neural tube, and *Snail* expression originating in these cells expands antero-laterally to mark the lateral border of the neural plate, and persists into the curling neural folds. *Snail* expression was also noted in cells that give rise to the sensory vesicle and sensory pigment cells in both *H. roretzi* and *C. intestinalis*, and in descendants of the b8.19 cells in *H. roretzi*, which give rise to the dorsal row of caudal neural tube ependymal cells. Given their origin from the neural plate border, it is perhaps more plausible that these *Snail*-expressing cells represent antecedents to the vertebrate neural crest.

Origin of neural crest-derived skeletogenic cell types

Following their migration, neural crest cells differentiate into a diversity of cell and tissue types. Among the best characterized of these are the craniofacial skeletal tissues of jawed vertebrates. Neural crest cells give rise to the chondrocytes of primary and secondary cartilage, the osteoblasts/osteocytes of dermal and endochondral bone, and the odontoblasts of tooth and scale dentine. Note, however, that both cartilage and bone may also form from mesoderm, and that only dentine derives exclusively from neural crest (Hall, 2009). When considering the evolution of neural crest-derived skeletogenic cell types – and the evolution of osteocytes and chondrocytes, in particular – it is essential to consider the phylogenetic sequence of skeletal tissue occurrence, in order to determine whether gene regulatory interactions underlying particular cell types functioned primitively in mesoderm and were co-opted to the neural crest, or vice versa. Here, we consider the evolution of neural crest-derived odontoblasts and chondrocytes, as examples of cell types whose neural crest origin likely represent cases of novelty and co-option, respectively.

Odontoblasts/dentine

Odontodes – the developmental module that gives rise to dentine-secreting odontoblasts and enamel-secreting ameloblasts – are an ancient component of the vertebrate skeleton, quite possibly representing the first example of vertebrate skeletal biomineralization (Smith & Hall, 1990, 1993).

Phylogenetically, putative odontodes appear first in the fossil record at the base of the gnathostome (jawed vertebrate) stem, as the mineralized feeding elements of the conodont oral apparatus (Donoghue, 2002; Donoghue et al. 2006). Conodont elements are composed of basal tissue and crown tissue, and these tissues have been homologized with the dentine and enamel, respectively, of gnathostome teeth. Microstructurally diverse dentinous scales, denticles and tubercles are broadly distributed among various fossil taxa that sit on the gnathostome stem (reviewed by Smith & Hall, 1990; Sire et al. 2009). Among extant jawed vertebrates, odontodes are present both orally (as the oral teeth and pharyngeal denticles of bony and cartilaginous fishes) and dermally (as the dermal denticles of cartilaginous fishes and polypterid bony fishes). Given that, among extant jawed vertebrates, oral tooth odontoblasts are exclusively derived from neural crest (de Beer, 1947; Smith & Hall, 1990, 1993), it is generally assumed that all dentine-containing tissues in extant and extinct jawless vertebrates are similarly neural crest derived. However, this remains to be formally tested by neural crest lineage tracing in extant taxa with post-cranial dermal denticles (e.g. chondrichthyans; Miyake et al. 1999).

Dentine-secreting cells (odontoblasts) and bone-secreting cells (osteoblasts) are similar in many respects. Both cell types are polarized, exhibit cell processes and secrete common extracellular matrix proteins – e.g. type I collagen, bone sialoprotein (osteopontin), osteocalcin dentine matrix protein 1 and periostin (osteoblast-specific factor-2; Lu et al. 2001; Fraser et al. 2004; Kawasaki et al. 2004; Kruzynska-Freitag et al. 2004; Pääkkönen et al. 2007). However, while osteoblasts become surrounded by their secreted matrix (then becoming osteocytes; Franz-Odenaal et al. 2006), odontoblasts retreat into a pulp cavity as they secrete their matrix, leaving only their cell processes embedded within mineralized matrix.

Odontoblasts can also be distinguished from osteoblasts/osteocytes by their secretion of certain unique matrix products (e.g. dentin sialoprotein and dentin phosphoprotein – Butler & Ritchie, 1995), and by their expression of mechanosensitive ion channels, such as TRPV1 and voltage-gated sodium channels (Okumura et al. 2005; Allard et al. 2006). Whether odontoblasts functioned primitively in a sensory capacity (Baker, 2008; Magloire et al. 2009), or co-opted their sensory function coincident with the evolution of oral teeth, remains to be determined. However, given that there are no identifiable dentine or odontoblast homologues in non-vertebrate chordates – or in any invertebrate group – we can classify odontoblasts as a vertebrate novelty, and as a novel neural crest cell fate. Indeed, consistent with the proposal for the continued evolution of neural crest-derived skeletal tissues, Kawasaki et al. (2004, 2005) have provided evidence from phylogenetic analyses of extracellular matrix proteins for the independent evolution of dentine in teleost fish and in tetrapods.

Chondrocytes/cartilage

Cartilage is an avascular, cellular supporting tissue with an extracellular matrix composed of fibrous proteins (typically, but not exclusively, type II collagen) and proteoglycans reviewed in Hall, 2005c). Regardless of germ layer origin, cartilage development is first detectable, histologically, as a condensation of preskeletal mesenchyme. Condensed mesenchymal cells proliferate, begin to secrete cartilage extracellular matrix, and subsequently undergo differentiation into chondrocytes (Hall & Miyake, 2000).

Developmental genetic and transcriptomic studies have highlighted a number of transcription factors and signalling molecules that function at different stages of cartilage condensation and differentiation. For example, members of the *SoxE* (*Sox9*) and *SoxD* (*Sox5/6*) families, as well as *Twist1/2* and *It2/3* are expressed in post-migratory cranial neural crest cells, and are required, in some capacity, for subsequent chondrogenic differentiation of these neural crest cells. Another group of transcription factors, including *Barx1/2*, *Cart1*, *Alx3/4*, *Bapx1* and *Runx1/2/3*, which are expressed in pre-cartilaginous condensations, appear to be

upstream of genes encoding cartilage extracellular matrix proteins, such as *Col2a* and *Aggrecan*. While the regulatory interactions among these factors remain poorly understood, a preliminary GRN for vertebrate chondrogenesis has been put forward (Cameron et al. 2009; Cole, 2011).

While ascidians lack cartilage or cartilage-like tissues, cephalochordates possess an acellular collagenous skeletal tissue that supports their pharyngeal arches. This collagenous tissue is secreted by pharyngeal mesoderm, and fills both the primary gill bars and secondary (tongue) gill bars of the pharyngeal basket (Fig. 3). This tissue is most likely homologous with the endodermally secreted acellular gill bar skeleton of enteropneust hemichordates (Fig. 3); both tissues have been classified as 'cartilage-like' based largely on their fibrillar protein composition, which includes type II collagen. Given this shared matrix property – and the location of this secreted skeleton in cephalochordate/hemichordate pharyngeal arches – it has been variously suggested that this skeleton represents an antecedent to the neural crest-derived vertebrate pharyngeal endoskeleton. This would imply that the cartilage developmental program (or, more specifically, the type II collagen developmental program) was co-opted to the neural crest from germ layers that primitively expressed type II collagen in non-vertebrate deuterostomes – e.g. endoderm (Rychel et al. 2006; Rychel & Swalla, 2007) or mesoderm (Meulemans & Bronner-Fraser, 2007).

Meulemans & Bronner-Fraser (2007) sought to determine whether the vertebrate cartilage developmental program was: first, co-opted to the neural crest as a conserved network; or second, assembled *de novo* in the neural crest by co-option of multiple factors from different germ layers or tissues. To this end, they analysed the expression patterns of 11 amphioxus orthologues of genes involved in vertebrate neural crest cell chondrogenesis. They noted that

while *AmphiTwist*, *AmphiEts*, *AmphiAlx* and *AmphiColA* (an orthologue of vertebrate *Col2a1*) are co-expressed in amphioxus pharyngeal mesoderm, expression of *AmphiSoxE* and *AmphiSoxD* was excluded from the pharyngeal mesoderm (though was expressed in the neural tube and the pharyngeal endoderm, respectively). *AmphiTwist*, *AmphiEts*, *AmphiSoxE*, *AmphiSoxD* and *AmphiColA* are also expressed in the notochord. Meulemans and Bronner-Fraser conclude that cellular cartilage is a vertebrate novelty resulting from the *de novo* assembly of a cartilage program in neural crest cells by co-option of individual components that primitively functioned in mesoderm.

While co-option of a neural crest chondrogenic developmental program from mesoderm seems reasonable, there is evidence that the extent to which this network was assembled prior to the origin of vertebrates – and, indeed, prior to the origin of chordates – is much greater than is currently appreciated. Tissues fitting the definition of cartilage (as stated above) clearly did not originate in vertebrates, but are, in fact, well represented in several major lineages of protostome invertebrates, including brachiopods, annelids, molluscs and arthropods (Cole & Hall, 2004a, 2009; Hall, 2005c; Cole, 2011). Most studies on the origin of vertebrate cartilage suffer from a lack of consideration of the many protostome invertebrate cartilages, and so consider the acellular collagenous skeleton of the cephalochordates and/or enteropneust hemichordate pharynx as the earliest tissue potentially recognizable as cartilage (or cartilage-like; de Beer, 1937; Zhang & Cohn, 2006; Meulemans & Bronner-Fraser, 2007; Donoghue et al. 2008). As discussed previously, this conclusion is based predominantly on the presence of type II collagen. However, *Col2a1* expression is not unique to cartilage; notochord, epithelial basement membranes and the vitreous humor of the eye all express *Col2a1*, but none of these tissues would be classified as cartilage (or

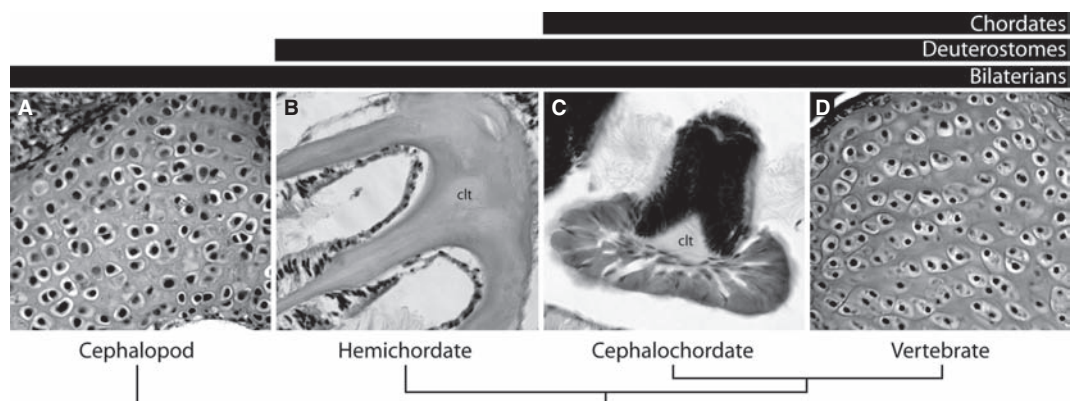


Fig. 3 (A) The cellular funnel cartilage of the short-finned squid *Illex illecebrosus* resembles the cellular cartilage of vertebrates in terms of its development via a mesenchymal condensation, chondrocyte cell morphology and some shared extracellular matrix components. This tissue is distinct from the acellular collagenous 'cartilage-like tissue' (clt) of (B) hemichordates (represented here by *Saccoglossus kowalevskii*) or (C) amphioxus (represented here by *Branchiostoma lanceolatum*). (D) Hyaline cartilage of the dwarf African clawed toad *Hymenochirus boettgeri* for comparison. It is likely that the neural crest co-opted a mesodermal chondrogenic program that predates the origin of deuterostomes. Images (A) and (D) provided by Dr Alison Cole.

even 'cartilage-like' – with the exception, perhaps, of the notochord).

Cephalopod molluscs possess cartilages that are remarkably similar to, and share a number of histological, developmental and biochemical properties with vertebrate cartilage. For example, the developing funnel cartilage of the cuttlefish *Sepia officinalis* is first visible as a mesenchymal condensation underneath a cuboidal epithelium. This condensation subsequently differentiates into a cartilage that closely resembles vertebrate hyaline cartilage, with an extracellular matrix that is rich in mucopolysaccharides. *Sepia officinalis* cartilage matrix stains positively both for fibrillar collagen and for chondroitin sulphate (two biochemical features of vertebrate cartilage extracellular matrix), and cephalopod chondrocytes are effectively indistinguishable from the chondrocytes of vertebrate hyaline cartilage in terms of cell morphology (Fig. 3). A detailed molecular assessment of *Sepia* cartilage and chondrocyte differentiation is anxiously awaited, as is an assessment of the development of other protostome invertebrate cartilages (e.g. the branchial cartilage of the horseshoe crab, *Limulus polyphemus*, and the feeding tentacle cartilage of sabellid polychaetes; Cole & Hall, 2004b).

Based on these data, and given the striking developmental and histological similarities between certain protostome cartilages (e.g. cephalopod hyaline cartilage) and vertebrate cartilages (Fig. 3), it seems inescapable that mesodermally derived cellular cartilage has a much deeper (i.e. pre-deuterostome) origin in metazoan phylogeny than having arisen with vertebrates. Homology of invertebrate and vertebrate cartilages implies either a widespread loss of cartilage in many invertebrate lineages, or the parallel evolution of cartilage and cartilage-like tissues in brachiopods, annelids, molluscs and arthropods (Cole & Hall, 2004b). It would also follow that a well-developed chondrogenic developmental program functioned to generate mesodermally derived cellular cartilage prior to the origin of vertebrates, and that this program was co-opted to the neural crest in vertebrates. Finally, while the acellular collagenous pharyngeal skeletal tissue of amphioxus and hemichordates is likely a primitive feature of the deuterostome pharynx that has been lost in echinoderms, ascidians and vertebrates, it may best be interpreted as the independent evolution of a type II collagen containing 'cartilage-like' tissue that converges on vertebrate cellular cartilage (Gillis et al. in press).

Conclusion

If we define a neural crest cell as a multipotent cell that delaminates from the neural tube and migrates peripherally to differentiate into one of the many cell types discussed in the Introduction (as seems reasonable, and as has been done by countless authors before us), it is immediately apparent that no known urochordate or cephalochordate cells meet these criteria. Strictly speaking, this would render

neural crest cells a *bona fide* novelty of the vertebrate crown group (Hall, 2005b; Hall & Kerney, 2011).

If, however, we consider cell populations in non-vertebrate chordates that meet some (though not all) criteria of the neural crest definition, then it is possible to recognize 'latent homologues' of the neural crest outside of vertebrates. It is important to recognize the neural crest as a continuous character that exists in multiple ordered states, and not simply a binary character that is either 'present' or 'absent'. *Snail*-expressing cells at the border of the neural plate in cephalochordates and ascidians likely represent the extent of neural crest elaboration in extant non-vertebrate chordates, with the origin of migratory neural crest cells from this region occurring along the vertebrate stem. Recognition of latent neural crest homologues allows for a more nuanced sequence for the evolution of the neural crest and of neural crest cells, with a gradual step-wise acquisition of a distinct neural crest cell identity, migratory ability and differentiated cell fates through chordate phylogeny – though, nevertheless, with the majority of neural crest cell fates acquired, either by innovation or co-option along the vertebrate stem (Donoghue et al. 2008).

Acknowledgements

Research support from the Natural Sciences and Engineering Research Council (NSERC) of Canada (Grant A5056 to BKH) is gratefully acknowledged. JAG was supported by a Royal Society Newton International Postdoctoral Fellowship, and by a NSERC postdoctoral fellowship. Images of *Illex* and *Hymenochirus* cartilage in Fig. 3 were kindly provided by Dr Alison Cole. This is reviewed journal paper number 300 from this laboratory.

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