SPOTLIGHT

Animal models for studying neural crest development: is the mouse different?

Elias H. Barriga\(^1\), Paul A. Trainor\(^2\)\(^,\)\(^3\), Marianne Bronner\(^4\)\(^,*\) and Roberto Mayor\(^1\)\(^,*\)

ABSTRACT

The neural crest is a uniquely vertebrate cell type and has been well studied in a number of model systems. Zebrafish, \textit{Xenopus} and chick embryos largely show consistent requirements for specific genes in early steps of neural crest development. By contrast, knockouts of homologous genes in the mouse often do not exhibit comparable early neural crest phenotypes. In this Spotlight article, we discuss these species-specific differences, suggest possible explanations for the divergent phenotypes in mouse and urge the community to consider these issues and the need for further research in complementary systems.

KEY WORDS: Neural crest, Mouse, Zebrafish, \textit{Xenopus}, Chicken, Gene knockout

Introduction

The neural crest is a multipotent stem cell population unique to vertebrates that contributes to development of the peripheral nervous system, craniofacial skeleton, adrenal gland, cardiac outflow tract, enteric ganglia of the gut, and other tissues (LeDouarin, 1982; Bronner and LeDouarin, 2012; Bronner, 2012; Mayor and Theveneau, 2013; Bhatt et al., 2013). The multipotency and migratory ability of the neural crest render it a cell type of great interest for regenerative medicine, as a therapeutic target for craniofacial defects (Trainor and Andrews, 2013) and for providing insights into vertebrate evolution (Simões-Costa and Bronner, 2013; Parker et al., 2014).

Many different vertebrates have emerged for studying neural crest biology in the past century: first amphibians and birds, followed by mouse, teleosts and, more recently, basal vertebrates such as lamprey and hagfish (Aybar and Mayor, 2002; Trainor, 2005; Sauka-Spengler et al., 2007; Ota et al., 2007). Loss-of-function analyses in animal models (primarily chick, \textit{Xenopus} and zebrafish) have established a pan-vertebrate neural crest gene regulatory network (GRN), in which inhibition of individual transcriptional components leads to severe neural crest induction phenotypes (Simões-Costa and Bronner, 2015). Surprisingly, however, mouse mutants of the same genes often lack comparable phenotypes, at least at early stages of neural crest development. Here, we explore possible reasons for these apparent discrepancies between mouse and non-mammalian models.

Methodologies to study genes involved in neural crest development

Dominant-negative constructs, antisense morpholinos and RNAs have been the methods of choice for transiently perturbing neural crest development in chick, \textit{Xenopus} and zebrafish embryos. In addition, several zebrafish neural crest mutants have emerged from forward genetic screens (Chakrabarti et al., 1983; Driever et al., 1996; Haffter et al., 1996). These loss-of-function approaches have yielded largely similar neural crest phenotypes and congruity, with only relatively minor differences, across non-mammalian species. Although TALENs and CRISPR-Cas9 are currently being employed across a wide variety of species to generate knockout animals (Peng et al., 2014), it is currently unknown whether they will generate similar results in neural crest studies across multiple species.

The mouse offers a powerful genetic model for studying gene function during development. Indeed, many mouse mutants exhibit neural crest phenotypes, particularly during craniofacial development, leading to important discoveries linked to human craniofacial malformations (Chai and Maxson, 2006; Sakai and Trainor, 2009; Trainor and Andrews, 2013; He and Soriano, 2013; Fantauzzi and Soriano, 2014). Strategies for generating mutants include spontaneous or radiation-induced mutations and gene targeting to produce null mice or conditional knockouts, using Cre/loxP or Flp/FRT technology.

There are several examples in which null mice completely lacking neural crest genes (e.g. \textit{Fgf8}, \textit{Pax3}, \textit{Pax7}, \textit{Notch1}) do not exhibit a failure of neural crest cell induction comparable to those observed in non-mammalian species (Conway et al., 1997; Frank et al., 2002). We describe potential reasons for these differences further below. To study mouse neural crest development in a spatiotemporal manner, the most widely used genetic tool for conditional gene knockout has been \textit{Wnt1-Cre} (Danielian et al., 1998). However, this may not be ideal for early neural crest development for several reasons. First, \textit{Wnt1} expression is not restricted to the neural crest domain but rather marks dorsal neural stem cells that contribute to both central nervous system and neural crest progenitors (McMahon et al., 1992). Second, in avian embryos, expression of \textit{Wnt1} commences well after neural crest induction (Basch et al., 2006). If the same is true in mice (which has not yet been determined), \textit{Wnt1-Cre} may initiate too late to elicit a neural crest induction phenotype. Third, the \textit{Wnt1-Cre} line was recently found to elicit activation of \textit{Wnt} signalling in ectopic locations (Lewis et al., 2013), particularly the midbrain, which might affect the interpretation of results related to the cranial neural crest.

Differences in loss-of-function phenotypes between mouse and non-mammalian models

Clear discrepancies exist in the loss-of-function phenotypes observed between non-mammalian and mouse models.
Table 1. Comparison of early neural crest (NC) phenotypes generated in loss-of-function experiments between non-mammalian and mouse models

<table>
<thead>
<tr>
<th>Gene/pathway</th>
<th>NC phenotype in non-mammalian models</th>
<th>NC phenotype in mouse</th>
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<tr>
<td><strong>NC inductive factors</strong></td>
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<tr>
<td>Wnt1 (canonical)</td>
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<tr>
<td>Wnt1</td>
<td>Impaired NC induction in Xenopus (mRNA [1]) and chick (Δ−/− [2])</td>
<td>No NC induction phenotype (KO [3,4])</td>
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<tr>
<td>Wnt3a</td>
<td>Impaired NC induction in Xenopus (MO [5]) and impaired cardiac NC formation in zebrafish (MO, mRNA [8]); NC expansion in chick and zebrafish gain-of-function experiments (mRNA [6,7])</td>
<td>No NC induction phenotype (KO [4,8-13])</td>
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<tr>
<td>Wnt8</td>
<td>Impaired NC induction in Xenopus (Δ−/− [14,15]) and zebrafish (MO [16])</td>
<td>No NC induction phenotype (KO [4])</td>
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<tr>
<td>Wnt1/Wnt3a</td>
<td>NA</td>
<td>No NC induction phenotype, late effect on NC maintenance (KO [3])</td>
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<td><strong>Retinoic acid (RARs)</strong></td>
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<tr>
<td>Fgf8</td>
<td>Impaired NC induction in Xenopus (Δ−/− [20]; MO [21]) and cranial patterning defects in zebrafish (mu−/− [22])</td>
<td>No NC induction phenotype, late effect on migratory cardiac NC maintenance (KO [23])</td>
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<tr>
<td>Notch</td>
<td>Impaired NC induction in Xenopus (Δ−/− [24], chick (Δ−/− [25]) and zebrafish (mu−/− [26,27]))</td>
<td>No NC induction phenotype, late effect on NC migration, proliferation and differentiation (KO−/− [28])</td>
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<td><strong>NC GRN SoxE</strong></td>
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<tr>
<td>Sox8</td>
<td>Impaired NC induction in Xenopus (MO [29]) and chick (MO [30])</td>
<td>No NC induction phenotype (KO [31])</td>
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<td>Sox9</td>
<td>Impaired NC induction in zebrafish (MO+mu−/− [32]) and Xenopus (MO [33,34]); impaired NC maintenance in chick (mRNA [35]; Δ−/− [36]; MO [37])</td>
<td>No NC induction phenotype, late effect on migration and differentiation (KO−/− [38,39])</td>
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<tr>
<td>Tfp2</td>
<td>Impaired NC induction in Xenopus (mu−/− [40]), Xenopus (MO [41,42])</td>
<td>No NC induction phenotype, late effect on NC differentiation (KO [43,44])</td>
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<tr>
<td>Foxd3</td>
<td>Impaired NC induction in zebrafish (MO [45]; mu−/− [46]), Xenopus (Δ−/− [47,48]) and chick (MO [49])</td>
<td>No NC induction phenotype, late effect on NC maintenance (KO−/− [50])</td>
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<td>Pax3/7</td>
<td>Impaired NC induction in Xenopus (MO [51]) and chick (MO [52]); partial inhibition of NC induction in zebrafish (MO [53])</td>
<td>No NC induction phenotype with respect to cranial and cardiac NC, but impaired trunk NC induction; late effect on cardiac NC migration (mu−/− [54])</td>
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<td>Msx1/2</td>
<td>Impaired NC induction in Xenopus (Δ−/− [55]); impaired NC induction in zebrafish when both msxb and msxc are inhibited (MO [56]); NC expansion in chick gain of function (57,58)</td>
<td>No NC induction phenotype, late effect on migration and differentiation in the double KO Msx1−/−;Msx2−/− [59]</td>
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<td>Snail1/2</td>
<td>Impaired NC induction in Xenopus (Δ−/− [60,61]; MO [62]); early migration phenotype in chick (AS [63]; MO [64])</td>
<td>No NC induction phenotype, early lethality (KO [65,66])</td>
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<td><strong>NC migration factors</strong></td>
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<tr>
<td>Wnt11</td>
<td>Impaired early NC migration in zebrafish (mu−/− [67]) and Xenopus (MO [68,69])</td>
<td>No NC migration phenotype (KO [4,70])</td>
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<td>Dvl</td>
<td>Impaired early NC migration in zebrafish (Δ−/− [67]), Xenopus (MO [69]; Δ−/− [71]) and chick (Δ−/− [72])</td>
<td>No early NC migration phenotype, late effect on cardiac NC differentiation (KO [4,73])</td>
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<tr>
<td>Vang1/2</td>
<td>Impaired NC migration in zebrafish (mu−/− [74]) and Xenopus (MO [69])</td>
<td>No NC induction or migration phenotype (KO, KO−/− [75])</td>
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<tr>
<td>Sdc4</td>
<td>Impaired NC migration in zebrafish and Xenopus (MO [74])</td>
<td>No NC induction or migration phenotype, late effect on cranial cartilage (KO [76])</td>
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<td>N-cad/Cdh2</td>
<td>Impaired NC migration in zebrafish (MO [78]; Δ−/− [77]), Xenopus (MO [78]) and chick (BA [79]; Δ−/− [80])</td>
<td>No early NC migration phenotype, late effect on cardiac NC differentiation (KO−/− [81])</td>
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</table>

These are examples of genes that show a clear discrepancy in loss-of-function phenotypes between non-mammalian (in at least two species) and mouse. Many other examples that show a similar discrepancy with mouse are not included here because they have been studied in only one non-mammalian species. MO, morpholino; AS, antisense oligonucleotide; mu−/− or mu−/+, homozygous or heterozygous mutant; Δ−/−, dominant negative; KO, full knockout; KO−/−, Wnt1-Cre KO; BA, blocking antibody; mRNA, gain-of-function. NA, not applicable. RAR, retinoic acid receptor.
Summarised in Table 1 are those cases in which the same gene has been studied in more than one non-mammalian species. Key examples are discussed below.

There is strong evidence that canonical Wnt signalling is essential for neural crest induction. Although the requirement of a specific Wnt ligand can vary among species, the inhibition of Wnt signalling, their receptors or downstream components has a dramatic effect on neural crest formation in chick, Xenopus and zebrafish (Saint-Jeannet et al., 1997; LaBonne and Bronner-Fraser, 1998; Garcia-Castro et al., 2002; Lewis et al., 2004; Sun et al., 2008; Patthey et al., 2009; Steventon et al., 2009; Elkouby et al., 2010). Surprisingly, however, knockout of canonical Wnt genes in mice does not affect neural crest induction (Takada et al., 1994; Greco et al., 1996; Yoshikawa et al., 1997; Ikeya et al., 1997; Lee et al., 2000; Ikeya and Takada, 2001; Rodriguez-Mari et al., 2005; Nakaya et al., 2005; van Amerongen and Berns, 2006).

During the induction process, the neural crest GRN becomes activated (Betancur et al., 2010a; Steventon et al., 2005). This transcriptional cascade is highly conserved, even in the basal jawless vertebrate lamprey (Sauka-Spengler et al., 2007; Nikitina and Bronner-Fraser, 2009; Nikitina et al., 2008). Two of the key genes are Snail1/2 and Foxd3, both of which are required for neural crest induction in zebrafish, Xenopus and chick (Nieto et al., 1994; LaBonne and Bronner-Fraser, 2000; Pohl and Knöchel, 2001; Sasai et al., 2001; Aybar et al., 2003; Lister et al., 2006; Stewart et al., 2006; Taneyhill et al., 2007; Shi et al., 2011; Fairchild et al., 2014). However, neural crest induction is unaffected in Snail1/2 or Foxd3 knockout mice (Jiang et al., 1998, Murray and Gridley, 2006, Teng et al., 2008), although Foxd3 knockouts exhibit a defect in late neural crest maintenance (Teng et al., 2008).

A defining feature of the neural crest is its migratory capacity. Many of the cellular mechanisms underlying migration are conserved across species; for example, in Xenopus, zebrafish and chick, non-canonical Wnt signalling is essential for normal neural crest migration (De Calisto et al., 2005; Carmona-Fontaine et al., 2008; Matthews et al., 2008a; Matthews et al., 2008b; Rios et al., 2011; Banerjee et al., 2011). By contrast, inhibition of non-canonical Wnt signalling in mouse does not affect migration (Majumdar et al., 2003; van Amerongen and Berns, 2006; Pryor et al., 2014).

Thus, key factors implicated in the induction and migration steps of neural crest development in non-mammalian species appear to be dispensable for those stages in mouse, at least as assayed by the most commonly used methodologies. This raises the intriguing possibility that the mechanisms of early neural crest development are divergent in the mouse lineage, or that there is a higher redundancy of genes in mouse compared with non-mammalian species. These and other alternatives are discussed below.

Possible explanations for the differences between mouse and other vertebrates

As detailed in Table 1, genes required for different steps of early neural crest development, from induction to migration, are largely conserved between avians, amphibians and fish, whereas specific knockouts of homologous mouse genes only have later neural crest defects. Several possible explanations may account for these differences.

First, there may be limited conservation of neural crest genes between mouse and non-mammalian organisms. It is well known that there is a greater temporal separation between neurulation and neural crest cell induction in mice than in frogs, fish and (to a lesser extent) avians. Thus, there might be fundamental differences in the temporal induction of neural crest cells in non-mammalian versus mammalian species, perhaps accompanied by differences in the neural crest GRN. However, given that the expression and function of neural crest GRN components are conserved between fish, frog, birds and lamprey (Simões-Costa and Bronner, 2013), this seems unlikely and non-parsimonious. The relevance of these differences, if any, should be further explored to gain a deeper understanding of the conservation and diversity of neural crest cell induction mechanisms across species. This highlights the importance of developing other mammalian model systems, such as the rabbit or pig (Vadasz et al., 2013).

Second, the Wnt1-Cre line might be inappropriate to study early stages of neural crest development, as the initiation of neural crest development, at least in non-mammalian species, occurs prior to the initiation of Wnt1 expression. In addition, even though Wnt1-Cre lineage tracing labels most neural crest cells in mice, if a gene of interest or prospective neural crest cell is present in a domain that is broader than that demarcated by Wnt1, then Wnt1-Cre may not excise the gene completely and adjacent cells might compensate. Thus, it is crucial to explore these issues further during mouse development to ascertain the degree of conservation and diversity of neural crest induction across species.

Third, technical limitations of the Wnt1-Cre line, such as ectopic activation of Wnt signalling, may confound some analyses, as increasing Wnt signalling could promote neural crest induction and thereby rescue the phenotype.

Fourth, there might be redundancy or compensation in the mouse. Many neural crest GRN transcription factors have been duplicated during evolution, perhaps leading to paralogous factors or the co-option of members of other gene families assuming the same function in the GRN. Like all extant models, the mouse is derived and has a particularly long phase of early development, perhaps enabling additional gene compensation and/or rewiring of its neural crest GRN.

Finally, non-specific off-target effects from transient knockdown techniques may affect the interpretation of phenotypes. Use of morpholinos or dominant negatives is less specific than the production of null or conditional mice, perhaps partially accounting for differences in phenotypes. However, the reproducibility of most phenotypes across species, together with important controls such as rescue experiments and comparison with zebrafish mutants where they exist, makes this less likely.

Conclusions

Regardless of the underlying reasons for the differences between mouse and other species, we recommend exercising caution in interpreting negative results from knockout mice regarding a role in early neural crest development. The absence of an obvious phenotype does not necessarily mean that the gene of interest is not normally involved in the process. It could highlight a redundant function in mice rather than a fundamental difference with other species. To date, it is unknown whether differences between mouse and non-mammalian species reflect peculiarities of mouse development or are generally true for mammals. This is a particularly important question because the mouse is typically considered a better model for human development (and developmental disease) than non-mammalian species. Future neural crest studies using other mammalian species are therefore needed to clarify the degree of conservation within the mammalian lineage, and hopefully to provide insight into human neural crest development and defects thereof.
In the mouse, studies using a new Wnt1-Cre line that corrects ectopic expression (Lewis et al., 2013) will be very informative, as will the development of other Cre drivers that function earlier in development at epiblast stages and are restricted to the prospective neural crest; hence, considerable attention from the field needs to be devoted to developing new spatiotemporally appropriate lines. Equally, transient knockdown techniques suffer from problems including dilution over time and a requirement for careful controls to guarantee specificity. In the long term, analogous knockdown experiments must be performed across numerous species, including other mammals, to systematically examine the role of a gene of interest and its paralogues. The recent advent of CRISPR-Cas9 will facilitate comparative studies of this kind across a range of important vertebrates to definitively establish key players in neural crest development and GRN changes during vertebrate evolution. This will help establish the degree of redundancy versus species specificity in the molecular and cellular mechanisms governing neural crest induction and migration. Although an overlap of core GRNs components is expected, there might also be many surprises.

The availability of new technologies that enable analysis across a wide spectrum of species makes for exciting times in the field. Such studies are essential to determine which animal model or combination of models is best suited for understanding neural crest development, evolution and disease.

Competing interests
The authors declare no competing or financial interests.

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