

Chapter 5

Conclusions

5.1 Summary

The experimental studies presented in this thesis provide the first molecular insights into the cellular processes of assembly, and aggregation of neural crest and placodal cells into discrete cranial sensory ganglia. The results show that throughout trigeminal gangliogenesis (starting from ingression of placodal neurons to forming a condensed ganglion), neural crest and placodal cells are highly intermingled *in vivo* and *in vitro*.

To test their interactions, we performed classical tissue ablation experiments with several key improvements. We were able to make clearer conclusions about their relationship during gangliogenesis since we performed ablation at the stages that minimize regeneration, analyzed the ablated embryos at the time of ganglion formation, and most importantly, carefully distinguished the two cell types by cell or molecular markers which provided information on both the extent of ablation and also the causal effects on the other cell population. Results show that after ablation of neural crest, placodal neurons failed to integrate, instead forming separated aggregates and aberrant central and peripheral axonal projections. On the other hand, ablation of the placodal ectoderm led to loss of ganglion, whereby neural crest cells failed to coalesce, showing that the presence of placodal neurons is essential. This provides the first insight into the role of the placodal tissue on neural crest, which had been difficult to obtain due to regeneration issues noted by previous investigators (Hamburger, 1961; Stark et al., 1997), as well as the lack of use of markers to distinguish the two cell types. The data demonstrate that interactions between neural crest

and placodal cells are necessary to drive ganglion formation and are highly interdependent. Bi-directional neural crest–placode signaling likely mediates this process.

The concurrent expression of Slit1 in the migratory neural crest and its cognate receptor Robo2 in the placodal cells during trigeminal ganglion assembly prompted us to ask whether this ligand–receptor pair may mediate neural crest–placode interaction. Loss-of-function of either Robo2 or Slit1 *in vivo* resulted in severely disorganized assembly of placodal neurons into dispersed or aberrantly condensed ganglion, consistent with the effects of neural crest ablation. Furthermore, the aberrant pattern of placodal neurons by blocking Robo2 function caused wild type neural crest cells to also coalesce abnormally. The results establish a critical role of Slit1–Robo2 signaling in organizing placodal neurons and underscores the reciprocal nature of neural crest–placode interaction. A striking defect in Slit1–Robo2 deficient embryos is the abnormal coalescence of ganglion, suggesting a role for cell adhesion in this process.

As a possible downstream mechanism, the function of the cell–cell adhesion molecule N-cadherin was tested during trigeminal gangliogenesis. Our results reveal a role for N-cadherin in mediating aggregation of placodal neurons into the ganglion downstream of Slit1–Robo2. We show that Slit1–Robo2 interaction can positively regulate N-cadherin mediated placodal adhesion by modulating N-cadherin expression. Since neural crest and placodal neurons are highly intermixed, condensation of ganglia may require adhesion of not only placode–placode, but also crest–crest and crest–placode cells. Our results also suggest that another adhesion molecule, Cadherin-7, may play a complementary role with N-cadherin in driving ganglion coalescence. Finally, the mechanisms of Slit1–Robo2 and N-cadherin may be general for all cranial ganglia of dual origin, as the expression and function of these molecules in the epibranchial regions closely resemble that in the trigeminal ganglion.

In summary, the results of this thesis have identified several key molecular players involved in neural crest–placode formation of the chick cranial sensory ganglia. We hypothesize that the process of cranial gangliogenesis can be divided into at least five distinct but overlapping steps—1) neural crest migration to the site of ganglion assembly, 2) cell fate specification and differentiation of placodal cells into neurons in the ectoderm, 3) ingression of placodal neurons into the ganglion anlage, 4) interactions between neural crest and placodal neurons at the border of ectoderm and mesenchyme, and within the mesenchyme, and 5) the condensation of the intermixed neural crest cells and placodal neurons into discrete ganglion structure. It is interesting to note that several of these steps are interactive. For example, the production and ingression of placodal neurons from the surface ectoderm continues throughout most of gangliogenesis while the ganglion is condensing, and interactions between neural crest and placode underlie this entire process.

We show that Robo2–Slit1 mediated neural crest– placode interaction has an important role in several of these steps at times of placodal ingression and coalescence (see Fig. 1 for a schematic summary). The downstream mechanisms of Robo2 dependent signaling in mediating ingression is not clear, but we elucidate a critical role for N-cadherin in placodal aggregation and suggest that ganglion coalescence may be driven by a novel interplay of two distinct cadherins, Cadherin-7 and N-cadherin, from which we propose two possible models (see Fig. 2). Finally, three points of difference between the early (during gangliogenesis) and late (in the nearly mature ganglion at embryonic day 12 (D'Amico-Martel and Noden, 1983)) stages of trigeminal ganglion development can be emphasized. First, the description of the segregation of placodal cells to the distal region as in the nearly mature ganglion does not apply during gangliogenesis since placodal cells are intermixed with neural crest cells throughout almost the entire ganglion region. Second, neural crest cells remain undifferentiated through gangliogenesis at least up to stage 18. Third, placodal neurons during gangliogenesis have generally short processes such that

disorganization of axonal projections was accompanied by displacement of neuronal cell bodies in the same pattern. This could have interesting implications on how similar signals may mediate both migration of early trigeminal neuronal cells and guidance of their growing axons.

The results provide the first molecular insights into the roles of a putative signaling by Slit–Robo and cadherin-mediated aggregation that underlie neural crest–placode formation of the cranial sensory ganglia. They demonstrate the importance of heterotypic cell interactions during cranial gangliogenesis for cellular condensation, casting light on the critical interplay of cell–cell communication and cell adhesion in formation of complex structures in the developing vertebrate embryo.

5.2 Future perspectives

The work presented in this thesis lays a foundation for many more questions about the fascinating process of cell–cell interactions and morphogenesis during cranial sensory ganglia formation. Many aspects of neural crest–placode interactions remain to be investigated to fully understand the intricate and complex process of cranial ganglia assembly. In this thesis, I have identified a critical role for Slit1–Robo2 in mediating one aspect of neural crest–placode interactions: the signaling from neural crest to placodal cells for proper placodal cell organization. However, other aspects of their interactions remain unknown at a molecular level. These include the mechanisms underlying the reciprocal or reverse signaling from placode to neural crest cells, whether multiple signaling pathways are involved in their communication, and finally if there are direct protein interactions bridging neural crest–placode cell–cell contacts. Because Robo2 is not expressed by neural

crest cells, the data suggest that the reciprocal signaling would involve a Robo2 independent pathway. Although we cannot rule out the possibility that reverse signaling is mediated by Slit–Robo interactions but through a different Robo receptor, this seems unlikely for several reasons. Robo1 does not appear to be a candidate receptor for neural crest–placode interaction during trigeminal gangliogenesis, since its expression was not detected strongly or specifically in either neural crest or placodal cells at times of ganglion assembly (st.12–18). The possible functions for Robo3 and Robo4 were not examined here, but based on their expressions in other systems, they appear to be restricted to the central nervous system (Sabatier et al., 2004) and endothelial cells (Park et al., 2003; Suchting et al., 2005) respectively. Therefore, it is more likely that the reciprocal signaling relies on other yet unknown signaling molecules.

The precise way in which Slit1–Robo2 signaling regulates trigeminal ganglion assembly over time remains to be determined. A range of defects was found after Slit1–Robo2 perturbation including defects in placodal ingression, organization, coalescence, and axonal guidance of cranial ganglia. This suggests that Slit1–Robo2 may have a range of different spatiotemporally regulated functions to participate in proper ganglion formation, or its actions at earlier events led consequentially to the later defects. Since the expression of Robo2 begins by about stages 11–12 in the trigeminal placodes and that perturbation of Robo2 just prior to ingression at mostly stages 9–10 did not appear to affect placodal cell differentiation or cell number, we suspect that its role in placodal ingression would represent its earliest action. We provide two possible models for Slit1–Robo2 actions that may explain the different defects. First, the delayed ingression caused by blocking Robo2 led subsequently to abnormal organization and coalescence of the placodal ganglia, or second, different actions of Robo2 may mediate placodal migration from the surface ectoderm versus organization and coalescence of cells into ganglion. We favor the latter model since we find that although ingression occurs at later stages of

ganglion assembly, the organization of the ganglion remains chaotic over time, or alternatively, we think that the combination of both models may explain the different defects. Further understanding of the downstream and intracellular events activated by Robo2 in the different placodal regions as well as testing the consequence of ganglion assembly when Robo2 is blocked only in the ingressed placodal cells would clarify this point.

The conventional model assumes that Robo–Slit signaling involves binding of the receptor and ligand that mediates its actions; however other form of interactions between the ligand and receptor and between the receptors themselves may exist. This is especially relevant to our finding that Robo2 signaling plays a critical role for coalescence of placodal neurons, suggesting a role in placodal cell–cell adhesion. We have so far demonstrated a role for N-cadherin in placodal cell adhesion as discussed in Chapter 3 and its implicated link with Slit1–Robo2. However, there are at least two other possible models for Robo2 interactions that may also mediate placode–placode adhesion.

First, we cannot rule out the possibility that binding of Robo2 to itself also is involved in placode–placode adhesion. This is consistent with findings that Robos can bind homophilically *in vitro* and that Slit independent functions of Robos have been implicated in other systems (Hivert et al., 2002; Parsons et al., 2003). Second, an interesting model called the ‘Slit sandwich’ by Kraut and Zinn (2004), proposes that a Robo–Slit–Robo interaction mediates signaling between the visceral mesoderm and chordotonal neurons that blocks migration of the latter in the fruit fly (Kraut and Zinn, 2004). A similar scenario may apply to placode interactions in our system. Accordingly, the interaction between placodal cells may be mediated by binding of Robo2 from one cell to its cognate ligand, Slit1, extracellularly, which in turn binds to Robo2 on the neighboring cell. This mechanism may explain the role of the Robo2 in cell adhesion, but not its function in promoting placodal ingression. However, it is not clear if a single Slit can bind

to two Robo receptors. Alternatively, the ‘Slit sandwich’ may involve dimerization of Slit to bind Robos on neighboring cells. These additional scenarios provide alternative models for Slit1 and Robo2 interactions during trigeminal gangliogenesis that depart from conventional view of a ligand–receptor binding. Uncovering the molecular interactions of Slit1 and Robo2 at different times and in different regions of the forming ganglion (e.g. surface placodal ectoderm versus ingressed placodal cells) is important for understanding how this signaling mechanism drives proper ganglion assembly.

Here, I present six possible future directions that stem from this study for addressing some of these open questions. The study of neural crest–placode interactions would not only elucidate a critical process during development of the peripheral nervous system, but also serves as an excellent new model to study and unravel cell–cell signaling and subsequent cellular changes *in vivo* that have been traditionally studied *in vitro*.

First, uncovering the cadherin-based mechanism of ganglion coalescence would provide important insights into the process of cellular condensation (thus organ formation) and potentially novel cadherin interactions *in vivo*. As discussed in Chapter 4, the aggregation of neural crest and placodal neurons may rely on either a homotypic or a mix of homotypic and heterotypic cadherin interactions. This study may also elucidate whether cadherin binding may mediate direct neural crest–placode cell–cell contacts. Exploration of other classical and non-classical cadherins during ganglion formation may also elucidate additional molecular players.

Second, examining Slit1–Robo2 interaction at the protein level during gangliogenesis is a necessary step for understanding the function of this signaling pair more deeply. Previous Slit–Robo studies in *Drosophila* have shown interesting differences between mRNA and protein expressions during midline commissural axon crossing as well as specific localization of Slit and Robo proteins at the cell surfaces in the forming heart tube that provided important information about their functions that might otherwise be

obscured by only mRNA expression. Protein expression of Slit1 and Robo2 at the different stages of ganglion formation would provide information on the spatiotemporal pattern of when and where signaling might be occurring. This may be different from the uniform expression of Slit1 on the migratory neural crest stream and Robo2 on all the placodal cells that the mRNA expressions suggest. Along with immunoblot studies, this would also address whether proteolytic processing of Slit1 occurs in the neural crest, as has been shown for Slit2 in other systems, which may modulate its signaling activity. Furthermore, the role of heparan sulfate proteoglycans in regulating distribution and activity of Slit1 signaling could also be elucidated.

Third, exploring the downstream transduction of Slit–Robo signaling in the placodal neurons would elucidate the precise intracellular mechanisms dictating the placodal cellular changes during gangliogenesis. The interaction of Slit–Robo could elicit different outcomes at different steps of placodal development (e.g. placodal ingression versus placodal condensation). Our results showing a range of effects upon Robo2 inhibition are consistent with this possibility. Screening for changes in gene transcription downstream of Robo2 activation and inhibition and for presence and activities of candidate intracellular proteins (e.g. Rho GTPases, Abl tyrosine kinase, and other actin regulators, among others) would provide further information about the downstream events. This information in addition to functional experiments involving removal of the Slit source may also clarify whether Slit1 independent functions may also be present during gangliogenesis.

Fourth, little is known about the later development of the trigeminal ganglion and the role of Robo2 therein. An area ripe for exploration is the molecular and cellular mechanisms underlying the differential differentiation of neural crest cells into neurons in the proximal ganglion and glia along the entire region. It would be interesting to ask if interactions with placodal neurons have a role in this process. Furthermore, does the embryonic origin of the sensory neurons in the ganglion correlate with different neuronal

cell types of different functions (e.g. nociceptors, thermoreceptors, mechanoreceptors)? Does Robo2 signaling have a role in any of these events?

Fifth, it would be fruitful to dynamically analyze neural crest–placode interactions through the process of ganglion formation by time-lapse imaging in real time. This would uncover indispensable information about the dynamics and cellular processes underlying this developmental event. Imaging placodal neurons as they ingress, interact, and coalesce with neural crest cells in the living embryo in real time will be an exciting future objective that will provide direct insights into the sequence of events and cellular changes leading to ganglion formation.

Finally, exploring whether neural crest–placode interactions and the roles of Slit–Robo signaling and Cadherin dependent cell adhesion are conserved in mediating cranial sensory gangliogenesis in other vertebrate species (e.g. mouse, zebrafish, and *Xenopus*) promises to reveal the evolutionary significance of these mechanisms. In line with the possibility that Slit–Robo may have a conserved role in cranial gangliogenesis, Robo2 is expressed by the trigeminal ganglion in zebrafish (Lee et al., 2001) and mouse embryos (Ma and Tessier-Lavigne, 2007), but its role in ganglion formation is elusive. Taken together, the findings presented in this thesis motivate many exciting future investigations and research directions.

Figure 1. Multiple possible roles for Robo2 dependent signaling during cranial gangliogenesis. Three major defects are found in Robo2 deficient embryos as compared to wild type (WT) or control cases. First, ingression appears to be delayed in Robo2 inhibited placodal neurons at early stages of migration. At times of coalescence, Robo2 deficient placodal neurons do not assemble in their normal positions and are more disorganized while their axonal projections are affected in the same way. The aberrant organization of placodal neurons also caused non-cell-autonomous effects on the assembly of neural crest cells which mirrored the same abnormal placodal patterning. This is likely mediated by other, as yet unknown, molecules involved in the reciprocal signaling from placode to neural crest cells. Perturbation of Robo2 dependent signaling in placodal neurons causes a range of defects, which may suggest a role for Robo2 at different steps of ganglion formation in particular to cell migration and coalescence.

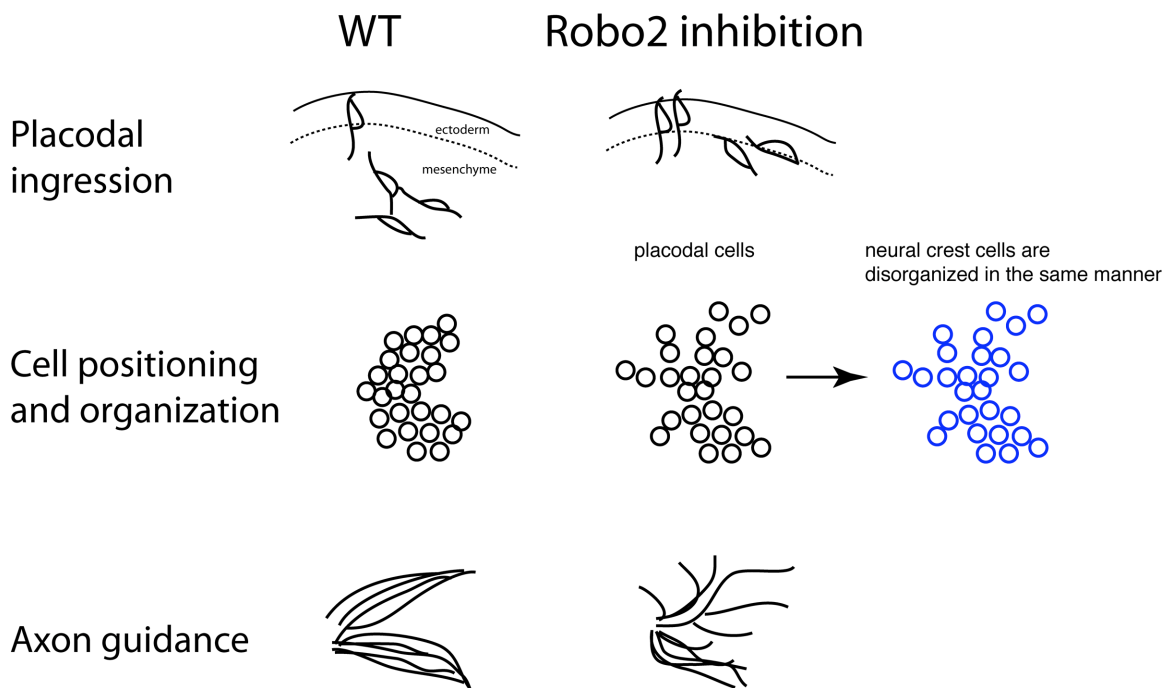
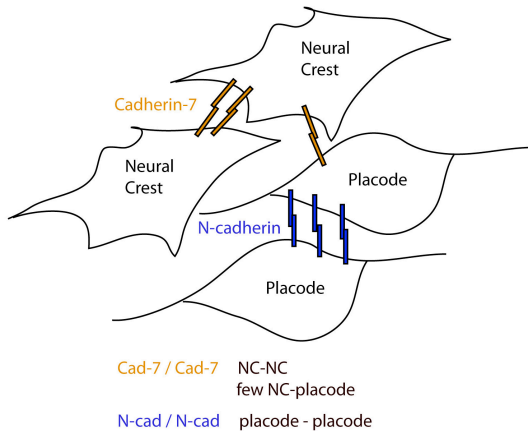


Figure 2. Two possible models for cadherin-based cellular condensation during trigeminal ganglion formation. Model 1 proposes that heterophilic binding between Cadherin-7 (Cad-7) and N-cadherin (N-cad) does not occur and therefore any neural crest–placode cell–cell adhesion would rely on a few number of Cad7–Cad7 interaction between the few placodal neurons that express the Cad-7 protein and the Cad-7 positive neural crest cells. Departing from the conventional view of the homophilic binding of cadherin molecules, Model 2 presents the alternative hypothesis, by which heterophilic interaction is present between Cad-7 and N-cad to mediate crest–placode adhesion.

Model 1: Homophilic cadherin interactions drive coalescence.



Model 2: Mix of heterophilic and homophilic cadherin interactions drive coalescence.

