# ONTOGENESIS OF MURINE NEURAL CREST DERIVATIVES

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# Summary

The neural crest is an epithelial domain within the neural and non-neural ectoderm. This domain is generated along the anterior-posterior axis-giving rise to many different cell types. The key difference between cranial and trunk neural crest is the ability of cranial neural crest to give rise to mesectoderm. However the origin of mesectoderm has not been unequivocally proven. Furthermore the issue of whether neural crest cells are pluripotent or fate restricted has also been controversial. In the current study we try to address the issue of whether cell fate and origin are linked in the neural crest and propose that there are two populations of delaminating cells with distinct cell fates.

To determine the origin of neural crest cells, we looked at the expression of neural crest markers within the neural and non-neural ectoderm defined by Sox1/N-cadherin and E-cadherin/L-CAM respectively. Cell fate of the neural domain was determined by Sox1 Cre mediated activation of reporter in the mouse or by DiI labeling of the two domains in the chick. We show that in cranial regions, there are two populations of cells delaminating within the neural fold. The first cells to delaminate originate from the non-neural ectoderm expressing E-cadherin/L-CAM; the second wave of cells delaminate from the neural ectoderm expressing Sox1/N-cadherin and the origin coincides with the fate that cells adopt – i.e., mesectoderm and neural derivatives respectively. Here we provide evidence that fate restriction occurs prior to cell delamination and that cell fate is linked to the time and site from

which cells delaminate. These observations have implications for understanding how neural crest are formed and patterned.

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# List of Abbreviation

| | |
|---|---|
| EMT | Epithelial to mesenchymal transition |
| BA | Branchial arch |
| BMP | Bone morphogenetic protein |
| FGF | Fibroblast growth factor |
| YFP | Yellow fluorescent protein |
| DIC | Differential Interference Contrast |
| DiI | 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate |
| Ecad | E-cadherin |
| Ncad | N-cadherin |
| FN | Fibronectin |
| **β**-DG | **β**-dystroglycan |

# Chapter 1: Introduction

## 1.1 The neural crest as a model system

This study tries to address the origin and fate of the neural crest, an embryonic cell population with exquisite migratory properties and great diversity of differentiation potential. This model is unique to vertebrates and mechanisms governing neural crest ontogeny have helped in the understanding of the processes involved in cranial-facial and heart syndromes as well as tumor invasion and metastasis. A review of the current literature of how neural crest is formed and lineage tracing experiments is detailed in the following sections to address these issues.

The neural Crest is also a unique model to understand emergence of vertebrates, since it is considered to contribute significantly to craniofacial structures. Most studies however focused on molecular and cellular mechanisms for cell specification, cell migration and cell differentiation. In particular this model system has been used:

1. To understand epithelial to mesenchymal transition (EMT). Neural crest cells are generated within the ectoderm and have to delaminate from the epithelia to form migratory mesenchymal cells.
2. To understand cell migration. Neural crest cells generated at different times take distinct pathways of migration, and this is controlled in part by differential expression of ligand/receptor pair by the cellular environment and neural crest cells. For example they utilize Slit/Robo

and Semaphorin/Plexin ligand/receptor pairs to guide their migration. In particular neural crest cells that form the enteric nervous system migrate from the pharynx to colonize the entire colon. The cells are guided by a gradient of GDNF within the gut, which activates c-Ret expressed by enteric neural crest cells.

3. To understand lineage restriction, neural crest can form a multitude of cell types: neurons, glia, melanocytes, smooth muscle cells, osteocytes, and chondrocytes.
4. To understand neurocristopathies. Defects in neural crest formation/migration cause a multitude of congenital defects like cleft palate or Hirschsprung disease (failure of crest cells to colonize and differentiate in the colon) and neurofibromatosis type 1 where there is a gene defect in NF1 results in and causes aberrant proliferation of neural crest derived glial cells.

## 1.2 General properties of neural crest

The term neural crest was first coined by Marshall to describe cells that delaminate from the neural ridge to form the cranial ganglia (Marshall, 1879). This group of cells is characterized by three properties:

1. They originate from the border between neural and non-neural ectoderm.
2. They undergo epithelial to mesenchymal transition.
3. They migrate away from the site of formation to their final destination and give rise to a multitude of different cell types

Neural crest cells are able to give rise to many different cell types; neurons, glia, endocrine cells, melanocytes, and connective tissue (Le Douarin and Kalcheim, 1999; Le Douarin et al., 2004). They are induced along the entire anterior posterior axis and can be subdivided into four regions (fig. 1.1):

1. Cranial neural crest
2. Cardiac and vagal neural crest
3. Trunk neural crest
4. Sacral neural crest

Cranial neural crest can be defined as the neural crest that is derived from the posterior prosencephalon to rhombomere 8. Cardiac neural crest is derived from rhombomere 6 to 8. Vagal neural crest cells are derived from the neural

tube between somites 1 to 7. Sacral neural crest cells originate from levels after somite 28 whereas trunk neural crest originates from levels between the vagal and sacral neural crest cells.

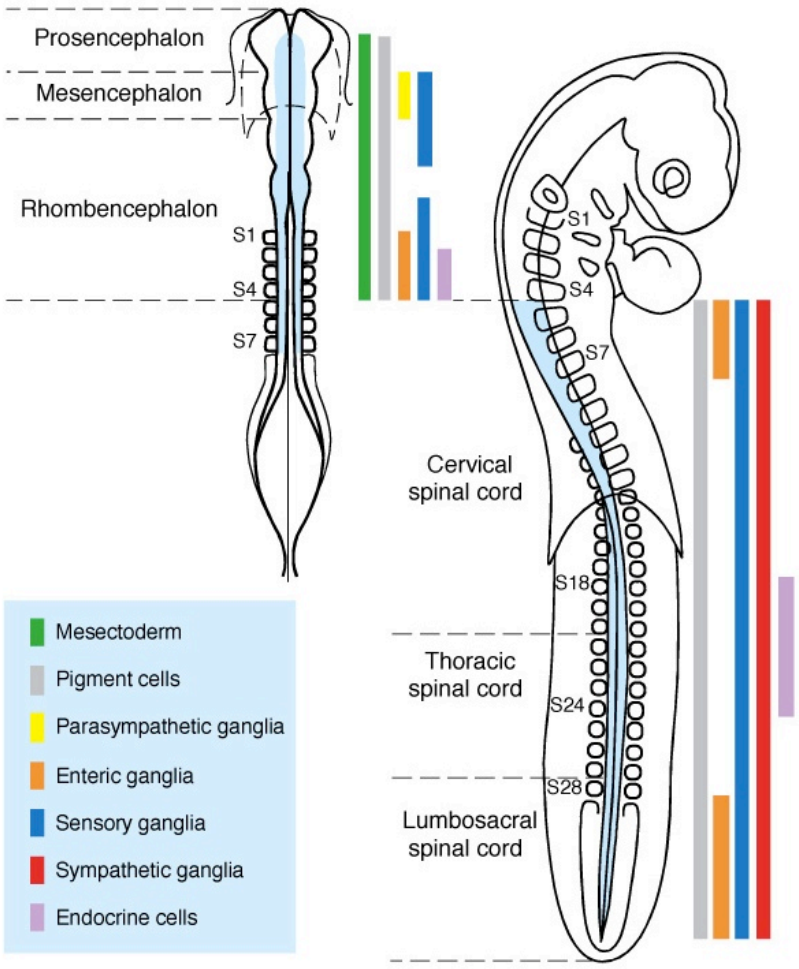


**Fig. 1.1: Fate map of neural crest cells along the anterior posterior axis.** Figure taken from a review by Le Douarin showing neural crest derivatives from different axial levels (Le Douarin et al., 2004).

The neural crest is a transient territory situated at the junction between the neural and non-neural ectoderm. Neural crest cells undergo EMT from this region and subsequently forms different cell types. As mentioned before, neural crest cells can be broadly categorized into cranial and trunk neural crest. Both the cranial and trunk neural crest give rise to neurons, glia and

melanocytes. However cranial neural crest has the unique property to form mesectoderm that subsequently differentiates into bone, cartilage and other connective tissue (fig. 1.1). Although it has been shown in amphibian and zebrafish that the trunk neural crest can also form mesectoderm in the dorsal fin (Raven, 1931; 1936; Smith et al., 1994).

At present it is unclear what accounts for this difference in the differentiation potential between cranial and trunk neural crest. It is also unclear when fate restriction occurs for all lineages; in particular it is still a matter of debate whether neural crest cells are pluripotent rather than being fate-restricted initially. Furthermore, some cells start to delaminate early from the lateral ectoderm and it has been speculated that these cells might give rise to mesectoderm, raising the issue that cell fate and origin may be linked.

## 1.3 Rationale for current study

### 1.3.1 Definition of the neural crest and origin of cranial mesenchyme - History

In the embryological literature of the 19th century, relationships among tissues were determined by morphology. For example, the fact that endodermal cells contain many yolk granules whereas ectodermal cells had fewer yolk granules allowed these two tissues to be distinguished early in development (Platt, 1893).

The first studies on the neural crest focused on the formation of the spinal ganglia (His, 1868; Balfour, 1876; Beard, 1888). Wilhelm His first identified the neural crest in 1868 as the source of spinal ganglia, however at that time he called these cells “Zwischenstrang”, the intermediate cord (His, 1868). In 1879, the term neural crest was first used by Marshall to describe cells that gave rise to the spinal and crania ganglia. He defined the neural crest as “the longitudinal ridge of cells which grows out of the neural canal and from which nerves, whether cranial or spinal, arise” (Marshall, 1879).

The origin of mesenchyme in the head was first briefly described by Kastschenko in 1888 (Kastschenko, 1888). Subsequently in 1893, Platt used the term mesectoderm to describe ectodermal cells that gave rise to mesodermal derivatives (visceral cartilage of the cranial skeleton of the mud puppy). She concluded that mesectoderm originated from the lateral non-neural ectoderm and was separate from the neural crest which was found in the dorsal neural ectoderm (Platt, 1893). This was very controversial at the

time because the dominating line of thought was the germ layer theory, which stated that bone and cartilage were exclusively derived from mesoderm and cannot be derived from the ectoderm.

However in 1921, Landacre disagreed with Platt's conclusion that it was the lateral ectoderm that gave rise to mesectoderm instead he concluded that it was the neural crest that gives rise to mesectoderm (Landacre, 1921). In his paper he presents a number of studies by other authors on the possibility of the lateral ectoderm origin of mesectoderm (Goronowitsch, 1893; Kupffer, 1895; Koltzoff, 1901) and discusses what are the possible reasons for the "erroneous" conclusions for the lateral ectoderm origin of mesectoderm. He also discusses the strong opposition from the dominant germ layer theory that bone and cartilage are derived from ectoderm (fig. 1.2).

| Lateral ectoderm origin of cranial mesenchyme | Neural crest origin of cranial mesenchyme | Germ Layer Theory |
|---|---|---|
| Platt JB 1893 | Dohrn A. 1902 | Rabl C. 1894 |
| Goronowitsch N 1893 | Brauer, August 1904 | Corning H.K. 1899 |
| Kupffer C.V. 1895 | Landacre FL. 1921 | Minot C.S. 1901 |
| Koltzoff NK 1902 | | Buchs Georg 1902 |

**Fig. 1.2: Summary of studies detailed in Landacre FL 1921 on what is the origin of the cranial mesenchyme.**

(Goronowitsch, 1893; Platt, 1893; Rabl, 1894; Kupffer, 1895; Corning, 1899; Koltzoff, 1901; Minot, 1901; Buchs, 1902; Dohrn, 1902; Brauer, 1904; Landacre, 1921)

The idea that bone and cartilage in the head were derived from ectoderm was further confirmed by ablation and vital dye studies between 1920 and 1940. Stone, Raven, Hörstadius and Sellman were among the first who used ablation of the neural fold to demonstrate that the cranial bone and cartilage originated from the ectoderm (Stone, 1926; 1929; Raven, 1931; 1936; Hörstadius and Sellman, 1941; 1946). These authors were primarily concerned with proving that ectoderm can give rise to mesodermal derivatives and disproving the dominant germ layer theory rather than defining exactly where within the ectoderm cells originated.

By the late 1940s, it was finally accepted that the bone and cartilage in the head originated from the ectoderm and that the germ layer theory was not applicable to this anlage (Hörstadius and Sellman, 1941; 1946; de Beer, 1947; Hörstadius, 1950). In the paper by de Beer, there is mention of the studies by Platt on the lateral ectoderm origin of mesectoderm but it is dismissed as not being substantiated by others and he agrees fully with the conclusion by Landacre that the neural crest gives rise to mesectoderm. These early studies set the stage for the subsequent studies of neural crest and also accorded the neural crest as a source of remarkable pluripotent cells with very broad differentiation potential, Refer to fig. 1.3 for a summary of milestones in the field of neural crest studies.

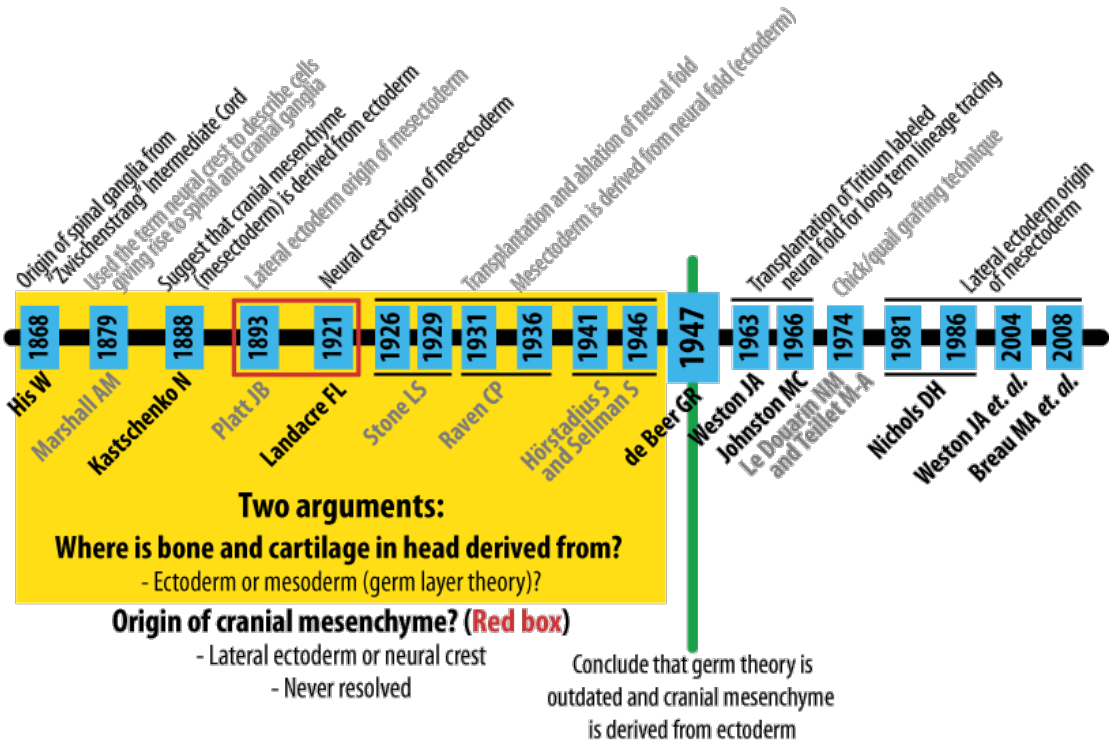


**Fig. 1.3: Milestones or landmarks in neural crest studies.**

Summary of the origin of neural crest (based on Hall BK and Landacre FL (Landacre, 1921; Hall, 2008)). With emphasis on the origin of the cranial mesenchyme as well as the initial debate on whether neural crest or lateral ectoderm gave rise to cranial mesenchyme (His, 1868; Marshall, 1879; Kastschenko, 1888; Platt, 1893; Landacre, 1921; Stone, 1926; 1929; Raven, 1931; 1936; Hörstadius and Sellman, 1941; 1946; de Beer, 1947; Weston, 1963; Johnston, 1966; Le Douarin and Teillet, 1974; Nichols, 1981; 1986; Weston et al., 2004; Breau et al., 2008).

So what led Platt and Landacre to come to different conclusions on the origin of the mesectoderm? In these studies, cell lineage was determined based on similarities in morphological appearance. In reassessing both studies, a key disagreement between the authors was how the neural fold was organized. Platt interpreted the neural fold as made up of two separate layers and cells were delaminating from distinct layers whereas Landacre described the neural crest as a single mass of cells within the neural fold (this will be elaborated in

greater detail in the discussion). Perhaps it was due to the need to disprove the dominating germ layer theory that authors failed to address the actual ectodermal domain that mesectoderm originated from (whether it originated from the lateral non-neural ectoderm or the neural crest).

### 1.3.2 Evidence for the lateral ectoderm origin of delaminating cells

From histological analysis of mouse neural crest cell delamination (Nichols, 1981; 1986), it was reported that in the cranial region of mouse, there is a temporal sequence of cell delamination from the neural fold epithelium. In the first study by Nichols, it was shown that the first cells delaminate from the non-neural ectoderm whilst later cells delaminate from the neural ectoderm. He also showed that the first cells to delaminate had higher levels of proteoglycans as shown by staining with toluidine blue. In the second study, Nichols went on to show that the cells that delaminate first end up dorsolateral to the pharynx whereas the cells that delaminate later form the cranial ganglia. These studies show that the neural fold region is heterogeneous and that there are two populations of neural crest cells in the head.

More recent work by Weston *et. al.* and Breau *et. al.*, have also revealed this heterogeneity of the neural fold (Weston et al., 2004; Breau et al., 2008). The authors were able to recapitulate the histological studies by Nichols using molecular markers. Weston *et. al.* looked at the expression of PDGFR-alpha (PDGFR**α)**, which is expressed in mesodermal derivatives (Morrison-Graham

et al., 1992; Orr-Urtreger et al., 1992), and in a subset of the delaminating cells within the neural fold. Incidentally in both studies, it was shown that these PDGFR**α** expressing cells also express E-cadherin and that these cells delaminate from the non-neural ectoderm. In the chicken cranial region, the neural fold also expresses PDGFR**α** (Endo et al., 2002). Based on these data, the authors propose that in the head there is a separate population of delaminating cells called “Metablast” that gives rise to the mesectoderm and that these cells are distinct from the neural crest which gives rise to neurons and pigment cells. Breau *et. al.* showed that cells initially delaminate from an E-cadherin positive domain in the neural fold. Based on the above studies as well as the initial studies that describe the origin of the neural crest, the first question that we would like to address is: “What is the neural fold?”

It is known that the first cells to delaminate from the cranial neural fold or dorsal neural tube gives rise to more ventral derivatives (Lumsden et al., 1991; Krispin et al., 2010). From these studies one can infer that time of delamination is a good indicator of cell fate and further implies that neural crest cells are fate restricted. However from grafting experiments, it seems that neural crest cells from different regions (orthotropic) and cells generated at different times (heterochronic) can compensate for each other and therefore they should have the same differentiation potential. How can these facts be reconciled?

Furthermore, it was known for a long time (DuShane, 1935; Erickson et al., 1992) that neural crest cells that are the last to delaminate generate melanocytes. Subsequently, there have been studies reporting the expression

of molecules implicated in melanocyte development by these late delaminating neural crest cells (Hayashi, 1993; Wehrle-Haller and Weston, 1995; Wilson et al., 2004). These reports of differential expression of markers further imply heterogeneity among neural crest cells.

The question of whether neural crest cells are pluripotent or fate restricted has important fundamental implications for how we consider the induction of the neural crest cells; do we consider the cells as individuals or as groups of cells? Determining when fate restriction occurs has important implication for understanding the induction of these cells by signaling molecules as well as transcriptional regulators. A diagram on the possible scenarios that fate restriction could happen in the neural crest is shown in fig. 1.4.

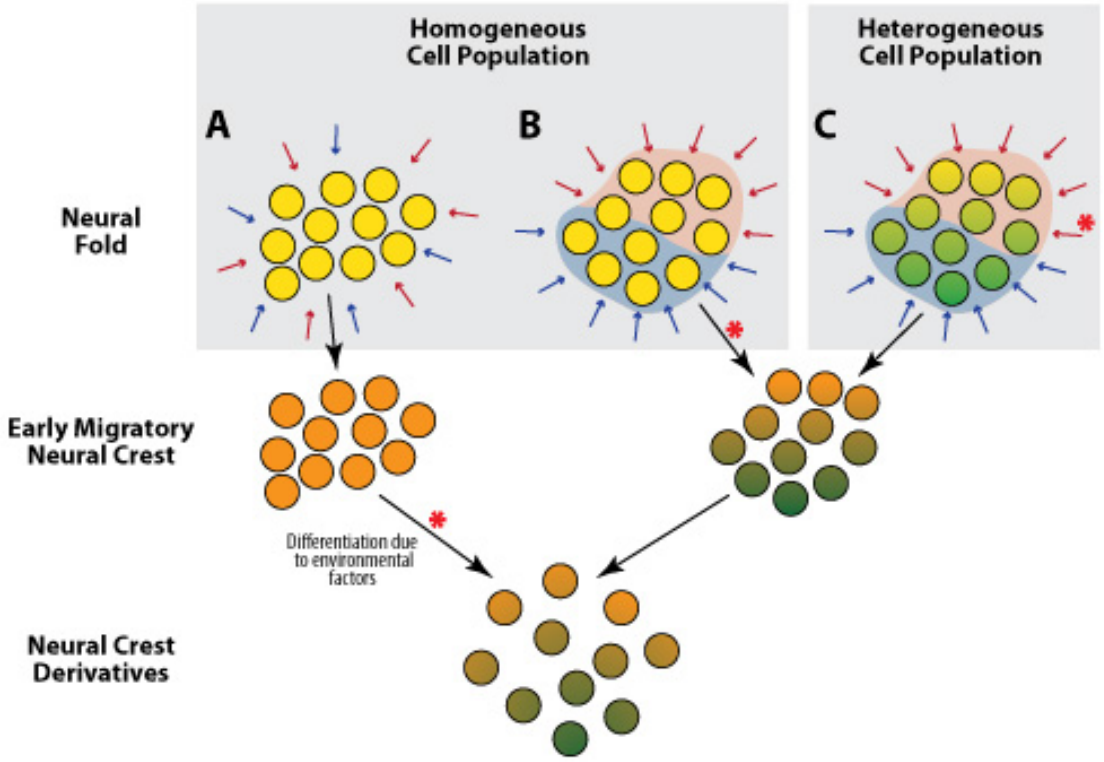


**Fig. 1.4: Different scenarios of how fate restriction occurs in neural crest cells.**

Arrows indicate signaling molecules and asterisk indicate time of fate restriction. (A) and (B), show that cells are initially homogeneous whereas (C)

show that the cells are already heterogeneous (denoted by gradient) in the neural fold.

Numerous experiments and observations have highlighted the difference between the cranial and trunk neural crest. These differences as well as the apparently unique ability of cranial neural crest to generate mesectoderm have raised the question of whether there is a link between origin and cell fate.

In the current study, we seek to define more clearly the neural fold by investigating the generation of neural crest cells in relation to how the neural and non-neural ectoderm segregate and also to determine if there are distinct populations of delaminating cells within the neural fold. We want to determine whether there is any correlation between fate and origin, and whether neural crest cells are pluripotent or fate restricted prior to delamination. In the following sections, we summarize what is currently known about how neural crest are formed and whether these cells are pluripotent or fate restricted. An important point to note is that we do not distinguish cells coming from different regions of the neural fold and we adopt the definition that the neural crest is an epithelial region between the neural and non-neural ectoderm.

## 1.4 Formation of neural crest cells

To investigate whether cell fate and origin are linked, there is a need to understand how the neural crest is specified. The neural crest is specified in the ectoderm and its formation can be considered in a stepwise manner:

1. Neural induction (patterning of the primitive ectoderm)
2. Induction of the neural crest
3. Delamination from the ectoderm

### 1.4.1 Neural induction

An essential step in the induction of the neural crest cells is the demarcation of the neural crest domain within the primitive ectoderm. The neural crest arises from the border of the neural and non-neural ectoderm. Not only is the neural crest induced spatially near the neural ectoderm, its induction is also temporally linked to the formation of the neural ectoderm. And it is for this reason that the study of neural crest formation has been greatly influenced by how neural ectoderm is induced.

Neural induction is the initial step in the formation of the central nervous system and it involves the induction of the naïve primitive ectoderm (ectoderm that does not go through gastrulation) to adopt a neural fate. This inductive process was elegantly demonstrated when Mangold transplanted the dorsal lip of the blastopore (Spemann's organizer) into the ventral side of a recipient embryo and showed that there was an induction of a second central nervous

system that was derived from the recipient embryo. This unequivocally showed that the organizer was able to induce a naïve primitive ectoderm to adopt a neural fate and the naïve ectoderm was responding to the signal coming from the organizer (Spemann and Mangold, 1924; 2001).

Subsequently it was shown that the naïve primitive ectoderm was induced to form neural ectoderm when it was transiently dissociated (Grunz and Tacke, 1989). This finding provided clues that neural induction was due to the inhibition of ubiquitous bone morphogenetic protein (BMP) signaling (Wilson and Edlund, 2001; Stern, 2005). This was the basis of the neural “default” model, because in the absence of BMP signaling primitive ectoderm adopted a neural ectoderm fate, suggesting that neural ectoderm was the default state.

As with most biological processes, it was soon found afterwards that other growth factors like fibroblast growth factor (FGF) and wingless-related MMTV integration site (Wnt) family members were also involved (Wilson and Edlund, 2001; Stern, 2005). Based on the expression pattern as well as loss of function assays, it was found that members of the SoxB1 family are expressed in the ectoderm and are important for the induction of the neural ectoderm (Uwanogho et al., 1995; Rex et al., 1997; Pevny et al., 1998; Uchikawa et al., 1999; Wood and Episkopou, 1999).

*Sox1*, *Sox2* and *Sox3* belong to the SoxB1 family (Uchikawa et al., 1999). Members of this family of transcription factors contain the DNA binding HMG domain. It was found that Sox3 is expressed in a broad domain within the epiblast prior to gastrulation (HH1) and expression is maintained in the neural

ectoderm but is lost in regions that are destined to form the definitive ectoderm. In contrast Sox2 is only expressed in the neural ectoderm at HH4 during early gastrulation when the neural ectoderm becomes thickened (Rex et al., 1997). In the chick, Sox1 is first expressed at HH7 in the neural ectoderm when the cells have committed to a neural fate (Uchikawa et al., 1999). In the mouse, Sox1 is first expressed in the neural ectoderm at the late head fold stage (Pevny et al., 1998; Wood and Episkopou, 1999).

It has been proposed based on the expression pattern of these factors as well as gain and loss of function experiments that the expression of the members of the SoxB1 family of genes reflects the state of neural induction. Prior to gastrulation, Sox3 is expressed in the entire epiblast and this reflects competence to form neural ectoderm. After gastrulation, expression of Sox3 in the neural ectoderm reflects the beginnings of neural induction whereas loss of Sox3 expression reflects a switch to a definitive ectodermal fate. The expression of Sox2 during early gastrulation reflects the very early signs of neural induction (thickening of the neural ectoderm) whereas Sox1 is expressed in the neural ectoderm when cells are committed to a neural fate (Scotting and Rex, 1996).

During neural induction, the initial flat epiblast undergoes morphogenesis to form the head fold. Naïve primitive ectoderm cells in the epiblast are initially cuboidal and upon neural induction, the cells thicken and become spindle shaped. In the chicken, neural induction is also accompanied by apical constriction and this is important for the formation of the neural tube and subsequent neural tube closure. This involves changes in cadherin

expression, in the chick the epiblast initially expresses L-CAM (E-cadherin ortholog) and upon neural induction, the neural ectoderm expresses N-CAM and N-cadherin (Thiery et al., 1982; Edelman et al., 1983). These markers are expressed once the neural ectoderm becomes morphologically distinct and are one of the earliest markers of neural induction.

Formation of the neural tube involves thickening of the neural ectoderm to form the neural plate, bending of the neural plate to form the neural fold, and subsequent fusion of the apposing neural fold. One of the consequences of neural tube closure is the separation of the neural and non-neural ectoderm. In mammals, cranial neural crest cells delaminate from the neural folds at a stage when the neural tube is not yet closed. This is a stage when the neural and non-neural ectoderm are continuous with each other and neural crest cells are formed at the border between the neural and non-neural ectoderm. This is in contrast to the situation in the trunk where neural crest cells delaminate from the dorsal region of the neural tube when the neural tube is already fully closed and physically separated from the non-neural ectoderm.

### 1.4.2 Induction of the neural crest – Gene regulatory network

Induction of the neural crest occurs in two steps. The first step is the formation of the border between the neural and non-neural ectoderm that is controlled by signaling molecules secreted by the paraxial mesoderm, neural and non-neural ectoderm. The second step involves the expression of a gene

regulatory network by the neural crest cells that are at the border of the neural and non-neural ectoderm.

The initial signaling events trigger the formation of the neural crest by turning on a gene regulatory network that consists of a number of transcription factors. These transcription factors can be grouped according to their spatial and temporal expression pattern as well as function. The first group that is expressed is the border specifier, these transcription factors are expressed at the border between the neural and non-neural ectoderm. Some of these transcription factors are direct targets of growth factors. This first group can be thought of as genes that are important for the segregation of the ectoderm into distinct regions and their expression is not restricted to the neural crest. The second group of transcription factors are those that are involved in specifying the neural crest cells (Betancur et al., 2010a). This second group is only expressed in the cells that are going to delaminate, and therefore expression is much more restricted.

Some of the first transcription factors to be expressed in the border of the neural and non-neural ectoderm are genes that respond to the growth factors that induce the neural crest. For example *Msx1* expression is a direct target of BMP signaling (Suzuki et al., 1997). There are a number of genes that are expressed at the border between the neural and non-neural ectoderm, *Pax3, Pax7, Msx1, Msx2 and Tfap2a*. The expression of these genes are not only restricted to the neural crest but also includes the dorsal part of the neural ectoderm, which specifies the sensory neurons of the spinal cord, as well as ectoderm, which gives rise to sensory placodes. The border of the neural

plate has been shown to be quite flexible in adopting other cell fates, being the only region that can be induced to form neural ectoderm, this is in contrast to cells that lie more laterally which are unable to be induced to form neural ectoderm (Streit and Stern, 1999).

In the following section, a short summary of the known expression patterns of these transcription factors as well as phenotypes of mutants is provided to try to understand what is the role of these molecules in neural crest formation?

*Pax3* and *Pax7* are both members of the paired box containing transcription factor family. There are a total of 9 family members and with the exception of two members they are all expressed in the central nervous system. In the mouse, Pax3 is expressed along the entire anterior posterior axis whereas Pax7 is expressed in the more anterior regions (Mansouri et al., 1996). In the chick, Pax3 is weakly expressed in the anterior regions until HH9, whereas in mouse Pax3 is expressed earlier when the head fold is formed. In chicken embryos, Pax7 is expressed at the border of the neural and non-neural ectoderm at HH5 (Otto et al., 2006). Pax3 and Pax7 are also expressed in the dorsal neural tube as well as in the presomitic mesoderm. Both Pax3 and Pax7 are expressed in the migratory neural crest cells as well. Pax3 has also been shown to be important for the formation of the trigeminal placode (Dude et al., 2009) and it is interesting to note that at HH8 in the chick, Pax3 is expressed in the ectoderm. Interestingly, it was shown that these Pax3 positive cells at this stage do not give rise to cells of the trigeminal placode (Xu et al., 2008).

It has been reported that neural crest cells are derived from an early population of Pax7 positive cells at HH4+ (Basch et al., 2006). This is a stage when the embryo is still undergoing gastrulation and neural ectoderm is also just beginning to be formed. It was also shown that the formation of the neural crest is dependent on the expression of Pax7. This demonstrates an early requirement of Pax7 function, possibly in the induction of the neural crest or a requirement of Pax7 for the proper segregation of the neural crest domain. However knockouts of *Pax7* in mice shows a relatively mild craniofacial defect (Mansouri et al., 1996). Interestingly, the mouse knockout of *Pax7* does not have any obvious deflects in neuronal derivatives.

The mutation of *Pax3* in the mouse Splotch mutant leads to a severe reduction in neural crest derivatives like melanocytes, neurons and cardiac neural crest (Auerbach, 1954). The defects are more severe in posterior than anterior regions; this might be due to compensation by *Pax7* expression in anterior region. The *Pax3* and *Pax7* double mutants was shown to have more severe defects than single mutants and Pax3 and Pax7 have redundant functions within the spinal cord (Mansouri and Gruss, 1998). It was further shown that when neural tube from *Pax3* mutant mice was transplanted into chicken embryos, the *Pax3* mutant neural tube was shown to give rise to neural crest derivatives like dorsal root ganglia or sympathetic ganglia (Serbedzija and McMahon, 1997). In this case, mutation of *Pax3* seems to be affecting the migration of the cells but does not have an effect on their formation since neural tube explants from the *Pax3* mutant can give rise to neural crest derivatives. Pax3 is expressed in the somite and was probably

affecting the ability of the neural crest to migrate in the somite (Serbedzija and McMahon, 1997).

Msx1 and Msx2 are homeo domain containing transcription factors. Msx1 is expressed at very early stages in the border of the neural and non-neural ectoderm. In the chicken Msx1 is expressed in the posterior region of the embryo at HH4, at this early stage the expression seems to be very similar to Pax7 (Chen et al., 1995). In the mouse, prior to gastrulation Msx1 is expressed in the extra-embryonic tissue but after gastrulation (E8.0), Msx1 is expressed in the future dorsal ectoderm and the neural epithelia. Expression in the dorsal ectoderm is lost at E8.5 (Houzelstein et al., 1997).

In chicken, apoptosis of neural crest in rhombomere three and five is mediated by Msx2 expression in this region. The authors show that misexpression of Msx2 in rhombomere two causes apoptosis of neural crest cells (Graham et al., 1994; Takahashi et al., 1998). Knockout of *Msx1* leads to cranial facial defects like cleft palate. The cleft palate defect could be due to disruption of neural crest formation or due to a later requirement of Msx1 for cell differentiation because Msx1 is expressed in the branchial arch (BA) one (Houzelstein et al., 1997).

In mouse, Tfap2a is expressed very early during embryogenesis. It is first expressed in the ectoderm and neural crest at E8.0. Prior to this, Tfap2a is expressed in the trophectoderm (Mitchell et al., 1991). The early expression of Tfap2a in the ectoderm and neural crest is also seen in the chicken (Shen et al., 1997). Mutation of this gene leads to severe cranial facial defects as well

as anencephaly (Schorle et al., 1996; Zhang et al., 1996). In these mutants the cranial ganglia are also hypoplastic. The important role for *Tfap2a* in neural crest seems to be conserved for most species analyzed. It was shown that mutations in *Tfap2a* in zebrafish also lead to cranial facial defects. In chicken, Tfap2a is expressed in the migrating neural crest cells and also in mesectoderm within the BA (Minarcik and Golden, 2003).

It was postulated that Tfap2a might have an important role in the formation of the border between the neural and non-neural ectoderm and therefore very important for the formation of the neural crest (Meulemans and Bronner-Fraser, 2002). Amphioxus, which belongs to cephalochordates, does not have neural crest cells whereas lampreys are jawless vertebrates that have neural crest cells. Although lampreys are jawless, they still have cranial neural crest, which give rise to cartilage in the skull (Langille and Hall, 1988; McCauley and Bronner-Fraser, 2003). Expression of Tfap2a in these two organisms is subtly different. While Tfap2a is expressed in the non-neural ectoderm in both organisms, only in lamprey is Tfap2a expressed in the dorsal neural tube - suggesting that Tfap2a might have been co-opted for the formation of the border/neural crest.

The second group of genes that are involved in the specification of neural crest are *Foxd3*, *Sox9*, *Sox10* and *Snail*. The expression pattern of these genes shows a more restricted pattern. They are only expressed in the cells that will delaminate and give rise to neural crest cells.

*Foxd3*, previously known as *Hfh2*, is a member of the winged helix/forkhead transcription factors. In the mouse, after gastrulation, Foxd3 is expressed in the neural crest prior to delamination as well as after delamination (Labosky and Kaestner, 1998). It is also expressed in the cranial ganglia but not in the mesectoderm (Mundell and Labosky, 2011). In the chicken, Foxd3 is also expressed in the neural crest relatively early at HH6 (Khudyakov and Bronner-Fraser, 2009).

In the mouse, conditional deletion of *Foxd3* in the neural crest leads to neural crest defects (Hanna et al., 2002; Teng et al., 2008; Mundell and Labosky, 2011). In these mutants, there is a drastic reduction of neural derivatives; cranial ganglia are severely reduced in size, the enteric nervous system is lost, and sympathetic and dorsal root ganglia are also reduced in size. Although there are cranial facial defects (BA one and two are reduced in size whereas BA three and four are most severely affected), the initial migration of the neural crest cells to the BA is not affected.

Foxd3 expression is maintained or lost in neural crest cells that adopt neural and mesenchymal cell fate, respectively (Mundell and Labosky, 2011). In neural crest conditional knockouts of *Foxd3*, these authors found ectopic formation of neural crest derived vascular smooth muscle cells in the descending aorta, smaller cranial ganglia and dorsal root ganglia as well as accelerated development of bone and cartilage. Based on these observations the authors speculate that Foxd3 has a role in determining cell fate since deletion of *Foxd3* caused a change from neuronal to mesenchymal cell fate.

*Sox9* and *Sox10* are also members of the Sry family of transcription factors and they belong to the SoxE subgroup. In chick, Sox9 is expressed relatively early in neural crest cells prior to delamination (HH7) whereas Sox10 is expressed just prior to the cell delaminating at HH9 (Sakai et al., 2006). The expression of Sox10 in neural crest is also relatively late (Anselme et al., 2007) compared to Sox9 (Barrionuevo et al., 2008) in the mouse. Sox9 and Sox10 are expressed in both the premigratory and migratory neural crest in both mouse and chicken.

*Sox9* has been shown to play a very essential role during bone development and the disruption of this gene in neural crest leads to defects in cartilage and endochondral bone formation but does not affect intramembranous bone formation or neural crest formation (Mori-Akiyama et al., 2003). Sox9 is expressed in chondrocytes and has been shown to regulate the expression of type II collagen (Wright et al., 1995; Ng et al., 1997) as well as other factors important for chondrocyte development.

*Sox10* has been shown to be very important in glial cell formation as well as the formation of the enteric nervous system. It was found that loss of *Sox10* leads to the loss of all glial cells as well as the enteric nervous system (Southard-Smith et al., 1998; Britsch et al., 2001), however neural crest cells are still able to form cranial mesectoderm. Sox10 is expressed in glial cells at later stages of embryogenesis. Through *in-vitro* culture of neural crest cells, it was found that Sox10 was important for the maintenance of the multipotentiality of the neural crest (Paratore et al., 2001; Kim et al., 2003).

Snail transcription factors are amongst the earliest markers that are expressed in the neural crest. In the mouse there are 5 members of the Snail/Scratch zinc-finger transcription factor superfamily (Barrallo-Gimeno and Nieto, 2009). Only Snail1 and Snail2 are expressed in the neural crest and it should be noted that in the chicken, Snail2 has a similar time of expression and function to Snail1 in mouse and *Xenopus* (Nieto et al., 1994; Sefton et al., 1998; Aybar et al., 2003). Snail1 was shown to be a transcriptional repressor of E-cadherin and therefore plays a major role in the execution of the EMT in the neural crest (Nieto et al., 1994; Cano et al., 2000).

It must be noted that Snail2 is not a specific neural crest marker as it is also expressed in the cells at the primitive streak as well as in mesodermal cells. Snail2 is required in the neural crest to mediate EMT. In chicken Snail2 is expressed in the neural crest at HH7+ and in *Xenopus* at stage 11. It was shown that Snail transcription factors have different roles in the formation of the neural crest (LaBonne and Bronner-Fraser, 1998; Aybar et al., 2003). If Snail activity is blocked at early stages then neural crest formation is diminished whereas blocking Snail activity at later stages led to defects in migration.

Other transcription factors like Id, Dlx, and Zic, have also been found to have a role in the specification of neural crest (Morales et al., 2005; Betancur et al., 2010a). Based on loss of function of these transcription factors as well as data from regulatory interactions, the relationship between these transcription factors and signaling molecules, can best be described as a gene regulatory network (Betancur et al., 2010a). Studies using systems biology approach like

microarray analysis have tried to address which set of genes is required for the formation of the neural crest (Gammill and Bronner-Fraser, 2002; 2003; Buchstaller et al., 2004).

It is noteworthy that in the majority of cases where these transcription factors are inactivated, the mutant phenotype either affects neuronal derivatives or mesectodermal derivatives but there are no cases where both types of derivatives are affected. This is very intriguing as the majority of these transcription factors are expressed along the entire anterior posterior axis as well as supposedly in all the neural crest cells.

### 1.4.3 Delamination from the ectoderm (Epithelial to mesenchymal transition)

The neural crest cells have to undergo EMT to delaminate from the ectoderm before engaging into individual cell migration. This transition is important for the transformation of epithelia cells, characterized by apical basal polarity and junctional complexes, to a mesenchymal phenotype where the cells have transient intercellular contacts and front-rear polarity. Prior to emigration from the ectoderm, neural crest cells are not distinguishable from other ectodermal cells, thus hampering their localization in the ectoderm.

EMT in neural crest cells involves the down-regulation of intercellular adhesion and extensive reorganization of the actin cytoskeleton to permit

subsequent cell migration. During EMT, the epithelial cell basement membrane must be partially degraded in order to promote their delamination.

In order to execute the EMT program, the cells express transcription factors like Snail transcription factors, which suppress the expression of type I Cadherins (important for the maintenance of cell to cell contact) and mediate the change in expression to type II Cadherins to enable cell migration. In the chick, neural crest cells change from expression of N-cadherin to Cadherin-6b before cell migration and to Cadherin-7 when they are migratory (Nakagawa and Takeichi, 1995; Coles et al., 2007; Park and Gumbiner, 2010). However in the mouse, neural crest cells change expression from N-cadherin to Cadherin-6 (Inoue et al., 1997). It is thought that the change in expression of type I to type II Cadherins is important for EMT. It was shown that type I and type II Cadherins exhibit very different adhesive properties and that expression of these Cadherins confer different effects on motility within the embryonic environment (Dufour et al., 1999; Chu et al., 2006; Coles et al., 2007; Park and Gumbiner, 2010).

It should also be noted that the way in which neural crest cells delaminate from the ectoderm differs between species, probably due to differences in neurulation. In mammals, the cranial neural crest cells delaminate from the ectoderm when the neural plate is still wide open (the neural tube is not yet formed) and the neural crest cells delaminate into the underlying mesenchyme. In the chick, because the neural fold is basically an epithelium that is folded onto itself, cranial neural crest cells migrates to their final

destination by moving between the neural tube and ectoderm before reaching sites with underlying mesenchyme.

In zebrafish, the cranial neural crest exists as two separate masses of cells next to the central neural keel and these cells delaminate and migrate towards the branchial arch (Eisen and Weston, 1993; Schilling and Kimmel, 1994). In *Xenopus*, cranial neural crest cells delaminate from the ectoderm when the cranial neural tube is still open (Mayor and Aybar, 2001). *Xenopus* cranial neural crest cells initially delaminate as a mass of cells with polarized pioneer cells (Carmona-Fontaine et al., 2008), which dissociate from each other at later stages. It should be noted that although *Xenopus* is an amphibian (anuran), neurulation in *Xenopus* is different from other amphibian (urodele) like salamander (Nieuwkoop, 1996). The mode of neural tube formation in the cranial region of the chicken is somewhat similar to how neural tube is formed in Urodeles (along the entire anterior posterior axis). Fig. 1.5 describes the different modes of neural crest formation in mouse and chicken.

Despite differences in morphology of the head in various animals (Duband et al., 1995), in general cranial neural crest delaminates from the ectoderm when the neural and non-neural ectoderm is not fully segregated from each other. This is in contrast to the trunk region where neural crest cells delaminate from the dorsal region of the neural tube, which is fully separated from the non-neural ectoderm. Cranial and trunk neural crest cells migrate in different spatiotemporal modes. Trunk neural crest cells migrate as individual cells dorsolaterally or ventromedially, whereas cranial neural crest cells migrate

just beneath the ectoderm as a stream of cells (this is especially true in *Xenopus*).

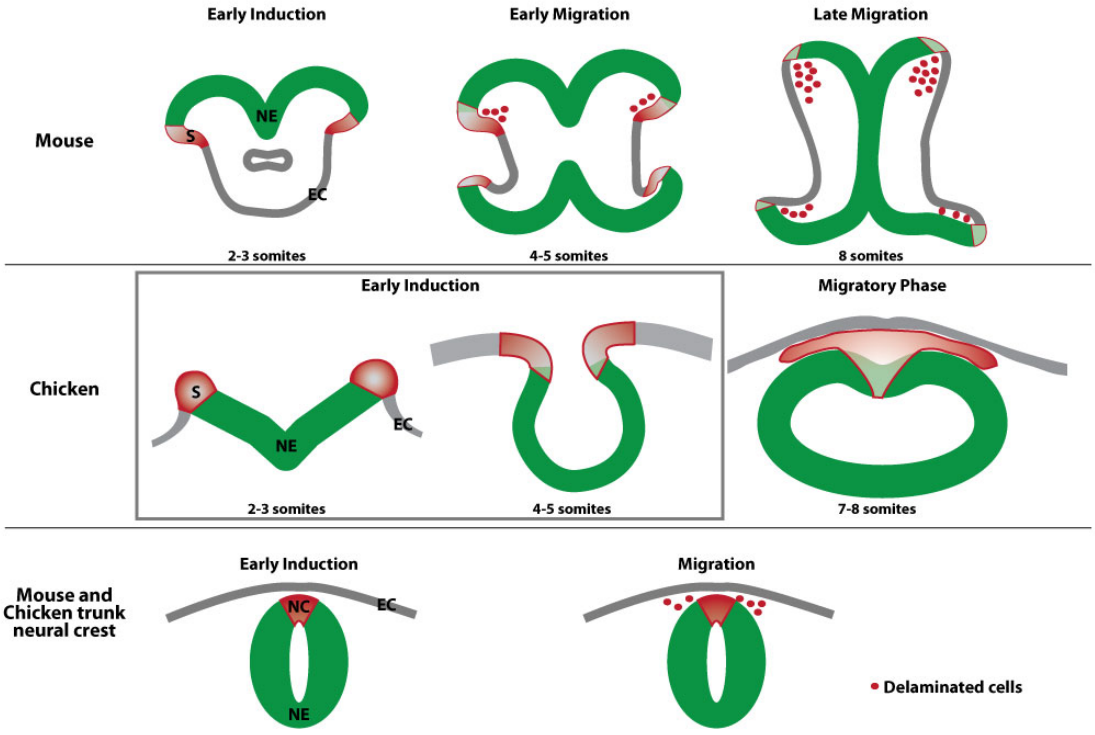


**Fig. 1.5: Different modes of neural crest formation/migration in mouse and chicken.**

Neural crest migration in cranial and trunk region of mouse and chicken. Note that in the chicken, cranial neural crest cells delaminate as a very compact mass of cells in contrast to mouse where the cells delaminate singly into the underlying mesenchyme. S, source of delaminating cells, NE, neural ectoderm, and EC, Ectoderm

Furthermore it must be emphasized that the way the cranial and trunk neural crest cells delaminate is very different – especially in chicken and amphibian where the majority of fate mapping experiments have been done. Cranial neural crest delaminate at stages when the neural tube is not yet fully fused whereas trunk neural crest cells delaminate from a fully closed neural tube (Duband et al., 1995). Because cranial neural crest cells delaminate before

neural tube is fully fused, grafts containing cranial neural crest are essentially neural folds, which contain both neural and non-neural ectoderm. This is in contrast to the trunk where the grafts contain only neural ectoderm from the neural tube.

## 1.5 Origin and cell fate of the neural crest

The basis of what is the origin of a particular cell type can be determined by observing the cell morphology between different tissues and determining their relationship based on similarity in appearance. Another approach is to introduce an extrinsic label into specific cells early during development and observing later in development what are the progenies of the labeled cells. In this way the origin as well as the fate of the cells can be determined. In the case of neural crest, Wilhelm His first described that the border of the neural ectoderm gave rise to spinal ganglia (His, 1868) but it was Marshall who coined the term neural crest in 1879 (Marshall, 1879).

Since then it has been shown that neural crest gives rise to a multitude of different anatomical structures including the jaws and ganglia. The origin and fate of the neural crest have been determined by a number of methods: labeling with vital dyes or viruses, genetic labeling with the Cre/loxP systems, ablation and grafting.

In the following section, a brief introduction of the methods for performing lineage analysis and a summary of data supporting or contradicting the idea that neural crest cells are pluripotent is presented.

### 1.5.1 Methods for lineage analysis

#### 1.5.1.1 Origins and fate map of neural crest - Labeling of cells with extrinsic labels.

In this method, an extrinsic label (usually a dye) is applied to the cells of interest and the fates of their progenies are tracked over time. The selection of the dye and method of labeling depends on the number of cells that are to be labeled. Nowadays the most common vital dyes used for labeling cells are DiI or labeled high molecular weight dextrans. Labeled dextrans have the advantage that they are retained within cells and therefore do not transfer to neighboring cells whereas DiI, a lipophilic small molecular weight compound labels the cell membrane and may transfer to neighboring cells. In the case of labeled dextrans, cells can either be labeled by electroporation or intracellular injections. With DiI, cells are labeled by contact with the dye; this is achieved by iontophoresis, injection of DiI solution or contact with the DiI crystal.

The advantage of labeling cells by an extrinsic label is that it allows for smaller number of cells to be labeled (this is especially the case when cells are injected with dextrans). The microinjection of dextran into cells allows the determination of what is the development potential of a single cell and construction of high-resolution fate map. The disadvantage of using these dyes to label cells is that the label is diluted each time the cell divides.

### 1.5.1.2 Origins and fate map of neural crest - Genetic labeling.

Viruses that are defective in replication, or constructs containing a reporter, can also be used to label cells. In its simplest form, a promoter driving the expression of a reporter like **β**-galactosidase or GFP is used to label specific cells. The promoter is only turned on in a discrete group of cells and the progeny of the labeled can be determined by expression of the reporter. The reporters used are usually quite stable and therefore can persist for a period of time even after expression has stopped.

A more elaborate and permanent way of labeling cells is achieved by utilizing a tissue specific promoter to drive expression of a recombinase to activate a constitutively expressed reporter, for example the Cre/loxP or Flp/frt systems. This is a two-component system with a tissue specific promoter driving the expression of the site-specific recombinase and a constitutively expressed reporter that has a stop cassette flanked by sequences recognized by the site-specific recombinase. Under basal conditions, the presence of the stop cassette, a strong translational stop signal, prevents the expression of the reporter. However, when the cell switches on the expression of the recombinase, the stop cassette is removed and the reporter is expressed. The expression of the reporter by the cell is constitutive and because progenies inherit the activated reporter, progenies also express the reporter constitutively.

There are variations of the two component genetic methods that allow smaller group of cells to be labeled, these include (i) the use of the site specific

recombinase to drive inter-chromosomal recombination, a much more inefficient process compared to intra-chromosomal recombination (Zong et al., 2005), and (ii) coupling inducible systems to the recombinase, for example fusing the estrogen hormone-binding domain to the recombinase – hence sequestering recombinase in the cytoplasm until it binds to tamoxifen which allows the recombinase to translocate to the nucleus to facilitate recombination (Vooijs, 2001). This later method allows labeling of cells at discrete time points (Branda and Dymecki, 2004).

The advantage of genetic labeling method is that it is very reproducible and with the use of different promoters, the origin of cells can be mapped onto the expression of different molecules thus enabling very high-resolution fate mapping.

The availability and selection of the promoter used for driving the reporter/recombinase is key to the success of this method since it determines which cells are labeled. A detailed knowledge of when and where the promoter is activated is required since spurious expression of the reporter/recombinase would result in a distinct group of cells being labeled and a wrong assignment of the cell fate of the labeled progeny. Wnt1 Cre, HtPA Cre or P0 Cre are typically used to label neural crest in the mouse and these lines have been used to confirm the derivatives originating from the neural crest in mouse (Yamauchi et al., 1999; Chai et al., 2000; Jiang et al., 2000; 2002; Pietri et al., 2003).

#### 1.5.1.3 Origins and fate map of neural crest – Grafting/ablation

Tissue ablation consists of surgically removing small areas of tissues at early stages. The origin of the missing territory in the operated embryo after surgery is attributed to the region that is removed. Tissue grafting, however, involves transferring small pieces of tissue between the host and donor (wherein the host and donor animals must have intrinsic or extrinsic differences) and the subsequent identity of the progeny of the graft is determined by observation of the label. Usually the graft is made at sites where the corresponding structure in the host is removed.

Weston and Johnston were among the first to use this method to track the neural crest over long periods of time with great resolution (Weston, 1963; Johnston, 1966). They used tritiated thymidine labeled chicken embryos as grafts. Subsequently, Le Douarin developed the chick/quail chimera system by taking advantage of the fact that quail cells have a distinctive nucleolus and thus could be distinguished from chicken cells (Le Douarin and Teillet, 1974), circumventing the need to label the donor. Initially in this method, the quail cells were detected by using the Feulgen method which stains the nucleolus however subsequently an antibody (QCPN) was developed that recognized the quail nucleus (Lance-Jones and Lagenaur, 1987).

The biggest advantage of the chick/quail chimaera system is that the label is permanent and thus the fates of progeny could be followed over prolonged periods of time. The disadvantage of this method is that it is technically challenging to perform micro-dissection and to maintain the grafted embryos

long enough after grafting. High-resolution fate maps are not possible since one cannot transfer a small amount of cells.

The ablation technique suffers from the disadvantage that sometimes the tissue that is ablated can form again. To circumvent this problem, larger areas of tissue need to be removed to prevent the regeneration.

### 1.5.2 Pluripotent versus fate restricted

The question of whether neural crest cells are pluripotent or fate restricted is a long-standing one. In order to answer this question, there is a need to be very specific in how cell fate is defined. Specification can be defined as the process whereby a cell during development distinguishes itself from other neighboring cells and differentiates into a particular cell type.

Because development is a continuum of differentiation whereby cells are initially pluripotent and through differentiation become fate restricted, it is necessary to be very strict about the time point, at which one wants to determine the differentiation potential. For example, during mouse gastrulation, formation of the primary germ layers by the inner cell mass involves loss of pluripotency. The mesoderm can form skeletal elements in the trunk but does not form neural tissue or skin. The mesoderm is fate restricted in comparison to the inner cell mass, which can give rise to all the cell types in the body.

Furthermore when considering the question of whether a cell is pluripotent or fate restricted, it is necessary to consider two aspects of cell fate: specification, and commitment. Specification occurs as a result of the interaction with the environment and expression of molecules that drive changes in cell fate. Commitment can be defined as the intrinsic inability of cells to change its adopted cell fate. Once cells are committed to a particular cell fate, it means that the cell can no longer differentiate into other types of cells. A key difference between specification and commitment is that

specification involves the activation or loss of molecules that can change cell fate whereas commitment involves the maintenance of the cell fate. There are caveats to this since reprogramming has been elegantly shown by Takahashi and Yamanaka (Takahashi and Yamanaka, 2006), when fibroblasts were reprogramed into pluripotent stem cells via introduction of transcription factors. These fibroblast-like cells under normal circumstances can only give rise to limited cell types and a limited number of progenies but after reprogramming into pluripotent stem cells, these cells can give rise to all cell types as well as can be maintained indefinitely in culture. In this case, re-specification of the fibroblasts was shown and commitment of the fibroblast to their cell fate explains why the reprograming efficiency was so low.

The question of whether neural crest cells are pluripotent or fate restricted at the time of migration was addressed in the 1980s. In the beginning, a number of laboratories used *in-vitro* culture methods to address this issue. Subsequently with the advent of cell labeling with extrinsic dyes and genetic labeling other laboratories tried to address this issue *in-vivo*.

There are distinct advantages and disadvantages of these two approaches. The advantage of *in-vitro* methods is that it is relatively easy to investigate whether single cells are pluripotent or not by simply performing limiting cell dilution. However this method suffers from the disadvantage that because the conditions for culturing the cells are artificial, there is a risk that the behavior of cells might not reflect what they would actually do *in-vivo*. Therefore the biggest advantage of *in-vivo* methods is that the behavior of cells within the

embryo can be most accurately assessed but the methods employed are more technically challenging.

#### 1.5.2.1 *In-vitro* fate mapping experiments

In 1980, Sieber-Blum *et. al.* conducted the first study to examine whether the chick neural crest cells in the trunk were pluripotent or fate restricted at the onset of migration (Sieber-Blum and Cohen, 1980). Neural crest cells were isolated by culturing neural tubes, at a stage prior to neural crest migration, in tissue culture. The neural tubes were subsequently removed from culture when the neural crest cells had migrated out. Neural crest cells were then analyzed by the clonal dilution method to test their developmental potential.

Due to the absence of good molecular markers at the time, the authors could only distinguish between pigmented (melanocytes) and non-pigmented cells (neurons or glia). They found a mixture of different types of colonies; colonies that gave rise to both pigmented and non-pigmented cells (28%) and colonies that gave rise to only pigmented cells (55%) or non-pigmented cells (17%). They concluded that some neural crest cells are pluripotent.

In 1993, Sieber-Blum *et. al.* published another study to address the issue of whether mouse neural crest cells were pluripotent or fate restricted at the time of migration (Ito and Sieber-Blum, 1991). Neural crest cells were isolated in the same way as in chicken embryos. They found that there were cells that were fate restricted and could only differentiate into melanocytes while others

differentiated into melanocytes, neurons and glia, much like what was found in the previous study. The conclusion for this study was similar to the previous study in chick.

Subsequent studies by Le Douarin *et. al.*, isolated neural crest cells from chicken embryos (both trunk and cranial neural crest) and used limiting cell dilution and *in-vitro* cell culture to determine whether the cells are pluripotent or fate restricted (Baroffio et al., 1988; 1991; Lahav et al., 1998; Trentin et al., 2004; Dupin et al., 2010). These studies looked at the differentiation potential of the cranial as well as trunk neural crest cells. To do that, cranial neural crest cells were isolated at approximately 10 somites at the mesencephalon and metencephalon levels. At this stage, the cranial neural crest cells have already started migrating as a mass of cells. The overlying ectoderm was also isolated along with the cranial neural crest cells during the procedure. For trunk neural crest cells, a similar approach as Siebler-Blum *et. al.* was adopted (Sieber-Blum and Cohen, 1980). Cell fate of the progeny was determined by immunohistological staining for markers of various cell fates.

The initial study by Le Douarin *et. al.* of cranial neural crest cells in 1988, showed that about 40% of the clones could form different types of neurons, 4% of the cells could form both neurons and melanocytes, 4% could form cartilage only, 1% could only form melanocytes, and 50% of the cells could form non-neuronal cells that were HNK1 positive (Baroffio et al., 1988). Subsequently in a follow-up study in 1991, they looked at a larger number of clones and found 10 clones out of 305 clones that gave rise to cartilage (Baroffio et al., 1991). Out of the 10 clones, 3 clones gave rise only to

cartilage, 5 gave rise to glia as well as cartilage and the remaining 2 could give rise to cartilage, neurons, glia and melanocytes. In this study the number of progenies each single isolated cell gave rise to was determined.

Another study conducted by Le Douarin *et. al.* in 1998, showed that the addition of growth factors like Endothelin3 to the cultured neural crest cells, promoted the survival and proliferation of glia and melanocyte precursors (Lahav et al., 1998). Subsequently in 2004 it was also shown by subcloning the isolated neural crest cells, that the multipotent cells renewed themselves (Trentin et al., 2004).

Based on these studies, Le Douarin *et. al.* concluded that neural crest cells undergoes gradual fate restriction to produce cells that are bipotent or single fate restricted cells and that the pluripotent progenitors can self renew. Furthermore, the authors concluded that the large number of cells generated by a pluripotent progenitor supported the notion that these pluripotent cells are stem cells (i.e. these cells could generate large number of progenies).

It must be emphasized that in these studies, cranial neural crest was isolated at a stage when cranial neural crest cells have already extensively migrated and mix with the underlying mesenchyme. Therefore it is possible that these investigators had not isolated a pure population of neural crest cells. The claim that the cells were pluripotent in the head was based on the occurrence of a small number of clones (2 clones out of 305 clones ~0.7%) that could differentiate into multiple lineages. Although they claimed that the large number of progenies generated by these clones was supportive evidence that

they were multipotent progenitors along with other progenitors with a more limited development potential. It is entirely possible that the large number of progenies was derived from a few cells rather than a single cell. Even if these two clones were bona fide pluripotent neural crest cells, an alternative explanation might be that there are only a few pluripotent neural crest cells within a majority of fate restricted neural crest cells.

Other studies concluded that trunk neural crest cells from rat embryos are pluripotent and that they are capable of self-renewal (Stemple and Anderson, 1992). This is in agreement with studies by Le Douarin *et. al.* In their study, they isolated neural crest cells that emigrated out of neural tube explants. A study by the same group also showed that pluripotent neural crest cells could be isolated from embryonic sciatic nerve which is ensheathed by Schwann cells, a neural crest derivative (Morrison et al., 1999).

A subsequent study by Henion and Weston in 1997, determined that a significant proportion of neural crest cells in the trunk of chicken embryos were already fate restricted when the cells commence their delamination from the neural tube (Henion and Weston, 1997). They cultured neural tube explants and isolated cells that emigrated out of the neural tube explant. Single cells were labeled at discrete time points to determine what the fates of individual clones were. They determined that even at early time points about 40% of neural crest cells gave rise to progeny of a single phenotype. Additionally, they found that the majority of neural crest cells initially gave rise to neurons but at later times gave rise to melanocytes.

In 2003 Luo *et. al.*, using live antibody staining for TrkC and c-Kit of neural tube explants and DiI labeling, showed that there are distinct populations of TrkC and c-Kit expressing cells and that these cells are fate restricted (Luo et al., 2003). Initially TrkC positive cells were able to give rise to both neurons and glia but subsequently at later times they gave rise only to neurons. This correlates well with what happens *in-vivo*. The advantage of these studies was that cells were maintained in a more native environment (neural crest cells were kept together although the environment they normally are in was removed).

The studies led by Le Douarin and Anderson conclude that neural crest cells are pluripotent whereas the studies by Sieber-Blum *et. al.*, and Henion and Weston conclude that the neural crest consist of some pluripotent cells and a significant portion of cells which are already fate restricted during early stages of their migration out of the neural tube. In the studies by Le Douarin and Anderson labs there were clones that gave rise to multiple cell fates as well as single cell fate, but the authors propose that the clones that gave rise to single cell fate were descendants of clones that gave rise to the multiple cell fates (Anderson, 1989). Without proof of phylogeny, it cannot be assumed that the clones that gave rise to single cell fate originated from cells with multiple cell fates and that there is gradual fate restriction happening. Therefore the different conclusions that were reached can be attributed to differences in interpretation of the results and the question of whether neural crest cells are fate restricted or pluripotent remains open.

As mentioned previously, a major flaw of *in-vitro* fate mapping experiments is the possibility that the adopted cell fate is due to differentiation induced by the foreign environment in which the cells are cultured. Another major problem with these *in-vitro* studies is the fact the neural crest is generated in a rostral to caudal manner. Therefore, when culturing tissue explants, a heterogeneous population of cells is obtained since cells from more rostral regions are more advanced in terms of migration as well as differentiation status. This is especially the case when cranial neural crest is cultured at a relatively late stage when they have already migrated out. Additionally these studies also raise the question of whether the neural crest cell is a homogenous population of cells.

#### 1.5.2.2 *In-vivo* fate mapping studies

Studies by Bronner-Fraser and Fraser in 1988 and 1989 demonstrated by labeling single cells in dorsal neural tube of chicken embryos in the trunk that trunk neural crest cells were pluripotent (Bronner-Fraser and Fraser, 1988; 1989). They labeled the cells between stages 10-17 (somites 8-28) or stages 11-18 (somites 12-33). Out of the 20 embryos with labeled neural crest derivatives, only 3 embryos gave rise to single derivatives. Based on this, authors concluded that the neural crest cell progenitors are pluripotent.

A study by Lumsden *et. al.* in 1991 investigated how cranial neural crest along the anterior/posterior axis gives rise to different segments of the crest derivatives (Lumsden et al., 1991). This was done by labeling cranial neural

crest at different times and anterior/posterior position, and determining the fates of the labeled cells. They showed that different regions of the cranial neural crest gave rise to distinct regions of neural crest derivatives; this is in agreement with what was found in the grafting experiments (Johnston, 1966; Le Lièvre and Le Douarin, 1975; Noden, 1975; Le Lièvre, 1978; Couly and Le Douarin, 1990). Another key finding of Lumsden's study was that neural crest cells gave rise to derivatives in a ventral to dorsal sequence, i.e. the cells that delaminate first form the most ventral derivatives. For example they found that labeling neural crest cells in the midbrain at HH8- gave rise to derivatives in the periocular and maxillary mesenchyme (more ventral derivatives) whereas labeling the same region at HH9-, in addition to giving rise to the parts of the periocular and branchial arch mesenchyme, the trigeminal ganglia (a more dorsal derivative) was labeled as well.

In contrast to the conclusion reached by Bronner-Fraser and Fraser, the results from Lumsden's study suggest that cranial neural crest cells are already fate restricted when they delaminate since labeling at earlier time points gives rise to a presumably mesectodermal cell fate. This is especially striking since no attempt was made to specifically label the early or late delaminating neural crest cells.

A recent paper (Krispin et al., 2010), demonstrate that the fate of the trunk neural crest could be predicted either by the relative position within the dorsal neural tube or the time that they delaminate. This is consistent with the fact that neural crest derivatives are colonized in a ventral to dorsal order (Serbedzija et al., 1989). Neural crest cells first migrate ventrally then

dorsolaterally (Erickson et al., 1992), cells that migrate dorsolaterally differentiate into melanocytes. Krispin *et. al.* showed that if cells that normally migrate ventrally are forced to migrate dorsolaterally, the cells still adopt their original neural fates. Therefore, the authors conclude that the neural crest cells are predetermined at an early stage even prior to delamination.

This study contradicts the findings of Bronner-Fraser and Fraser, which demonstrated that trunk neural crest cells are pluripotent. The reason provided by Krispin *et. al.* for the discrepancy was cell labeling by the other group were not as precise as their study (i.e. labeling was done over a longer time window and included a larger segment of the trunk), whereas in their study - labeling of neural crest cells was performed shortly before neural crest cells migrated out of the neural tube. This imprecision may have resulted in the labeling of neuroepithelial progenitor cells that give rise to both neuroepithelia as well as neural crest.

A study by Zirlinger *et. al.* in mice shows that Neurogenin2 expressing neural crest cells are predisposed to forming the dorsal root ganglia (Zirlinger et al., 2002). Neurogenin2 (Sommer et al., 1996) is expressed by neural crest cells in the trunk, prior to cell delamination, and in the dorsal root ganglia. Based on the expression of Neurogenin2, it was unclear whether all trunk neural crest cells expressed this molecule. In order to determine what neural crest cells expressing Neurogenin2 gave rise to; the authors generated a transgenic mouse with Neurogenin2 promoter driving a Tamoxifen inducible Cre. By crossing the inducible Cre line with a Rosa26 reporter line and inducing the activation of Cre recombinase at discrete time points (prior to neural crest cell

delamination), they demonstrated that Neurogenin2 expressing neural crest cells are predisposed to forming the dorsal root ganglia but not the sympathetic ganglia however within the dorsal root ganglia they differentiate into both neurons and glia at about 50% frequency. This demonstrates that the trunk neural crest was already fate restricted prior to delamination from the neural tube.

Another study using genetic fate mapping shows that there are two populations of premigratory neural crest progenitors (Wilson et al., 2004). It was shown that premigratory neural crest cells that express c-Kit give rise to melanocytes and these cells do not express p75 (a low affinity Neurotrophin receptor which has been used by other studies to isolate pluripotent neural crest cells). In chicken, it has also been shown that neural crest cells that migrate dorsolaterally express melanocyte markers (c-Kit and Mitf) prior to delamination and that these cells differentiate into melanocytes (Erickson and Goins, 1995; Wakamatsu et al., 1998).

Two studies in zebrafish also show that the neural crest cells are fate restricted prior to cell delamination (Raible and Eisen, 1994; Schilling and Kimmel, 1994). In these studies single premigratory neural crest cells in the head and trunk were labeled. In the head, the labeled neural crest cells only gave rise to single cell types (Schilling and Kimmel, 1994). In cranial regions of zebrafish embryos, the premigratory neural crest cells are found as 2 masses of cells that are lateral to the central neural keel. The study by Schilling and Kimmel show that the cells that are closest to the neural keel are fated to give rise to mesectodermal derivatives, and it is also assumed that

these cells would be the last to delaminate (lateral cells should delaminate first). Therefore it is quite strange that in cranial region of zebrafish, neural crest cells do not colonize derivatives in a ventral to dorsal sequence.

In the trunk most of the labeled neural crest cells also gave rise to single derivatives and the progenitors that gave rise to multiple derivatives did so by producing single lineage restricted progeny (Raible and Eisen, 1994). In this study they traced the relationships of the progenitor and the progeny produced. It must also be noted that the clones that gave rise to multiple lineages gave rise to more progenies, therefore it is possible that these labeled cells might be precursor cells that give rise to neural epithelia and neural crest.

Most of the *in-vivo* studies, except the single cell labeling done by Bronner-Fraser and Fraser, show that the neural crest cells are fate restricted at the time of delamination or that most neural crest cells are only capable of giving rise to limited derivatives and are thus, not pluripotent. Some of these studies even show that at the premigratory stage there are sub-populations of cells that differentially express certain molecules that have been implicated for cell fate determination and these cells subsequently differentiate into that particular cell fate. Therefore in this case, a strong argument against pluripotency of the neural crest cells is the initial heterogeneity of the whole population. If at the premigratory stage, neural crest cells already differentially express molecules that have a role in directing cell fate then it would suggest that lineage segregation has already occurred at early stages and there are no truly pluripotent neural crest cells. Although it is not possible to exclude the

possibility that a small population of the neural crest cells is pluripotent: these pluripotent neural crest cells would be few and far between.

### 1.5.3 Pluripotent or fate restricted - Conclusion

Findings from *in-vitro* clonal cultures have demonstrated the presence of clones that can give rise to all derivatives, whereas findings from *in-vivo* studies has shown that most of neural crest cells are fate restricted at the time of delamination (fig. 1.6). There are mounting evidence from work by Kalcheim *et. al.* that even in the trunk where all cells delaminate from the dorsal neural tube, neural crest cells with distinct cell fates delaminate in a timely and orderly fashion (Krispin et al., 2010). Normally in the trunk, neural crest cells first migrate ventral-medially to give rise to neurons and glia but even when these cells were forced to migrate dorsal-laterally these cells still adopted a neuronal fate. However the situation for cranial neural crest cells remains unclear.

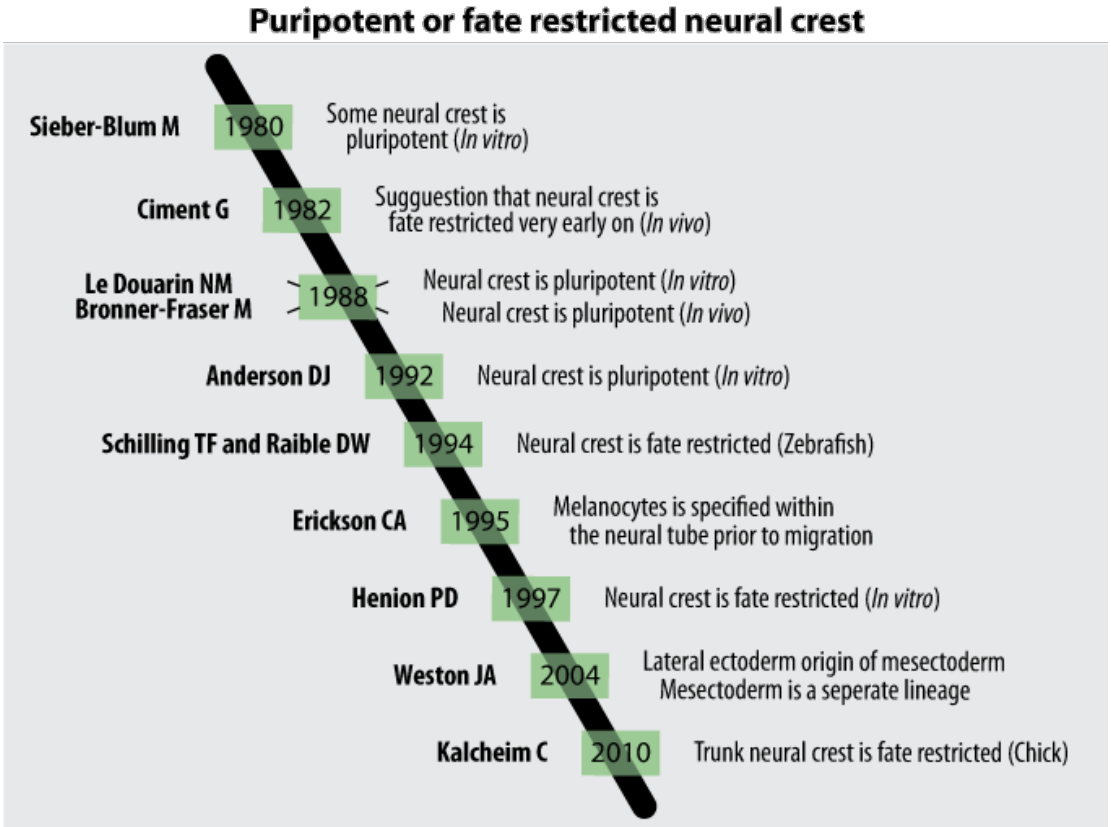


**Fig. 1.6: Summary of the debate over whether neural crest cells are pluripotent or fate restricted.**

(Sieber-Blum and Cohen, 1980; Ciment and Weston, 1982; Baroffio et al., 1988; Bronner-Fraser and Fraser, 1988; Stemple and Anderson, 1992; Raible and Eisen, 1994; Schilling and Kimmel, 1994; Erickson and Goins, 1995; Henion and Weston, 1997; Weston et al., 2004; Breau et al., 2008; Krispin et al., 2010).

The pluripotent neural crest cells found in *in-vitro* studies could simply be products of re-programing where an isolated cell reverts back to a more primitive state that is able to differentiate into multiple cell types. This possibility is unlikely as similar pluripotent neural crest cells have been isolated in other more differentiated tissue (Morrison et al., 1999; Wong et al., 2006). It would be interesting to determine where these pluripotent cells isolated from differentiated tissues originated. However as mentioned before, data from *in-vivo* cell labeling experiments suggest that most cells are lineage

restricted prior to cell delamination, the exception being the studies by Bronner-Fraser and Fraser (Bronner-Fraser and Fraser, 1988; 1991).

Another argument for the pluripotency of neural crest cells comes from heterochronic grafting experiments. Findings from these experiments suggest that the environment that the neural crest cells encounters during migration determines the cell fate of these cells. Grafts from mesencephalon levels containing late delaminating neural crest cells could give rise to the derivatives generated by early delaminating neural crest cells (Baker et al., 1997).

This is also the case for cardiac neural crest cells. It was shown that the early and late emigrating neural crest cells give rise to different derivatives but the late emigrating population can give rise to derivatives from early emigrating populations when the two populations are swapped (Boot et al., 2003). As mentioned before, neural crest cells that migrate out first give rise to more ventral derivatives. This seems to suggest that early or late delaminating neural crest cells contain cells that are not fate restricted and therefore can compensate for each other.

It is known from grafting experiments that neural crest cells from different axial levels can generate the derivatives of their new location when grafted into an ectopic location (Le Douarin and Teillet, 1974). In this study when neural crest cells that normally give rise to adrenal medullar (somites 18-24) were grafted into vagal regions (somites 1-7), they gave rise to vagal neural crest derived enteric neurons. This seems to suggest that neural crest cells from different

axial levels contain pluripotent cells that are not fate restricted and hence can give rise to derivatives found in the site into where they are grafted. Furthermore this may also imply that neural crest cells generated at different axial levels have the same developmental potential.

Studies where neural crest cells have been ablated show that when either cranial and cardiac neural crest domain is ablated within a discrete time point, neural crest cells are not lost but can be reformed by neighboring regions (Yntema and Hammond, 1945; McKee and Ferguson, 1984; Scherson et al., 1993; Buxton et al., 1997; Boot et al., 2003; Ezin et al., 2011). Scherson *et. al.* shows that when neural crest is removed, newly formed neural crest cells are derived from the adjoining ventral neural tube and not from posterior or anterior sites (Scherson et al., 1993).

In the case of cardiac neural crest ablation, surrounding tissue can only compensate for the ablation if ablation is carried out relatively early (i.e. compensation is lost if ablation is carried out after HH 9 (Ezin et al., 2011)). The scenario is different in the trunk where ablated neural crest cells will not be reformed (Yntema and Hammond, 1945). This also highlights another difference between cranial and trunk regions.

From the above grafting and ablation experiments it seems reasonable to suggest that the neural crest cell display a high level of plasticity, and neural crest cells at different regions have the same differentiation potential. It also shows that neighboring tissues, at least in the cranial and cardiac regions, retain the ability to form neural crest cells. Taken together this might mean

that inductive signals persist in these regions and when a segment of the neural fold is transferred to a new location, they may be induced to give rise to derivatives that are normally not formed by that particular segment. Therefore the plasticity of neural crest might not reflect that cells are pluripotent but rather neural crest cells are not irrevocably committed to a particular cell fate and are competent to respond to cues in the local environment. Alternatively, nearby surrounding regions may have the capacity to form neural crest cells but lack certain factors (Dickinson et al., 1995; Selleck and Bronner-Fraser, 1995). Recently it is becoming clearer from *in-vivo* lineage analysis that neural crest cells are most probably fate restricted prior to delamination.

#### 1.5.3.1 Unique skeletogenic potential of the cranial neural crest

The term mesectoderm was originally used by Platt to describe cells that originated from the ectoderm that gave rise to mesodermal derivatives. Mesectoderm is formed by cranial neural crest cells (from mesencephalon to anterior rhombencephalon) when they are ectopically grafted into the trunk (Le Douarin and Teillet, 1974). This suggests that the cranial neural crest is committed to a mesectodermal cell fate. When the reverse experiment is done, trunk neural crest does not form mesectoderm (Nakamura and Ayer-le Lievre, 1982). However, they find that if a unilateral graft of trunk regions (between somite 20-30) was grafted into cranial region with lateral half of the hindbrain intact, the graft can give rise to mesectoderm, but they do not give rise to cartilage or bone. This seems to suggest that the neural crest cells that

form cartilage and bone are intrinsically different from the other neural crest cells. In particular, these studies also highlight the key difference between trunk and cranial neural crest is actually bone and cartilage formation.

A study by McGonnell *et. al.* in chicken show that trunk neural crest cells have skeletogenic potential (McGonnell and Graham, 2002) and this was also shown by Abzhanov *et. al.* (Abzhanov et al., 2003). When explants of trunk neural tube of were cultured for long periods in media conducive for bone cartilage formation, trunk neural crest cells could give rise to bone and cartilage. Furthermore McGonnell and Graham show that *in-vivo*, when trunk neural crest cells can form bone or cartilage when directly transplanted into the branchial arch. The authors postulate that the inability of trunk neural crest to form bone or cartilage is due to aberrant migration of trunk neural crest in the head since they can form bone and cartilage when directly transplanted into the branchial arch.

The finding that trunk neural crest cells can only form cartilage or bone after prolonged culture whereas cranial neural crest readily form cartilage and bone, led Abzhanov *et. al*. to investigate whether trunk neural crest cells were "converting" to a more cranial neural crest state and found that this was the case. There was upregulation of markers that are expressed by cranial neural crest as well as downregulation of Hox genes that are normally expressed by these explants. Therefore it is highly likely that the ability of the trunk neural crest to form cartilage and bone is due to de-differentiation to a more cranial neural crest state and under normal circumstances the trunk neural crest cells is unable to form bone or cartilage.

Some data from grafting experiments seems to suggest that neural crest are pre-patterned (Couly et al., 1998). When grafts of rhombomere 4-6 (express Hoxa2) were transferred to posterior mesencephalon or rhombomere 1-2 (do not express Hox), they found that these neural crest cells derived from rhombomere 4-6 cannot give rise to bone and cartilage but are able to give rise to other mesectodermal and neural derivatives. This is in contrast to the reverse experiment, where they found that neural crest cells from posterior mesencephalon or rhombomere 1-2 grafted to rhombomere 4-6 gave rise to the hyoid, which is derived from neural crest cells from rhombomere 4-6.

This is in contrast to what was reported by Noden *et. al.* who observed that when mesencephalic neural crest was transplanted to rhombomere 4-6, an ectopic lower jaw was formed (Noden, 1983). In the study by Couly *et. al.*, duplication of the lower jaw was observed when mesencephalic or rhombomere 1-2 was grafted together with the neural tube. It was subsequently found that the isthmus was secreting FGF8, which was responsible for the different observations (Trainor and Krumlauf, 2001). FGF8 is able to inhibit the expression of Hoxa2 in rhombomere 4 and leads to the transformation of this region into behaving more like the neural crest cells originating from anterior Hox negative regions.

It was shown previously that the neural crest cells in cranial regions can be divided into 2 subpopulations, an anterior population that do not express Hox genes and a posterior population that express Hox genes. Homeotic genes (also known as Hox genes) are a group of homeobox containing transcription factors that are expressed by rhombomeres in the hindbrain, neural crest as

well as in the ectoderm (Hunt et al., 1991a; 1991b). Homeotic genes are important for the patterning of the anterior and posterior axis as well as the BA. Cranial neural crest gives rise to bones and cartilage of the lower jaw and the inner ear, BA1 gives rise to the lower jaw whereas BA2 gives rise to the bones of the inner ear. Neural crest cells that do not express Hox genes give rise to the cells in the BA1, whereas neural crest expressing Hox give rise to the cells within BA2.

In summary, based on all these experiments, it is reasonable to suggest that there is something unique about cranial neural crest from the mesencephalon and rhombomere 1-2 (which do not express Hox genes) since they form cartilage ectopically (Couly et al., 1998). Furthermore cranial neural crest from rhombomeres 4 and 6 that express Hox do not give rise to bone or cartilage when grafted into the mesencephalon or rhombomere 1-2 (Couly et al., 1998).

However two studies show by transplanting small numbers of cells instead of small tissue between different hindbrain segments, that cells can be reprogrammed to the identity of their new environment (Trainor and Krumlauf, 2000; Schilling et al., 2001). It was previously shown by Couly *et. al.*, that heterotopic grafts do not express the Hox genes of the new environment. This shows that the identity of the cells is dependent on community effects, how neighboring cells affect the behavior of each other, as well as the new environment. This might also explain the discrepancy between the results of the grafting experiments between Noden and Couly. Additionally given the fact that trunk regions can be induced to form mesectoderm only in the presence of an intact lateral hindbrain, one might speculate that there might

be inducers within cranial regions that are promoting the formation of the mesectoderm.

From these studies, it is at present unclear what accounts for the unique skeletogenic potential of the cranial neural crest and whether cranial and trunk neural crest cells have the same developmental potential. From the studies on the role of Hox genes in patterning the neural crest, it might be possible that the Hox genes have a role in determining whether cells can give rise to bone or cartilage.

# Chapter 2: Materials and Methods

## 2.1 Animal Welfare and housing

All experiments involving animals were reviewed and approved by the Institutional Animal Care and Use Committees of Agency for Science, Technology, and Research (A*STAR) Biomedical Science Institutes (IACUC No. 070250 and 100551). Mice were housed in the Biological Resource Center of the Biomedical Research Institute.

## 2.2 Genotyping of animals

DNA was extracted from tail clippings using the hotshot method of DNA extraction (Truett et al., 2000). In brief, 150µl of lysis buffer (25 mM NaOH, 0.2 mM EDTA) was added to the tail clippings and incubated for 30min at 95$^{o}$C. Samples were chilled on ice and 150µl neutralization buffer (40 mM Tris-HCl) was added. Samples were diluted with water to 500µl and 1µl of the DNA sample was used for the PCR genotyping.

Four strains of mice were used in the current study; Sox1 Cre; HtPa Cre; Gt(ROSA)26Sor$^{tm1.1(EYFP)}$; and Gt(ROSA)26Sor$^{tm1(LacZ)}$. Genotyping of Sox1 Cre mice was done by PCR using the primers Sox1 Cre F: 5'-gatcgctgccaggatatacg and Sox1 Cre R: 5'-ctctgaccagagtcatccttagc, and observing a band that is ~400bp. Genotyping of HtPA Cre was via PCR using

the primers HtPA Cre F: 5'-tgtctcctcttctttctctta and HtPA Cre R: 5'-cgcctgaagatatagaagata, and observing a ~450bp band. Genotyping of Gt(ROSA)26Sor$^{tm1.1(EYFP)}$; and Gt(ROSA)26Sor$^{tm1(LacZ)}$ was done by PCR using these 3 primers: Rosa1: 5'- aaagtcgctctgagttgttat, Rosa2: 5'-gcgaagagtttgtcctcaacc, and Rosa3: 5'-ggagcgggagaaatggatatg and observing a wild type band that was 600bp and a mutant band that was 300bp.

## 2.3 Generation of mouse embryos for lineage analysis

In order to obtain Sox1 Cre YFP or Sox1 Cre LacZ embryos for analysis, timed matings of Sox1 Cre and YFP or LacZ reporter mice were carried out. To determine the day of mating, vaginal plugs was checked the next couple of days and it was considered E0.5 on the day of observation of the vaginal plug.

## 2.4 Collection of early mouse embryos

Embryos were collected between E8.0 to E13.5. The embryos were carefully dissected from the decidual and the membranes covering the embryo were removed. Embryos were checked for yellow fluorescent protein (YFP) expression under a fluorescent stereomicroscope. The embryos were straightened before fixation for 30min at 4$^{o}$C in 4% PFA. For analysis of E9.5 and E10.5 embryos, the time of fixation was increased to 1hr, whereas for E13.5 embryos, the time of fixation was 2hrs.

## 2.5 Collection of early chicken embryos

Fertilized chicken eggs were obtained from Chew's Agriculture. The eggs were incubated in a 38 $^{o}$C incubator to the appropriate stages. Embryos were removed in Tyrode's saline. For ex-ovo culture of chicken embryos for lineage analysis, care was taken to ensure that the area opaca is intact. The embryos were fixed in 4% PFA for 30min at 4 $^{o}$C for cryo-sectioning.

## 2.6 Ex-ovo culture and labeling of the neural fold of early chicken embryos (Pastry culture)

After removing the embryos from the eggs, lateral regions of the neural fold (3-5 somite chicken embryo) or neural tube (6-8 somite chicken embryo) was labeled by DiI. The lateral region of the neural fold was labeled by applying a small drop of dye with a picospritzer (PLI-100, Harvard Apparatus) whereas the neural tube was labeled by injecting dye into the lumen of the closed neural tube. A picture of the labeled chicken embryo was taken to determine where the dye was applied. The embryos were cultured by pastry culture (Nagai et al., 2011) for 2 days at 38 $^{o}$C before being fixed in 4% PFA at 4 $^{o}$C overnight for analysis of where and what the initial labeled cells became. After 2 days of culture, generally the embryos developed to HH 16-18.

## 2.7 Embedding of embryos for cryo-sectioning

The embryos were briefly rinsed in PBS and equilibrated in 30% sucrose before orientating and freezing in Tissue-Tek® O.C.T. compound (Sakura Finetek, Torrance, CA, USA) in dry ice/ethanol bath. The tissue block was stored at -80 $^{o}$C before sectioning in cryostat. The sections were sectioned at 15mm thick.

## 2.8 Analysis of postnatal day 0 skull

For the analysis of X-gal stained skulls, newborn pups were decapitated. The skull was prepared for x-gal staining by removing the skin around to aid in penetration of the x-gal. To prepare whole mounts of the skull, the brain was removed as well so as to better visualize the stained bones in the skull since the entire brain was labeled in the Sox1 Cre animals. For sections, the brain was left in place. After removing the skin, the skull was briefly rinsed in PBS and fixed for 2hrs in PBS containing 0.02% Np40, 1% PFA, and 0.2% gluteraldehyde at 4$^{o}$C. The skulls were rinsed 2 times in staining solution (1x PBS, 0.2% NP40, and 2mM $MgCl_2$) without X-gal, $K_3Fe(CN)_6$, and $K_4Fe(CN)_6$.3H2O. Skulls were stained for 2 days at 4$^{o}$C with staining solution containing 1mg/ml Xgal, 5mM $K_3Fe(CN)_6$ and 5mM $K_4Fe(CN)_6$.3H2O. After staining, the skulls were washed 2x in PBS for 30min each. The skulls were then refixed in 4% PFA overnight.

For skulls that were sectioned, they were rinsed in PBS and soaked in 0.5M EDTA overnight with constant agitation before dehydration in an alcohol series and embedding in wax. The skull was sectioned at 8mm thick and sections counterstained with neutral red.

For whole mount x-gal stained skull, the skulls were rinsed with PBS and soaked in 2% KOH for 2 weeks to clear the soft tissue. The skulls were then transferred into 100% glycerol for 2 days, and this was repeated 2 times.

## 2.9 Immunostaining of sections

After sectioning, the O.C.T was removed by washing the slides with PBST (PBS containing 0.1% Triton X100) for 3 times (5min each time). Blocking was done by incubating the slides with blocking solution (PBST containing 10% normal donkey serum) for 2hrs at room temperature. Primary antibody was diluted in blocking solution and was added to the slides overnight at 4 $^{o}$C. The slides were washed by incubating the slides with PBST for 6min and this was repeated three times. Secondary antibody was diluted in blocking solution and incubated with the slides for 2hrs at room temperature before washing again with PBST for three times and 6min each time. For DAPI staining, PBST containing 0.2μg/ml of DAPI was added after secondary antibody and incubated for 10min before commencing washing.

## 2.10 Whole mount immunostaining

After fixation, the embryos were washed with PBS. Embryos were transferred to PBST (PBS containing 0.5% Triton-X100) for 10min. The embryos were then permeabilized by incubating them in acetone (kept at -20 $^{o}$C) for 45s, followed by washing for three times in PBST for 10min each time. Blocking solution (PBST containing 5% normal donkey serum, 1% DMSO) was added to the embryos and incubated overnight at 4$^{o}$C. This was followed by another overnight incubation with primary antibody (diluted in blocking solution). The embryos were washed three times with PBST for 1hr each time. Secondary antibody (diluted in blocking solution) was incubated overnight at 4$^{o}$C with the embryos. The embryos were then washed three times with PBST for 1hr each time. Finally the embryos were stored or imaged in PBS containing 50% glycerol.

## 2.11 Confocal imaging

After immunostaining, images were acquired with an Olympus fluoview FV1000 confocal microscope. Images were processed in an image processing software, Fiji.

## 2.12 Antibodies

Table 2.1: Antibodies used in this study:

| Name | Immunized Species | Reference |
|---|---|---|
| E-cadherin | Rat, monoclonal | Sigma (St. Louis, MO, USA) |
| L-CAM | Goat, polyclonal | Gift from Warren Gallin |
| Sox1 | Goat, polyclonal | R&D Systems, Minneapolis, MN, USA |
| NCD2 | Rat, monoclonal | R&D Systems |
| Fibronectin | Rabbit, polyclonal | Sigma |
| Laminin | Rabbit, polyclonal | Sigma |
| Sox9 | Rabbit, polyclonal | Millipore, Berllerica, MA, USA |
| Sox10 | Goat, polyclonal | Santa Cruz Biotechnology, Santa Cruz, CA |
| GFP | Chicken, polyclonal | Abcam, Cambridge, United Kingdom |
| Pax3 | Mouse, monoclonal | DSHB, Developmental Studies Hybridoma Bank, University of Iowa |
| Pax7 | Mouse, Monoclonal | DSHB |
| Msx1 | Mouse, monoclonal | DSHB |
| Ap2 | Mouse, monoclonal | DSHB |
| Snail2 | Mouse, monoclonal | DSHB |
| RhoB | Mouse, monoclonal | DSHB |
| β-Dystroglycan | Mouse, monoclonal | Novocastra laboratories (UK) |
| p75 | Rabbit, polyclonal | Promega, Madison, WI |

# Chapter 3: Characterization of mouse neural crest

## 3.1 Characterization of premigratory cells

In the following chapter, we try to determine where within the ectoderm cells are delaminating from and whether there is a link between origin and cell fate in mouse embryos. To investigate where cells are delaminating from, and whether the neural fold is phenotypically homogenous we wanted to determine the molecular characteristics of this region of the ectoderm. Immunostaining of mouse embryos with E-cadherin, Sox1 and neural crest markers (Sox9, Tfap2a and Pax7) would define distinct ectodermal domains: the non-neural ectoderm, neural ectoderm and where cells are delaminating from respectively.

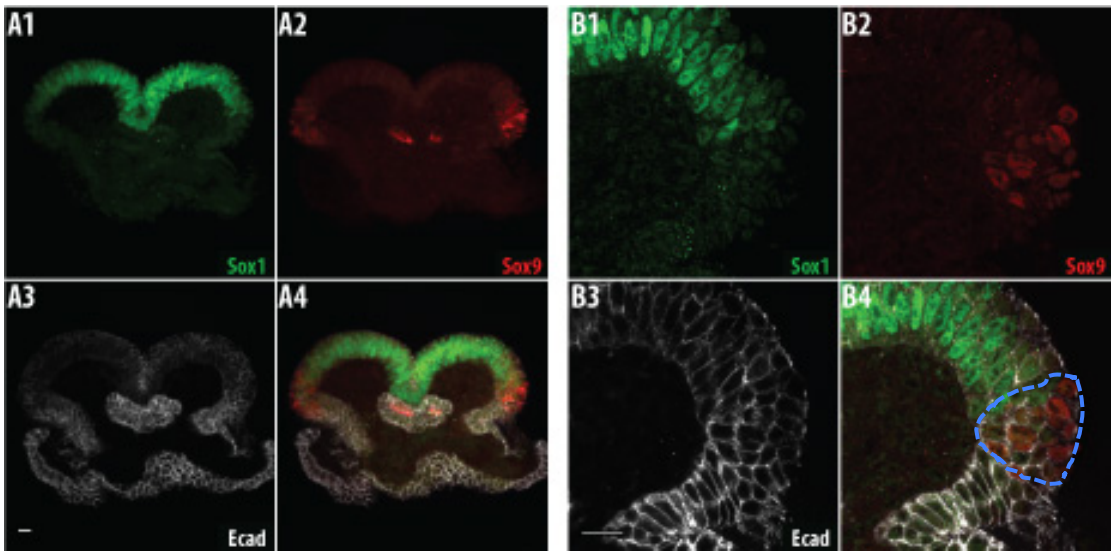


**Fig. 3.1: Expression of Sox9 within the non-neural ectoderm in the cranial region of E8.0 2 somite mouse embryos.**

Sox9 is expressed in the E-cadherin (Ecad) expressing non-neural ectoderm but not in the Sox1 expressing neural ectoderm (A-B). (B), magnified view of the neural fold. Blue dotted line; Sox9 expressing cells in the non-neural ectoderm. Scale bar is 20μm.

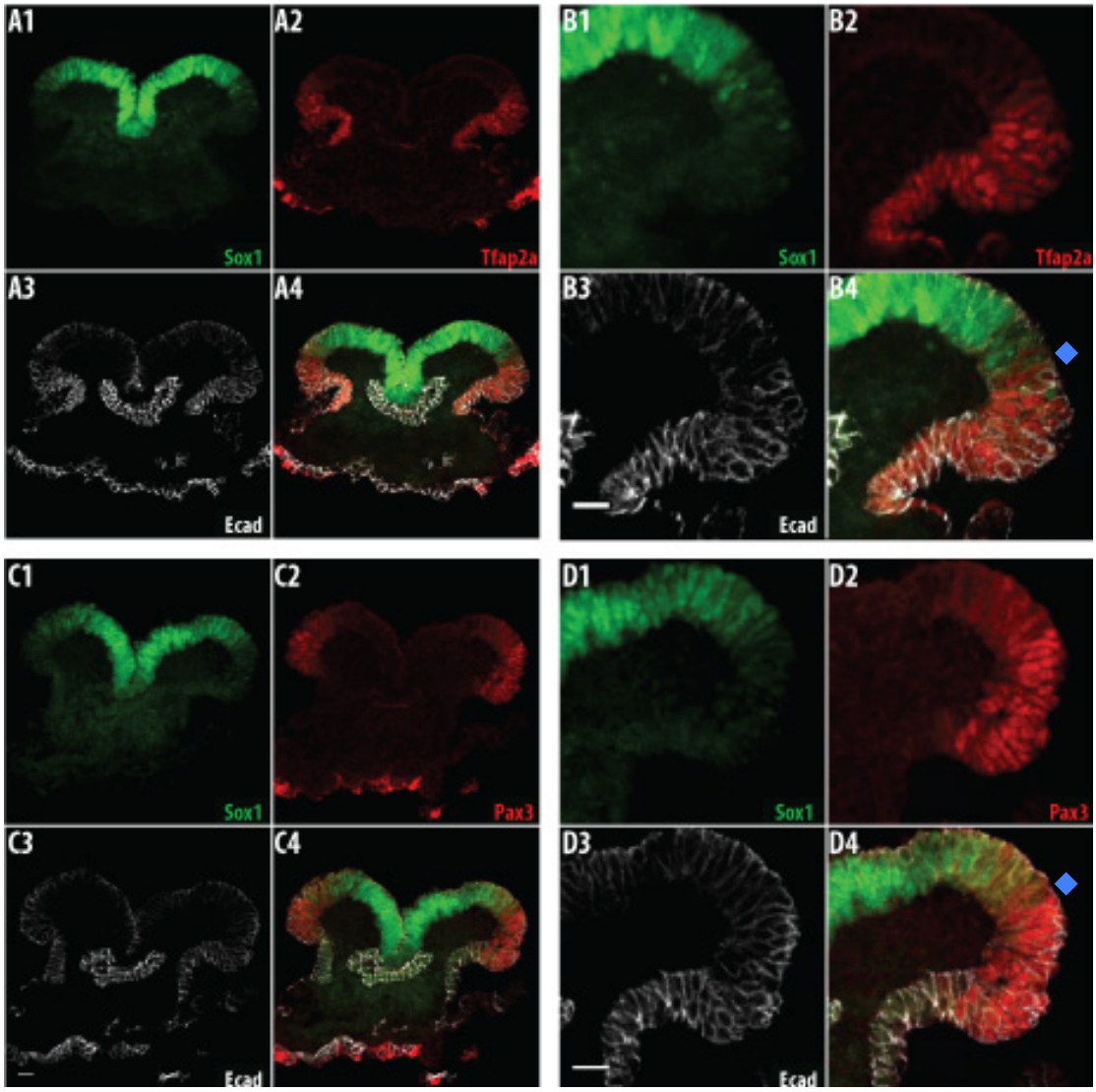


**Fig. 3.2: Expression of Tfap2a and Pax3 in the cranial region of E8.0 2 somite mouse embryos.**

Dorsal markers like Tfap2a and Pax3 are also expressed in the non-neural ectoderm and are not expressed in the neural ectoderm (A-D). (B, D) magnified view of the neural fold. Blue diamond; show the boundary between the neural (Sox1) and non-neural ectoderm (Ecad). Scale bar is 20μm.

Fig. 3.1 shows a wild-type mouse embryo at E8.0 with 2 somites, Sox9 (a neural crest marker) and E-cadherin are co-expressed by the same cells. At this stage Sox1 is already expressed in the neural ectoderm but there is no expression of Sox9 in the neural ectoderm. Other neural crest markers like Pax3 and Tfap2a at this stage show a very similar pattern of being expressed

in the non-neural ectoderm (fig. 3.2), although Pax3 is also expressed in the dorsal neural ectoderm expressing Sox1 (fig 3.2D), Pax3 has been shown to be important for the development of dorsal neuronal derivatives in the spinal cord (Mansouri and Gruss, 1998).

From the observation above, the neural crest markers (Sox9, Tfap2a and Pax3) are first expressed in the non-neural ectoderm. From fig. 3.1 and 3.2, the cells expressing the neural crest markers are still epithelial at this stage. It has been shown that the breakage of the basement membrane is an early event during EMT (Nakaya et al., 2008). From fig. 3.3 A-B, the basement membrane is still intact under cells expressing Tfap2a showing that these cells have not undergone EMT. As the cells still have not migrated out of the ectoderm, there is the possibility that these cells that are going to delaminate may be induced into neural ectoderm before delaminating from the neural fold. At ~3 somites when cells have not delaminated, there is reduced amounts of basement membrane beneath the Sox9 cells, these Sox9 positive cells are located in the non-neural ectoderm since they do not express Sox1.

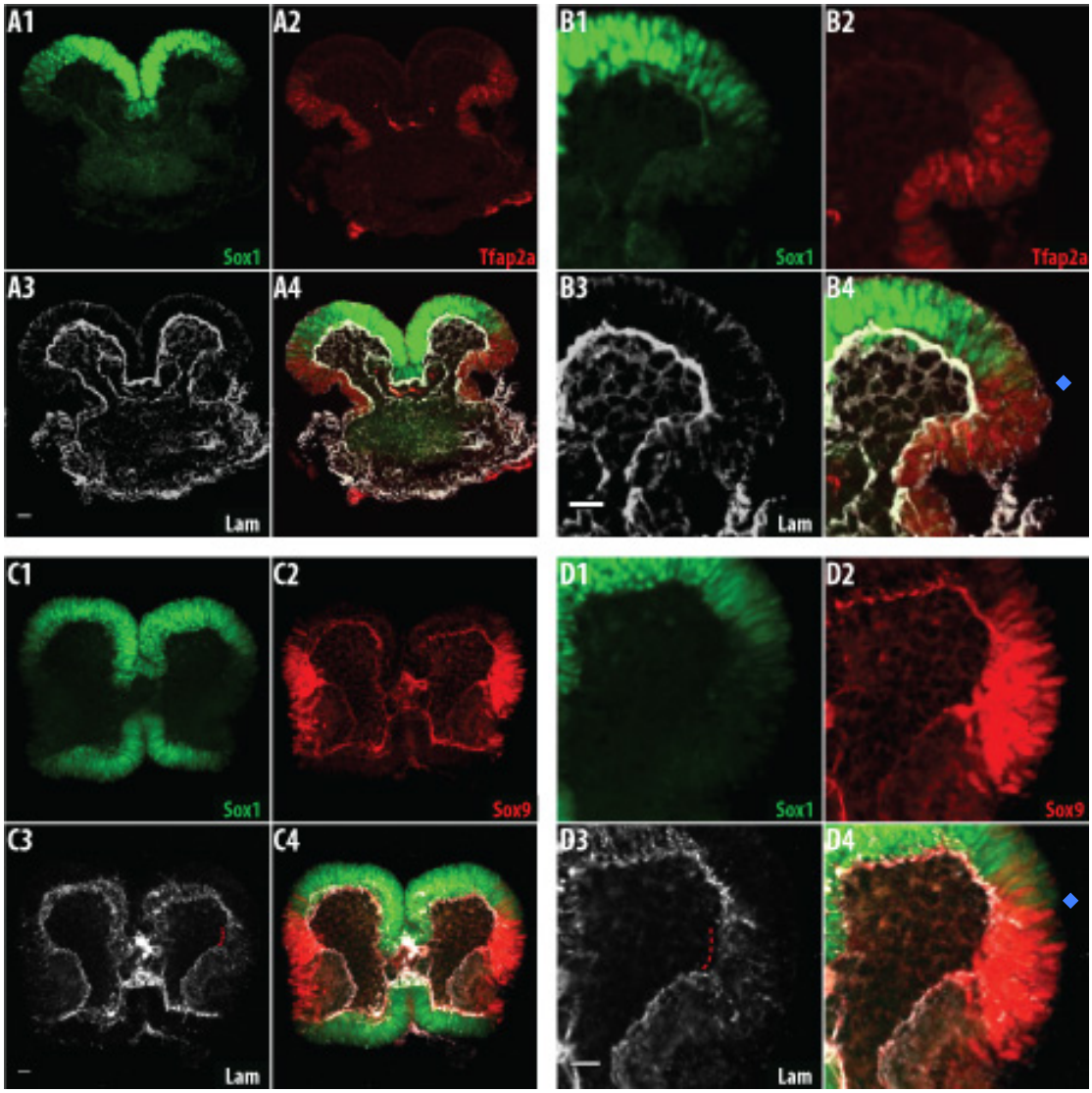


**Fig. 3.3: Appearance of the basement membrane underlying the neural crest in the cranial region of E8.0 mouse embryo.**

Neural crest cells have not started migrating out of the ectoderm (A-B). Sox9 cells in the non-neural ectoderm are beginning to delaminate as shown by the breakage of the basement beneath these cells (C, D). (A, B) 2 somite. (C, D), ~3somite, red dotted line shows reduced amounts of laminin under Sox9 expressing cells. (B, D), magnified view of the neural fold. Blue diamond; show the boundary of the neural ectoderm (Sox1). Scale bar is 20μm.

## 3.2 Characterization of cells that have delaminated

In embryos that are at E8.0 (4 somites), cells are beginning to delaminate from the ectoderm. At this stage, Sox9 is still expressed in the E-cadherin expressing non-neural ectoderm (fig. 3.4). At this stage there are a few Sox9 positive cells that also express Sox1 and recently delaminated Sox9 cells, which are just next to the ectoderm, have reduced amounts of E-cadherin. Consistent with the fact that these cells have just undergone EMT from the non-neural ectoderm (fig. 3.4B), since cells have to down-regulate E-cadherin in order to delaminate.

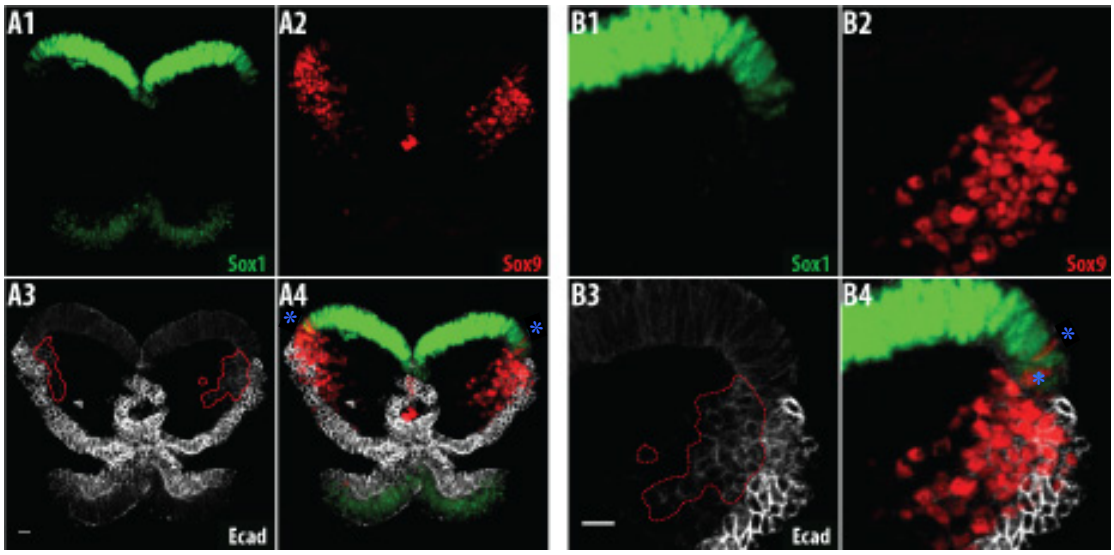


**Fig. 3.4: Expression of Sox9 within the non-neural ectoderm in the cranial region of E8.0 4 somite mouse embryos.**
During early cell delamination, Sox9 is expressed mainly in the E-cadherin expressing non-neural ectoderm and not in the Sox1 expressing neural ectoderm (A-B). Red dotted lines are cells that have delaminated but still express low levels of E-cadherin and blue asterisk are cells that are double positive for Sox9 and Sox1. (B) magnified view of the neural fold. Scale bar is 20µm.

Tfap2a expression within the neural fold shows a similar pattern to Sox9 at this stage, being mainly expressed in the non-neural ectoderm expressing E-cadherin (fig. 3.5). Furthermore, breakage of basement membrane is seen over the non-neural ectoderm (cells do not express Sox1 in this region). This means that cells are delaminating from the non-neural ectoderm (fig. 3.6).

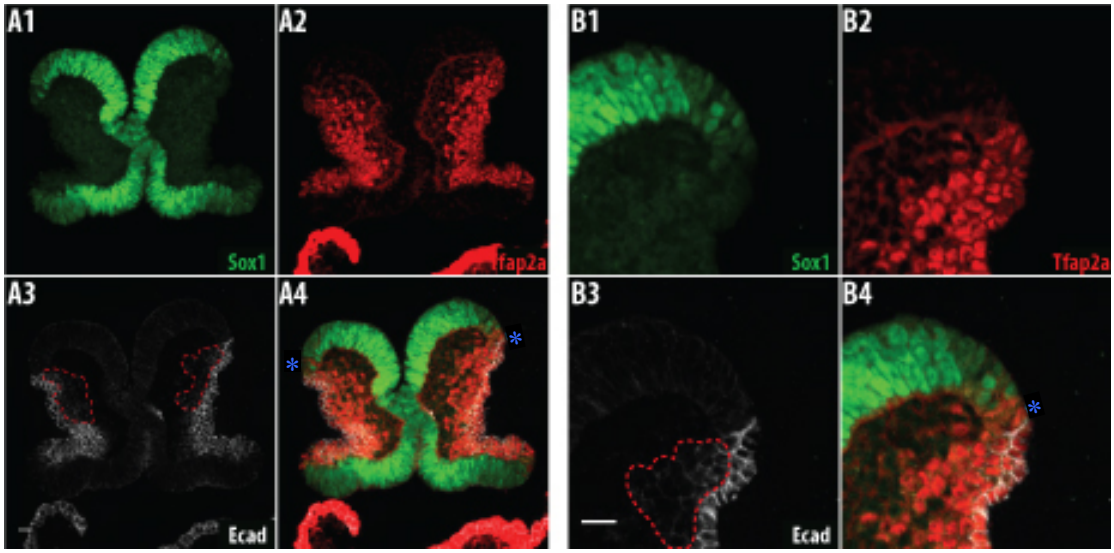


**Fig. 3.5: Expression of Tfap2a within the non-neural ectoderm in the cranial region of E8.0 4 somite mouse embryos.**

During early cell delamination, Tfap2a is expressed mainly in the E-cadherin expressing non-neural ectoderm and not in the Sox1 expressing neural ectoderm (A-B). Red dotted lines are cells that have delaminated but still express low levels of E-cadherin and blue asterisk are cells that are double positive for Tfap2a and Sox1. (B), magnified view of the neural fold. Scale bar is 20μm.

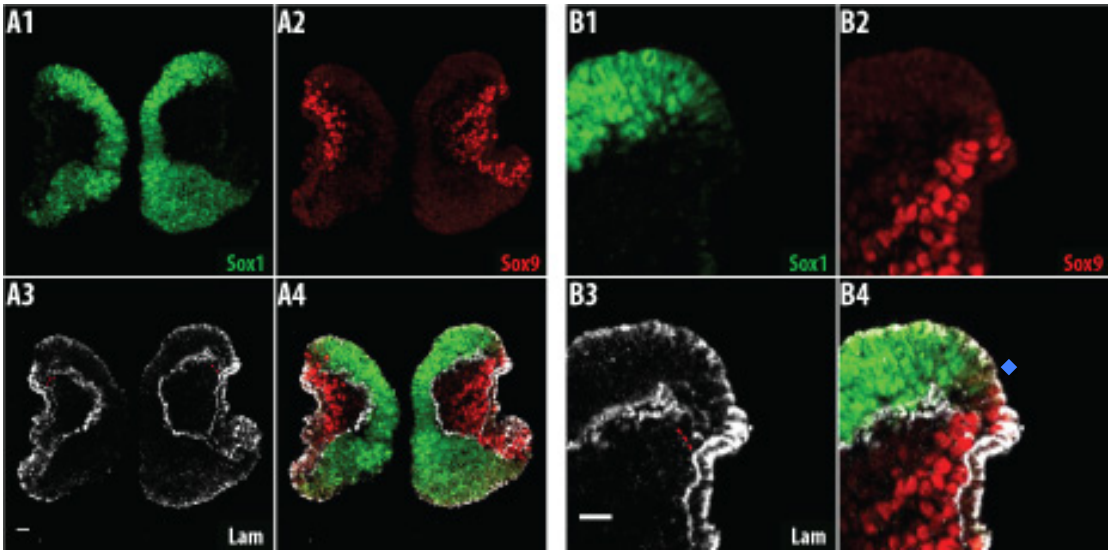


**Fig. 3.6: Disruption of the basement membrane in the cranial region of E8.0 4 somite mouse embryo occurs under the non-neural ectoderm.**
Cells have started migrating out of the ectoderm and breakage of basement membrane occurs under Sox9 cells that are located in the non-neural ectoderm (A, B). (A and B) 4 somites, red dotted line shows reduced amount of Laminin under Sox9 expressing cells. (B), magnified view of the neural fold. Blue diamond; show the boundary of the neural ectoderm (Sox1). Scale bar is 20μm.

In older embryos at E9.0 (8-10 somites), the border between the neural and non-neural ectoderm is more clearly defined. At this stage, Sox9 is expressed in the neural ectoderm (fig. 3.7). These cells are delaminating from a region of the neural ectoderm that express a lower level of Sox1, compared to more ventral parts of the neural ectoderm, and the cells that have just delaminated do not contain any E-cadherin.

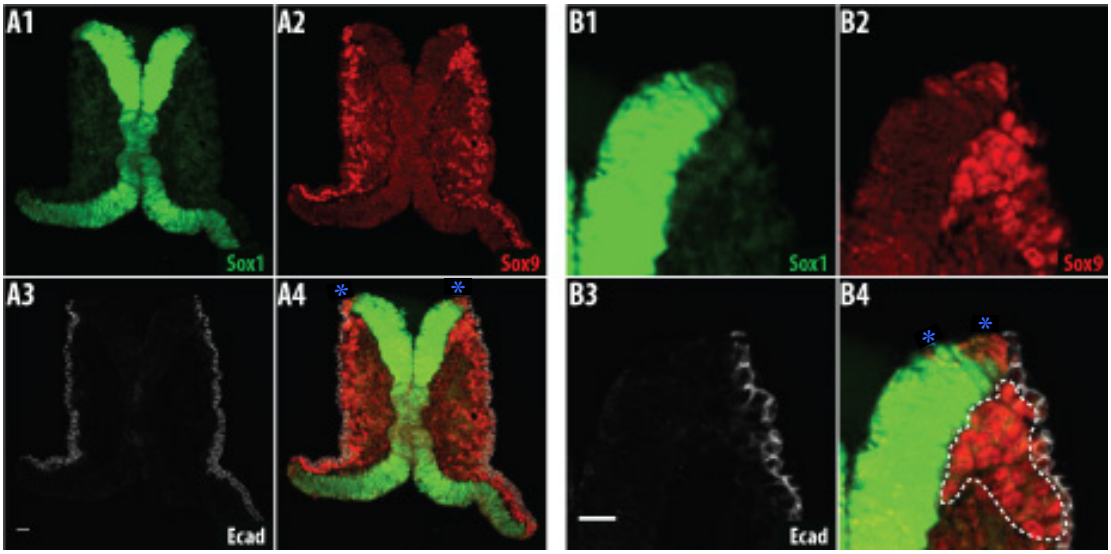


**Fig. 3.7: Expression of Sox9 within the neural ectoderm in the cranial region of E9.0 10 somite mouse embryos.**

Sox9 is expressed in the Sox1 expressing neural ectoderm but not in the non-neural ectoderm expressing E-cadherin (A-B). Blue asterisk are cells expressing both Sox9 and Sox1, and white dotted line are cells that have just delaminated which do not contain E-cadherin. Note that the dorsal most cells of the neural ectoderm express low levels of Sox1. (B), magnified view of the neural fold. Scale bar is 20μm.

Expression of other neural crest markers shows that they are now also expressed in the dorsal neural ectoderm. Tfap2a, which at earlier stages was mainly expressed in the E-cadherin positive non-neural ectoderm (fig. 3.2A and B), is now also expressed in the dorsal neural ectoderm (fig. 3.8A and B). Similarly for Pax3, which was at earlier stages expressed in the E-cadherin positive non-neural ectoderm (fig. 3.2C and D), is now solely expressed in the Sox1 positive neural ectoderm (fig. 3.8 C and D).

From the observations above, there is a shift in the expression pattern of the neural crest markers from the non-neural ectoderm to the neural ectoderm during initial and at later stages of cell delamination.

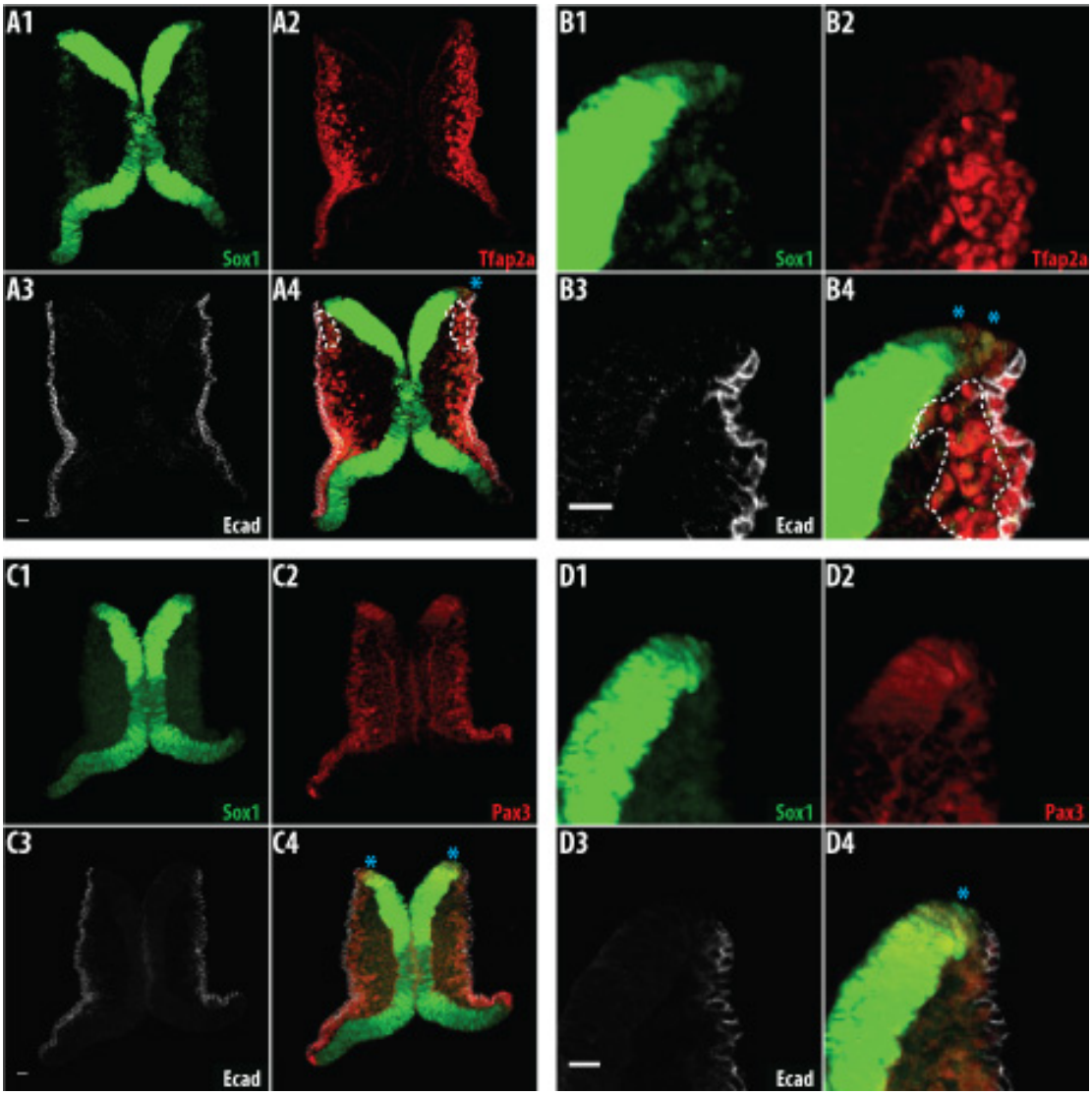


**Fig. 3.8: Expression of neural crest markers in the cranial region of E8.5 and E9.0 mouse embryos.**

Tfap2a and Pax3 are both expressed in the Sox1 expressing neural ectoderm (blue asterisk) at later stages of cell delamination (A-D). (A and B), Tfap2a expression in 6 somite embryo. White dotted line; cells that have recently delaminated do not express E-cadherin. (C and D), Pax3 expression in 10 somites embryo. (B and D), magnified view of the neural fold. Scale bar is 20μm.

## 3.3 Characterization of delaminating cells in the trunk.

In the trunk, the neural ectoderm and non-neural ectoderm are segregated prior to the delamination of the neural crest cells (fig. 3.9). Sox1 is expressed in the entire neural ectoderm, however expression is stronger in the ventral region compared to the dorsal region. At the dorsal region, there is lower expression of Sox1.

At this stage neural crest cells in the trunk have not started migrating out of the neural tube, Sox9 is only expressed in the neural ectoderm (fig. 3.9A) and it is the same case for other neural crest markers like Tfap2a (fig. 3.9B) and Pax3 (fig 3.9C). This is very different from the situation in the cranial region where prior to and during early cell delamination, these markers are first expressed in the non-neural ectoderm and only at later stages of migration, these markers begin to be expressed in the neural ectoderm.

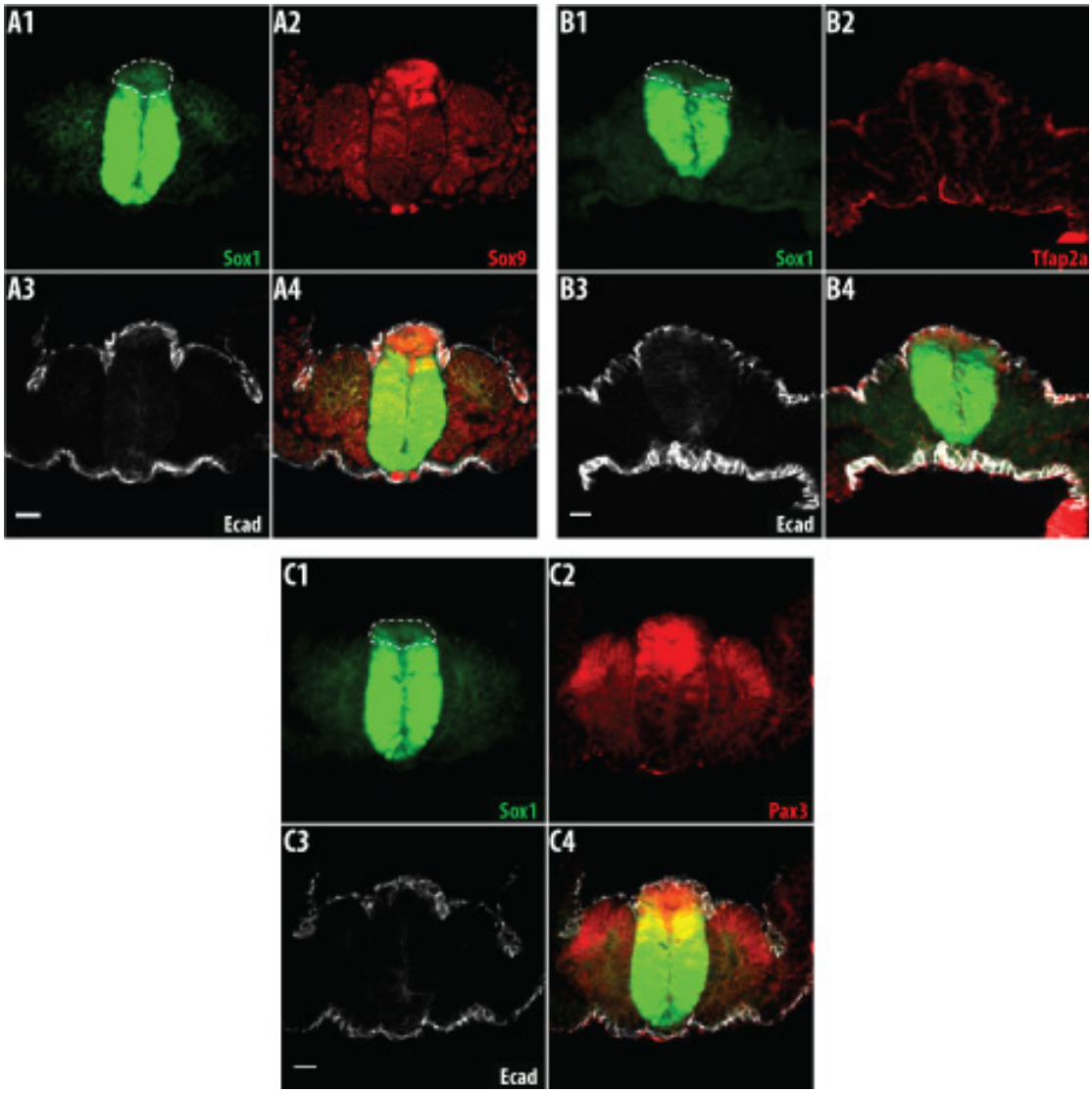


**Fig. 3.9: Expression of neural crest markers within the neural ectoderm in the trunk region of E9.0 mouse embryos.**

In 10-12 somite embryo, neural and non-neural ectoderm are segregated prior to neural crest delamination in the trunk. In this region, the dorsal neural expresses lower levels of Sox1 (white dotted line) and this is where the neural crest markers are expressed (A-C). (A), Sox9 expression in 10 somite trunk. (B), Tfap2a expression in 12 somite trunk. (C), Pax3 expression in 10 somite trunk. Scale bar is 20μm

## 3.4 Cells delaminating from the non-neural ectoderm are found mainly at the level of the midbrain.

It is known that the neural crest cells in the midbrain region are the first to delaminate. To have a better idea of where cells are delaminating from the non-neural ectoderm as well as how the border region looks like at different axial levels, whole mount immunostaining of Sox1, Sox9 and E-cadherin was performed on E8.5 embryos (5 somite). At this stage the Sox9 expressing cells are mainly found in the midbrain region (fig. 3.10) and some cells have already started to delaminate.

In the midbrain region (fig. 3.10A), there is a region where there is reduced expression of E-cadherin next to the Sox1 expressing neural ectoderm. At more posterior regions, there is no such region with reduced expression of E-cadherin and the neural ectoderm expressing Sox1 is tightly apposed to the non-neural ectoderm expressing E-cadherin. This together with fig. 3.4 shows that the border between the neural and non-neural ectoderm at the midbrain is not well defined at the earliest stage of neural crest migration (cells which are delaminating are found at this boundary). The region with reduced expression of E-cadherin corresponds to cells delaminating from the non-neural ectoderm (compare fig. 3.10A and fig. 3.10B). Fig. 3.10C shows an optical section through this region, with Sox9 cells delaminating from the non-neural ectoderm.

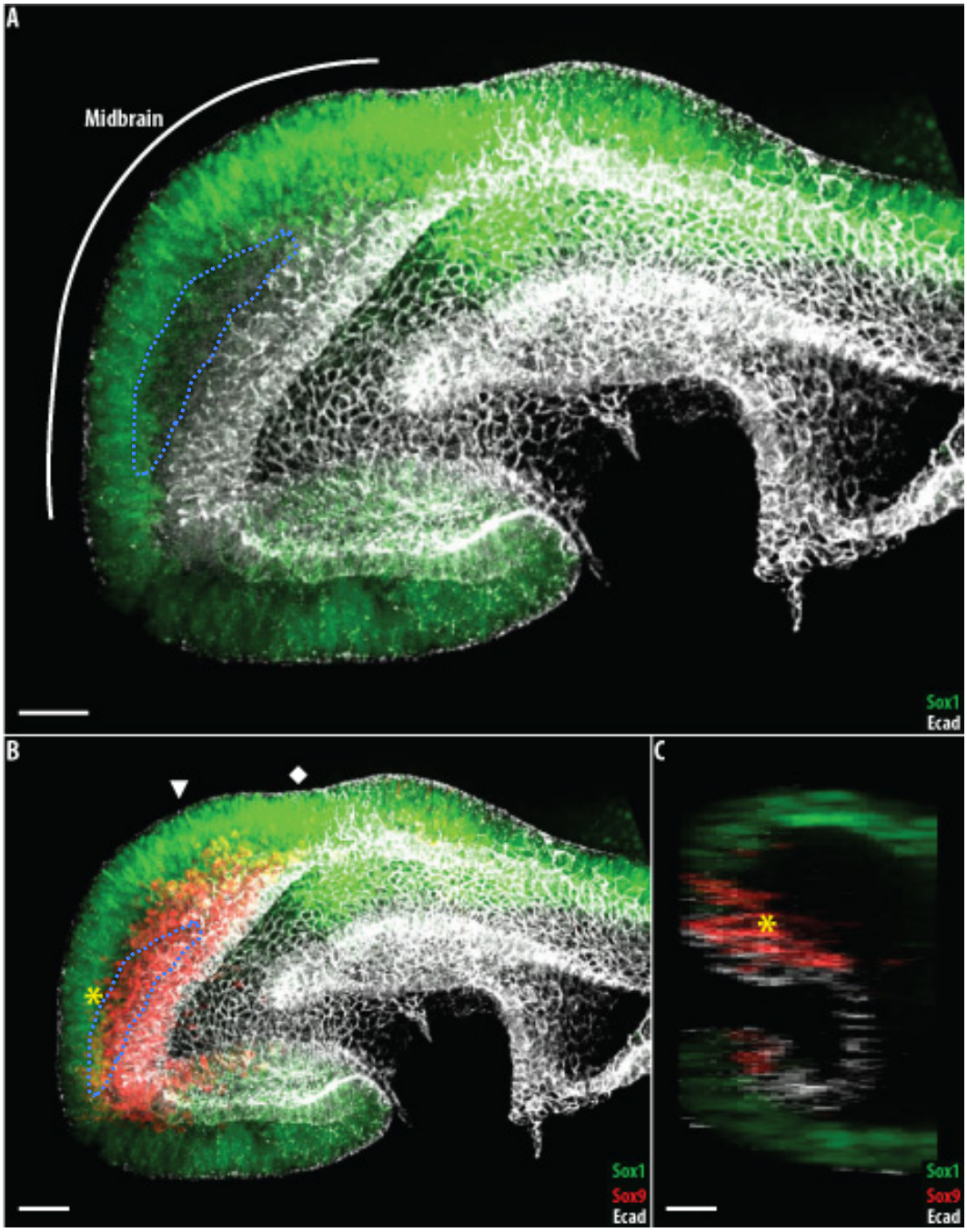


**Fig. 3.10: Whole-mount immunostains at midbrain level reveal that Sox9 labelled cells accumulate in an intermediate region between lateral non-neural and neural ectoderm.**

Confocal image of the cranial region of a whole mount immunostained 5 somites mouse embryo stained with Sox1, Sox9 and E-cadherin. Blue dotted line; region with reduced expression of E-cadherin (A and B). Yellow asterisk; Sox9 and E-cadherin double positive cells in the midbrain region (B and C). Diamond, marks the end of the midbrain region (C), Cross section of (B) at the triangle. Scale bar is 40µm

## 3.5 Characterization of derivatives in Sox1 Cre

Sox1 is expressed very early during neural development (Pevny et al., 1998; Wood and Episkopou, 1999). Interestingly, Takashima *et. al.* reported that in Sox1 Cre YFP embryos at E9.5, very little mesectoderm within BA1 was labeled (Takashima et al., 2007). This prompted us to request the mouse line from them for further analysis.

To ensure that the Sox1 Cre was acting at the earliest stages of neural induction, we crossed the Sox1 Cre mice with the Rosa26 YFP reporter line and looked at E8.0 (2-4 somites) embryos. Fig. 3.11A and B shows that YFP is expressed in a similar way to the endogenous Sox1 protein except that at these early stages, expression of the reporter is more mosaic (weak expression). This is probably due to the slight delay in activation of the YFP reporter compared to expression of Sox1 protein.

At early stages (fig. 3.11C and D) majority of the delaminating cells are not labeled. However due to the weak YFP signal, it is unclear whether there are some YFP positive cells which are delaminating (triangle in fig 3.11D, this will be discussed in greater detail in the later section). However at about 8 somites, the second population of cells delaminating from the neural ectoderm in the head is labeled (fig. 3.12). In the trunk of Sox1 stained embryos (fig. 3.9), the dorsal region weakly expresses Sox1, however in the Sox1 Cre YFP embryos, the entire neural ectoderm is labeled prior to neural crest cell delamination (fig. 3.13).

From these results, Sox1 Cre in the cranial region preferentially labels the cells that delaminate at later times from the neural ectoderm; however in the trunk it labels all delaminating cells.

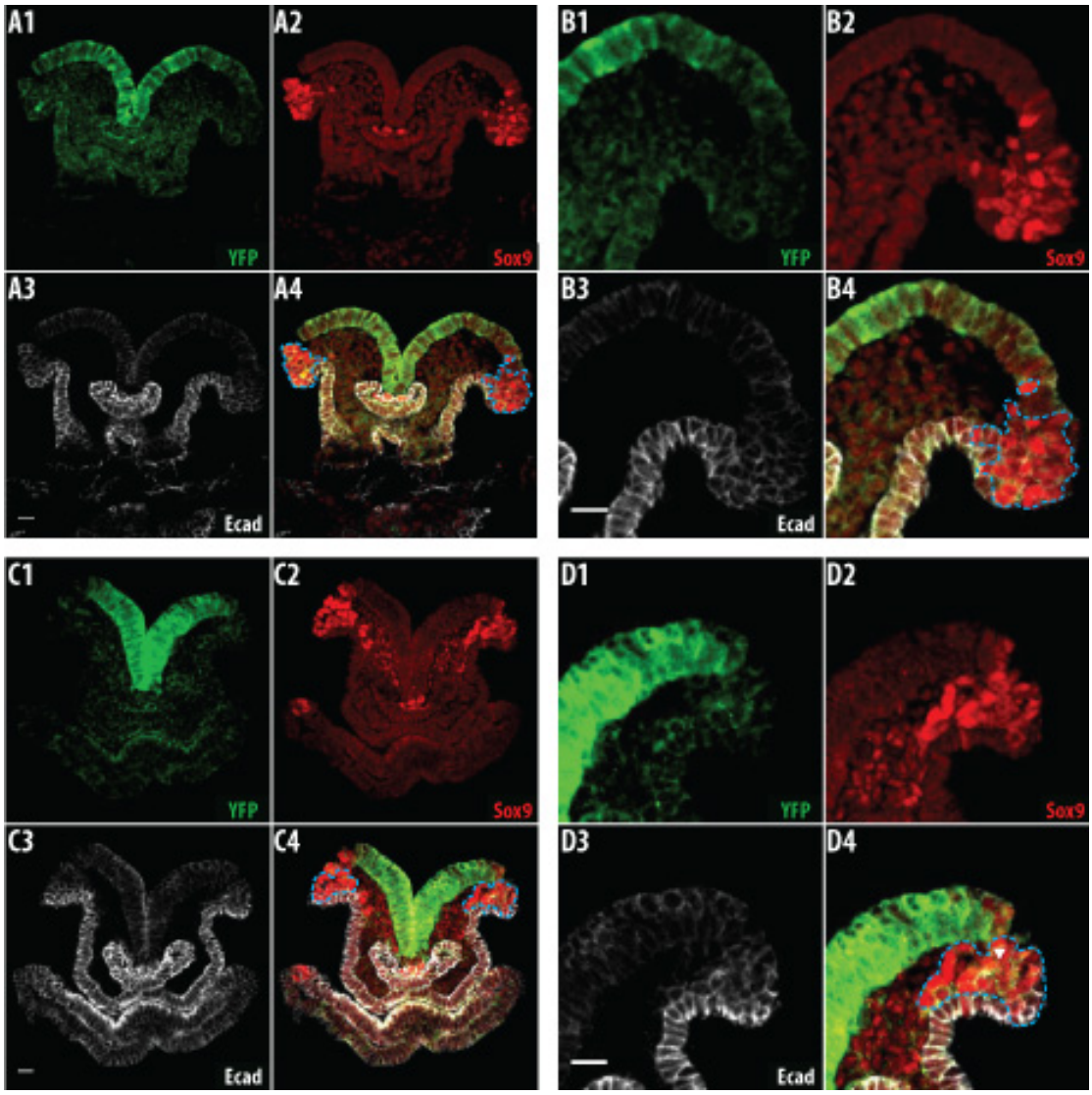


**Fig. 3.11: YFP is expressed in the neural ectoderm in Sox1 Cre YFP embryos at early stages of neural crest formation.**

YFP is expressed in the neural ectoderm prior to delamination of cells (A and B). When cells are beginning to delaminate, the majority of cells are not YFP positive (C and D). (A and B), 2 somite embryo. Blue dotted line; Sox9 expression in the non-neural ectoderm. (C and D), 4 somite embryo. Blue dotted line; Sox9 expressing cells which are delaminating. Due to the relatively weak YFP signal it is unclear whether some cells are YFP labeled (triangle). (B and D) magnified view of the neural fold. Scale bar is 20μm.

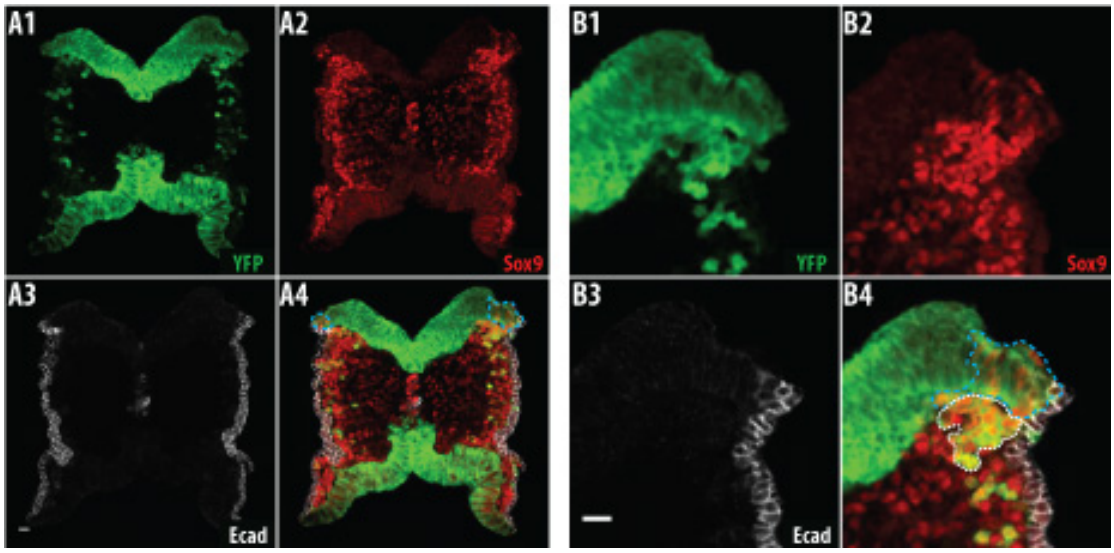


**Fig. 3.12: Sox1 Cre labels the second population of delaminating cells.**

At E9.0 (8 somites) when the second populations of cells are delaminating from the neural ectoderm, the dorsal neural tube is entirely labeled (A4 and B4, blue dotted line) and cells originating from this region are also labeled (B4, white dotted line). (B), magnified view of the neural fold Scale bar is 20μm

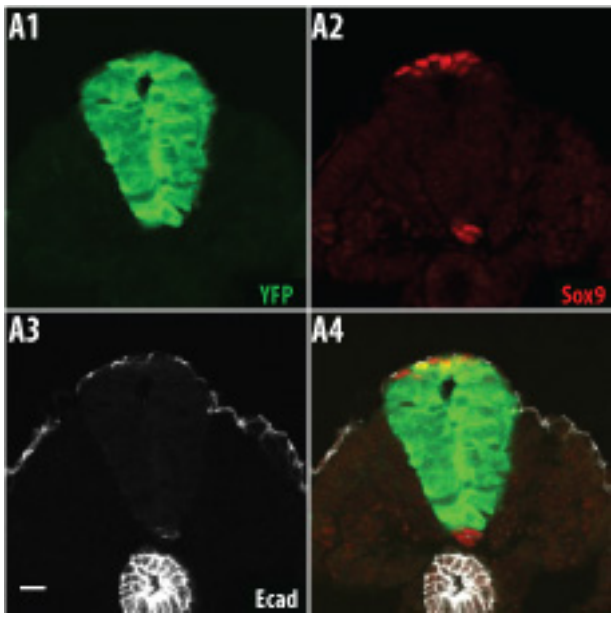


**Fig. 3.13: Sox1 Cre labels the neural tube in the trunk prior to delamination of cells**

In the trunk, the entire neural tube is YFP positive prior to neural crest delamination. (A), E9.5 (19 somite) Sox1 Cre YFP embryo. Scale bar is 20μm.

## 3.6 Derivatives labeled in Sox1 Cre animals

As shown in the previous section, we showed that the Sox1 Cre preferentially labels the cells that delaminate from the neural ectoderm; this allows us to determine whether there is a link between cell fate and origin.

From the previous sections, it was shown that Sox1 as well as the reporter is activated in the neural ectoderm of the trunk prior to any cell delaminating. This prompted us to determine whether the neural crest derivatives in the trunk are labeled. In the trunk, the dorsal root ganglia, sympathetic ganglia and melanocytes, which are derived from the neural crest, are labeled in the Sox1 Cre line (fig. 3.14).

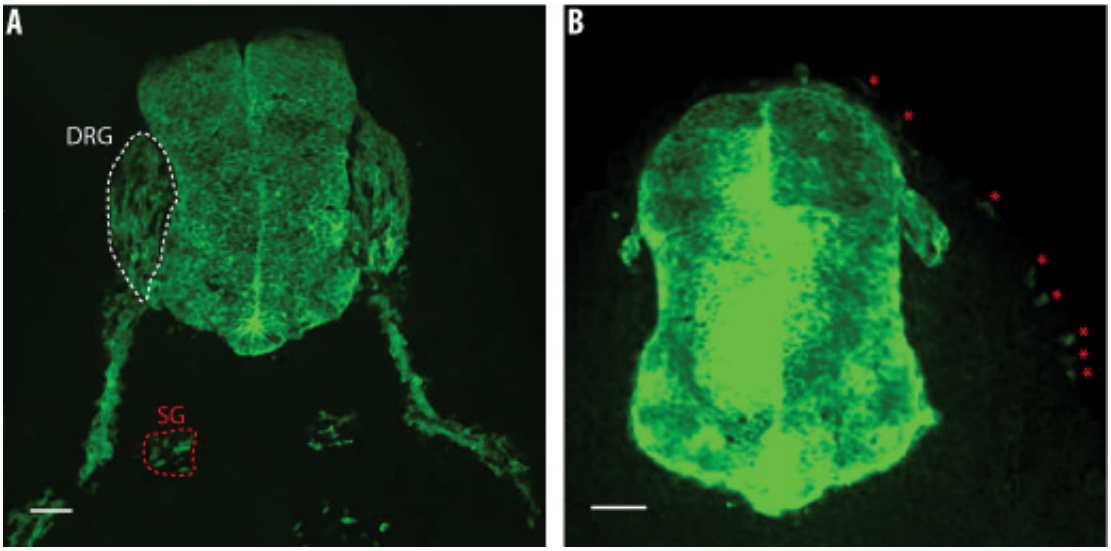


**Fig. 3.14: Labeled trunk neural crest derivatives in Sox1 Cre YFP embryos.**

E10.5 Sox1 Cre YFP embryo (A and B). DRG, dorsal root ganglion. SG, sympathetic ganglia. Red asterisk, melanocytes (B). Scale bar is 40μm

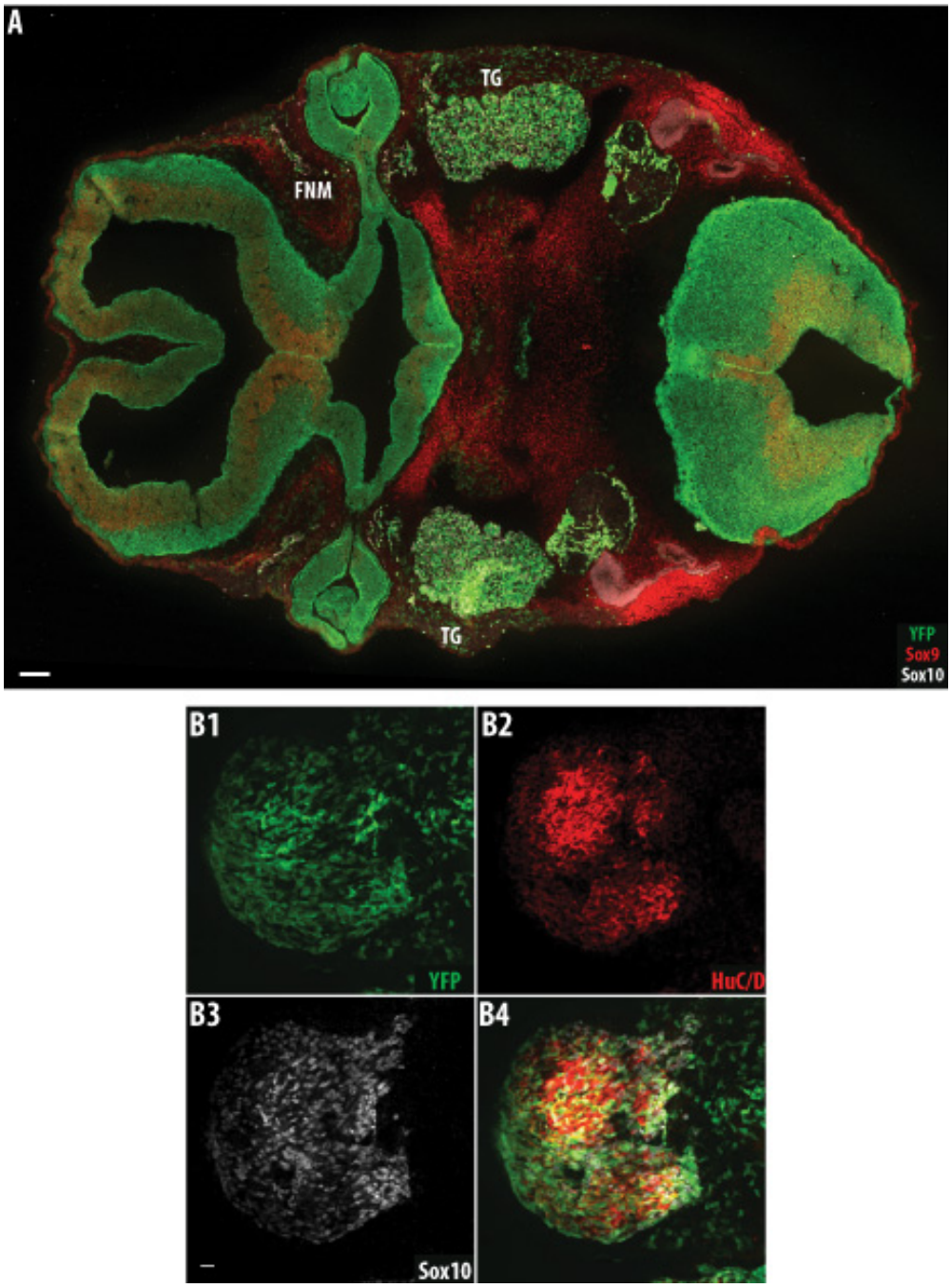


**Fig. 3.15: Glial cells within the trigeminal ganglia are well labeled in Sox1 Cre YFP embryos.**

E11.5 Sox1 Cre YFP embryo (A and B). (A), Cranial region containing the trigeminal ganglia. FNM, frontal nasal process. TG, trigeminal ganglia. (B), trigeminal ganglia. Neurons express HuC/D whereas glia expresses Sox10. Glial cells as well as some neurons in the trigeminal ganglia, are derived from neural crest, is labeled in Sox1 Cre. There are also a few YFP cells that express HuC/D (yellow cells in B4). Scale bar in A and B are 100μm and 20μm respectively.

Cross-section through the cranial region showed that the trigeminal ganglion is well labeled in Sox1 Cre YFP embryos. Glial cells in the trigeminal ganglia express Sox10 and are derived from the neural crest. These cells also express YFP showing that in the Sox1 Cre line (fig. 3.15), they originate from the neural ectoderm. In contrast, most of the neurons in the trigeminal ganglia are not labeled in the Sox1 Cre line (fig. 3.15B), this is consistent with previous data that most neurons (express HuC/D) in the cranial ganglia are derived from the cranial placode and not neural crest derived (D'Amico-Martel and Noden, 1983),

To determine whether mesectoderm, in particular bone and cartilage, are labeled in the Sox1 Cre, we looked at the BA1 of E11.5 embryos. This stage was chosen as bone and cartilage precursors begin to express Sox9 (Akiyama et al., 2005).

In fig. 3.16, there are YFP expressing cells within BA1 but most of these cells do not express Sox9. This is most prominent in more ventral regions of BA1 (compare fig. 3.16A and fig. 3.16B). To further confirm that bone and cartilage from BA1 are not labeled, we looked at the skull from Sox1 Cre LacZ embryos at later stages. These skull preparations show that the mandible, which is derived from BA1, is not extensively labeled (fig. 3.17).

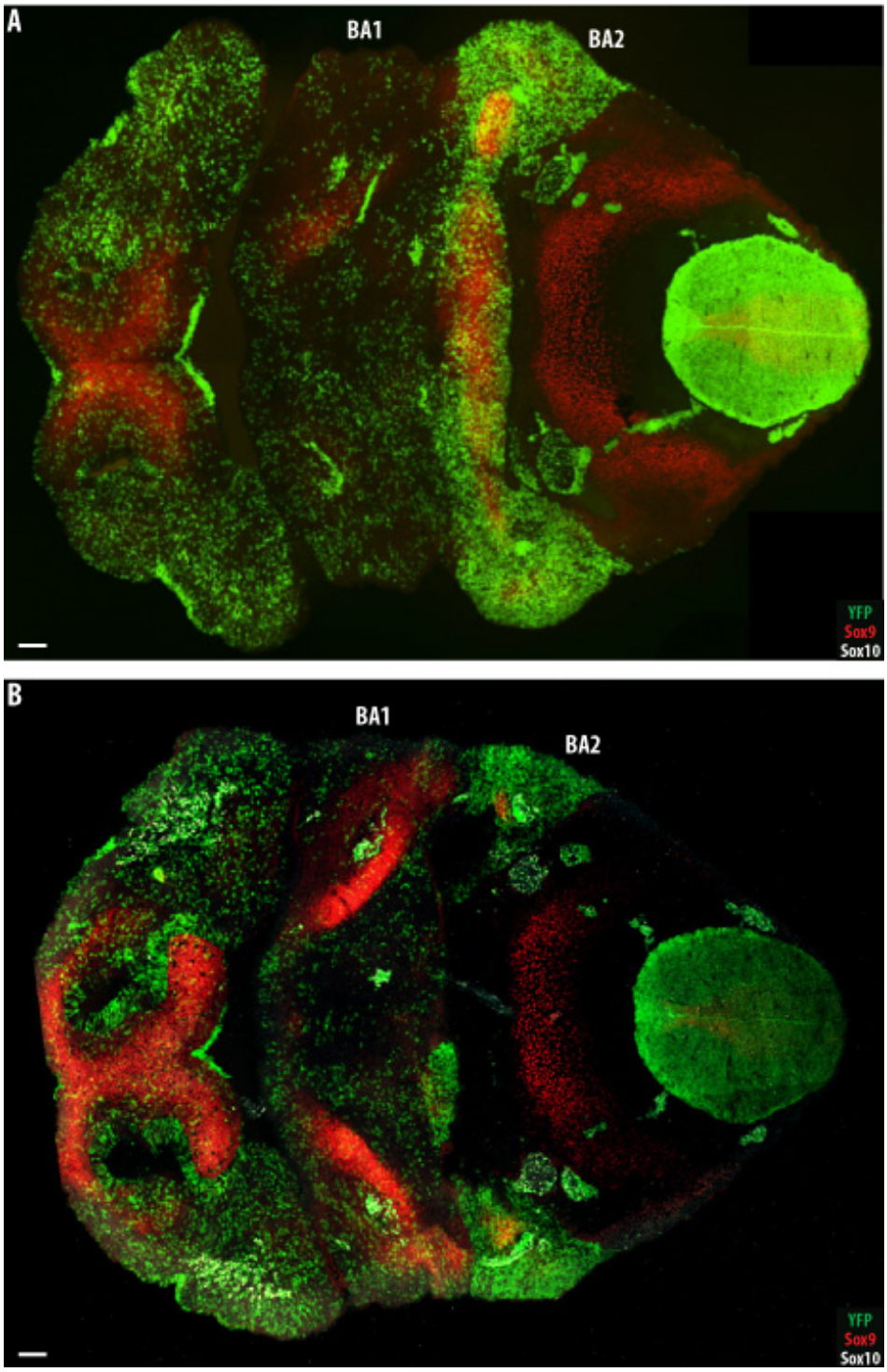


**Fig. 3.16: Cartilage/bone precursors are not well labeled in the Sox1 Cre YFP embryos.**

Cross-section of the head in E11.5 Sox1 Cre YFP embryos. BA1, branchial arch one. BA2, branchial arch two (A and B). (A), is a cross section that is more caudal than (B). Few cells expressing YFP (green) are found in the Sox9 expressing regions (red). Scale bar is 100µm.

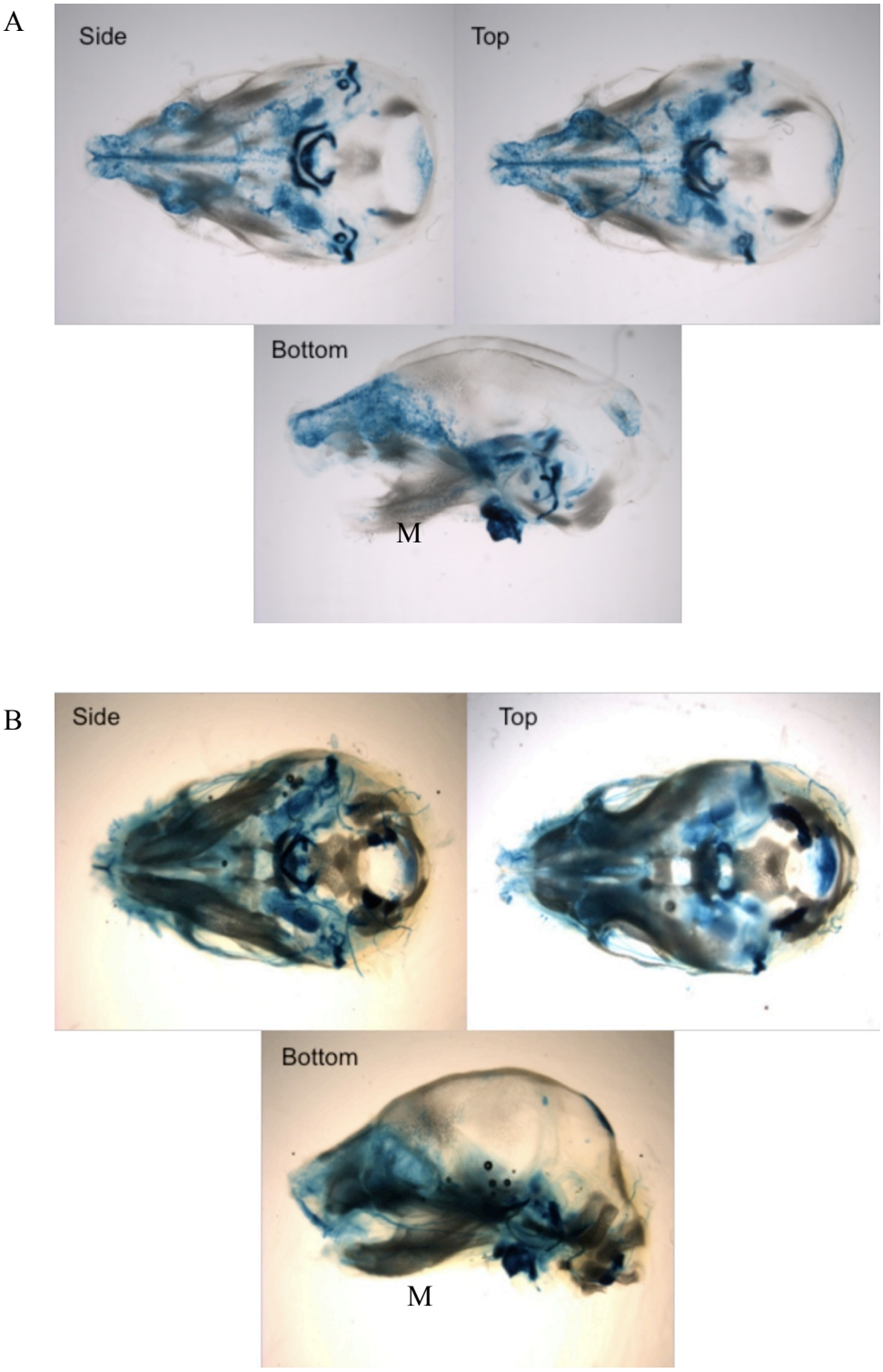


**Fig. 3.17: Skull preparations of Sox1 Cre LacZ embryos.**

The mandible is derived from neural crest cells that originate from the mesencephalon however X-gal staining in the mandible is not obvious in Sox1 Cre LacZ embryos. (A), E16.5 skull preparation. (B), E18.5 skull preparation. M, Mandible that is derived from BA1.

To quantitate what is the contribution of Sox1 Cre to neuronal versus mesectodermal derivatives, we counted the number of YFP positive cells in the trigeminal ganglion as well as BA1 at E9.0 (~15somites). Consecutive sections from Sox1 Cre/YFP embryos were collected and the sections were stained with Tfap2a, Sox10 and YFP to show the crest derivatives in the trigeminal ganglion as well as BA1. Crest derivatives in the trigeminal ganglia as well as mesectodermal express Tfap2a as well as Sox10; in addition Tfap2a is also expressed in the ectoderm. Fig. 3.18A shows the regions that was counted to generate the graph in fig. 3.18B. Fig. 3.18B shows the percentage of YFP labeled cells in the trigeminal ganglia versus BA1. From fig. 3.18B, the trigeminal ganglion is much better labeled compared to BA1.

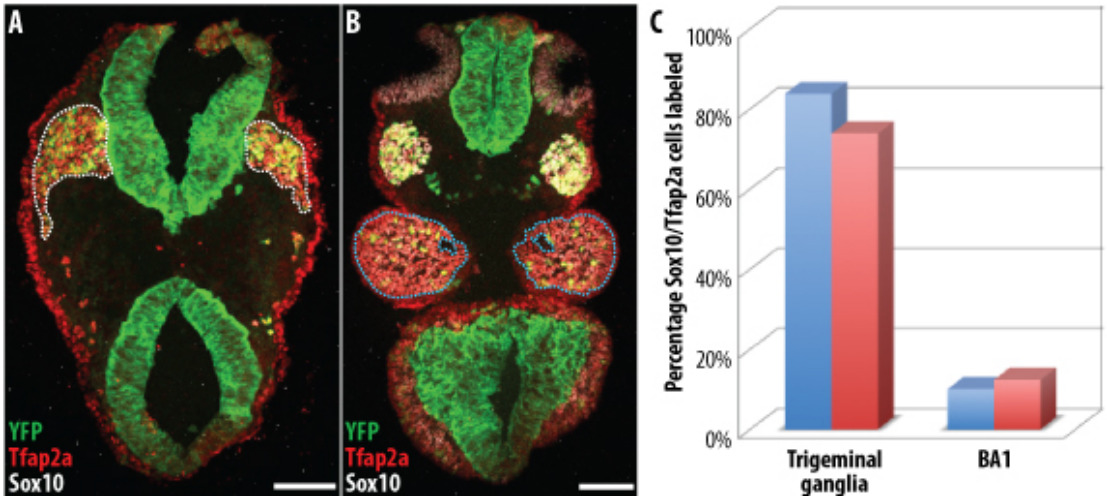


**Fig. 3.18: Quantification of YFP labeled cells in the trigeminal ganglia and branchial arch.**

The number of YFP, Tfap2a and Sox10 in the trigeminal ganglia (A) and branchial arch one (B) was counted to calculate the percentage of YFP labeled cells in each of these regions (C). (A), white dotted line; trigeminal ganglia. (B), blue dotted line; BA1. Scale bar in (A) and (B) are 100 µm. (C), each bar represents one animal.

In Sox1 Cre YFP embryos, neuronal derivatives are well labeled, whereas very few mesectodermal derivatives are labeled (fig. 3.16 and fig. 3.18). This coupled with the finding that in Sox1 Cre the second population of cells originating from the neural ectoderm is labeled lead us to conclude that the first population of cells that are delaminating from the non-neural ectoderm are giving rise to mesectodermal derivatives whereas the second population of cells originating from the neural ectoderm give rise to neuronal derivatives. This is in contrast to the trunk where all the derivatives from the neural crest are labeled.

However the presence of YFP expressing cells within BA1 as well as in other regions (branchial arch two in fig. 3.16) suggests that perhaps cells originating from neural ectoderm might also be capable of giving rise to mesectoderm. There are two possibilities about the origin of these cells, the first possibility is that they originate from the neural ectoderm and the second possibility is due to ectopic activation of Cre in regions that do not normally express Sox1.

When the expression of Sox1 protein (fig. 3.2A and B) is compared to reporter activation (fig. 3.11A and B), there is a graded expression of Sox1 protein in the dorsal region of the neural fold; this is in contrast to the activation of YFP in the Sox1 Cre YFP embryos. Furthermore at early stages of delamination, sporadic cells expressing YFP can be seen in areas, which are clearly expressing E-cadherin (fig. 3.19) (Cells express YFP, Sox9 and E-cadherin, these cells will be referred to as triple positive cells in the subsequent sections). Although we cannot rule out the possibility that the labeled

mesectoderm originates from the neural ectoderm, we think that at least some of the labeled mesectoderm originates from the triple positive cells.

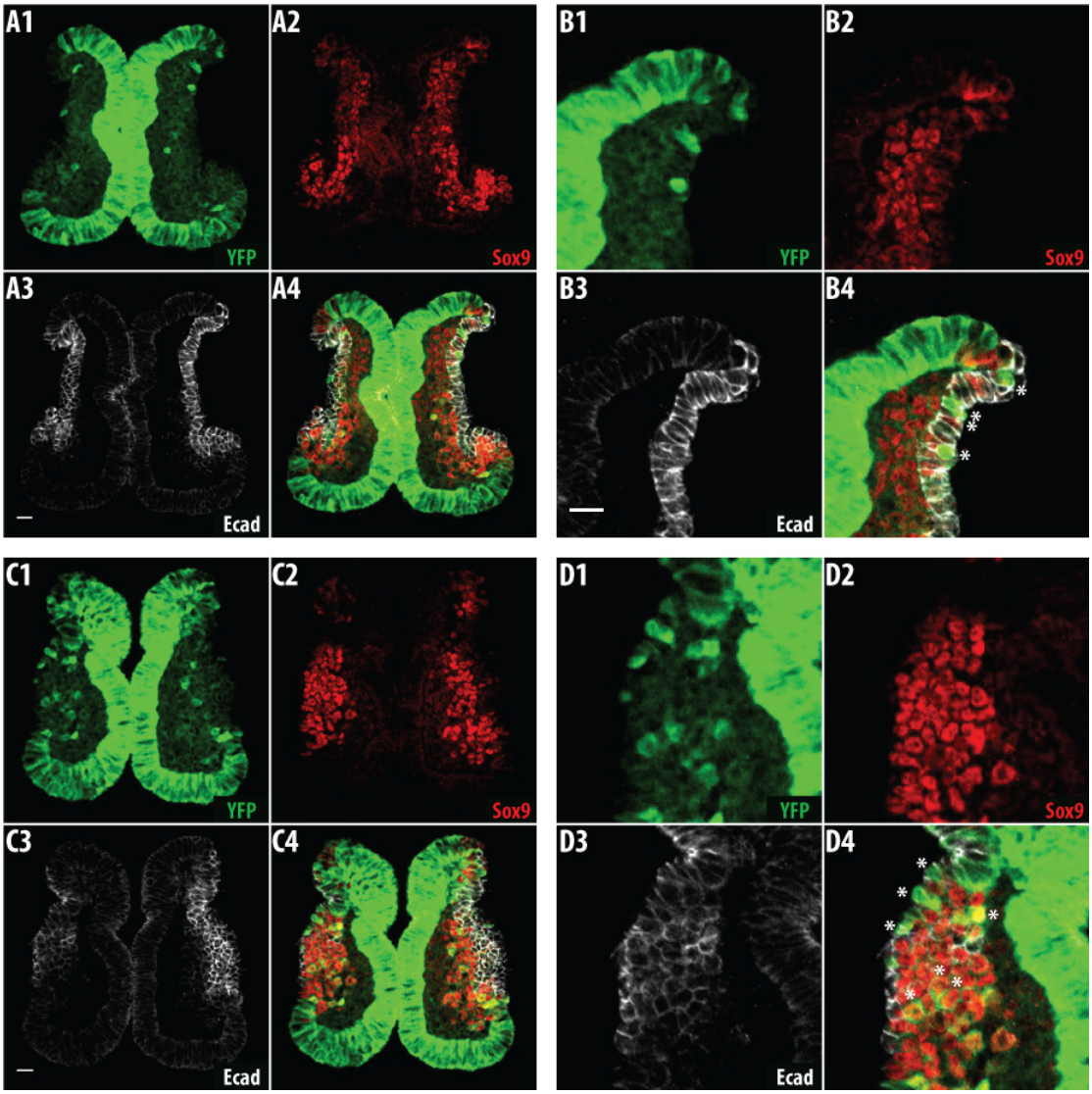


**Fig. 3.19: Aberrant activation of YFP in the non-neural ectoderm in Sox1 Cre YFP embryos.**

E8.5 (7 somites) Sox1 Cre YFP embryo. 2 serial sections of the same embryo; (A and B) is more posterior to (C and D). White asterisk, triple positive cells. (B and D) magnified view of the neural fold Scale bar is 20μm

An explanation for the appearance of the triple positive cells is probably due to the way that Cre works. Activation of the reporter reflects a threshold of Cre

activity that is needed to remove the upstream stop codon (an on or off state); it is unable to recapitulate a graded response. Furthermore it is also possible that under normal circumstances cells expressing a low level of Sox1 that ends up in the non-neural ectoderm will simply turn off Sox1 expression and convert to a non-neural ectoderm fate (boundary concept, (Dahmann et al., 2011)) and because activation of the reporter is permanent, it does not reflect this kind of dynamic expression.

# Chapter 4: Characterization of delaminating cells in chicken embryos

## 4.1 Expression profiles of neural crest markers in chicken

Due to the presence of the triple positive cells and the presence of YFP labeled mesectoderm in Sox1 Cre YFP embryos, we searched for another approach to determine whether there are cells delaminating from the non-neural ectoderm and whether these cells give rise to mesectodermal derivatives. The study by Lumsden *et. al.* had already shown in the chick that labeling the neural crest at early stages gave rise to mesectodermal derivatives whereas labeling the neural crest at later stages gives rise to neuronal and mesectodermal derivatives (Lumsden et al., 1991). What is remarkable in this study is that no specific region was being targeted and seems to suggest that the neural crest is fate restricted.

To determine whether cells are delaminating from the neural or non-neural ectoderm, we looked at the expression of L-CAM (chicken E-cadherin ortholog) and N-cadherin which are expressed in the non-neural and neural ectoderm respectively (Edelman et al., 1983; Hatta and Takeichi, 1986). Both of these molecules are expressed very early during embryogenesis, and we looked at the expression of Snail2, Pax7, Msx1/2, Sox9 and Tfap2a to determine in which domain these cells are delaminating from.

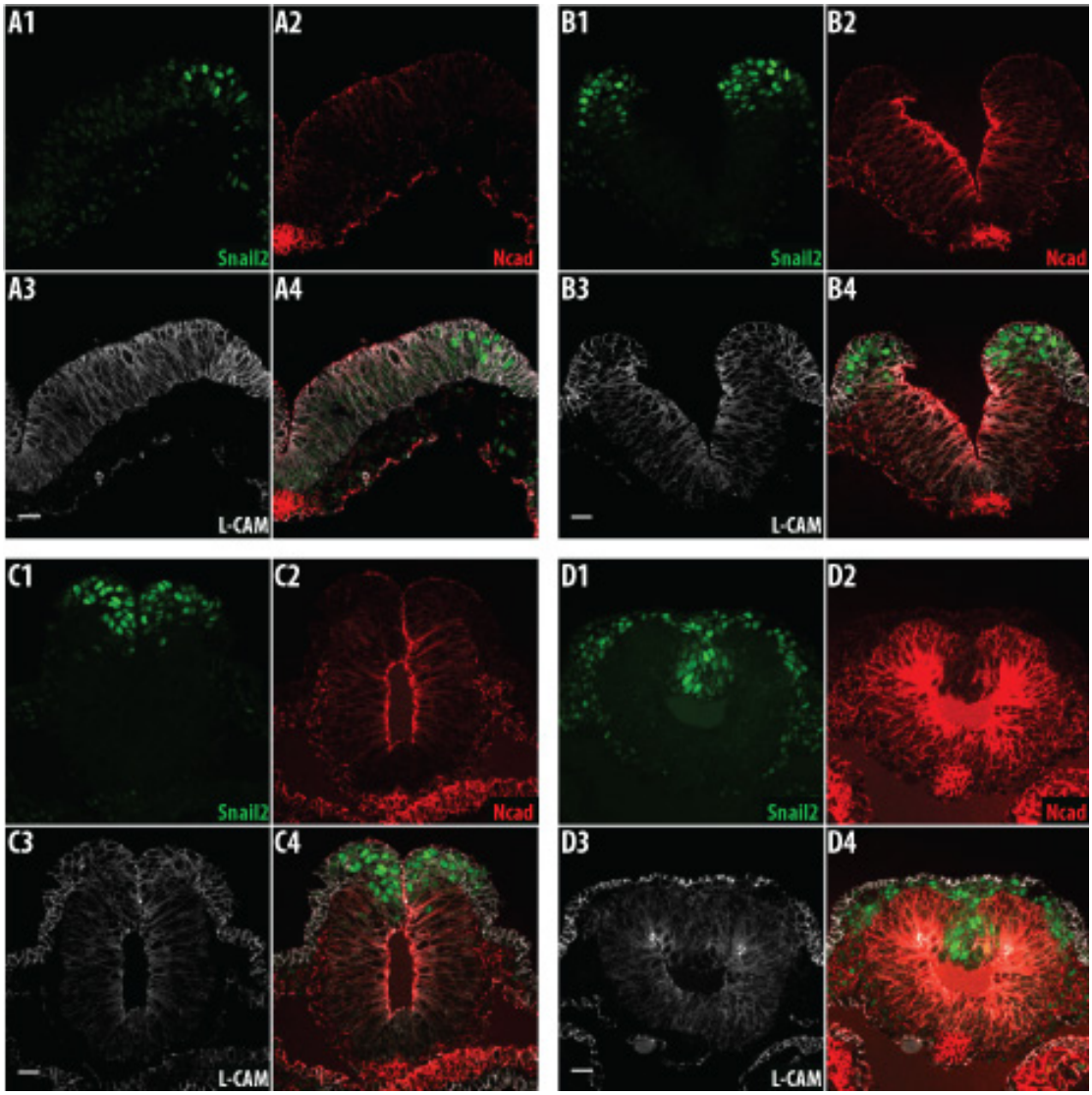


**Fig. 4.1: Formation and delamination of cells from the midbrain region of chicken embryos.**

Snail2 expressing neural crest cells in the neural fold (A-D), magnified views of (A, C and D) are shown in fig. 4.2. N-cadherin (Ncad) is initially only localized to the apical surface (A), however from ~3 somites onwards it is also localized on the lateral surface of cells (B-D). (A), 1-2 somites. (B), 3 somites. (C), 5 somites. (D), 7-8 somites. Scale bar is 20µm.

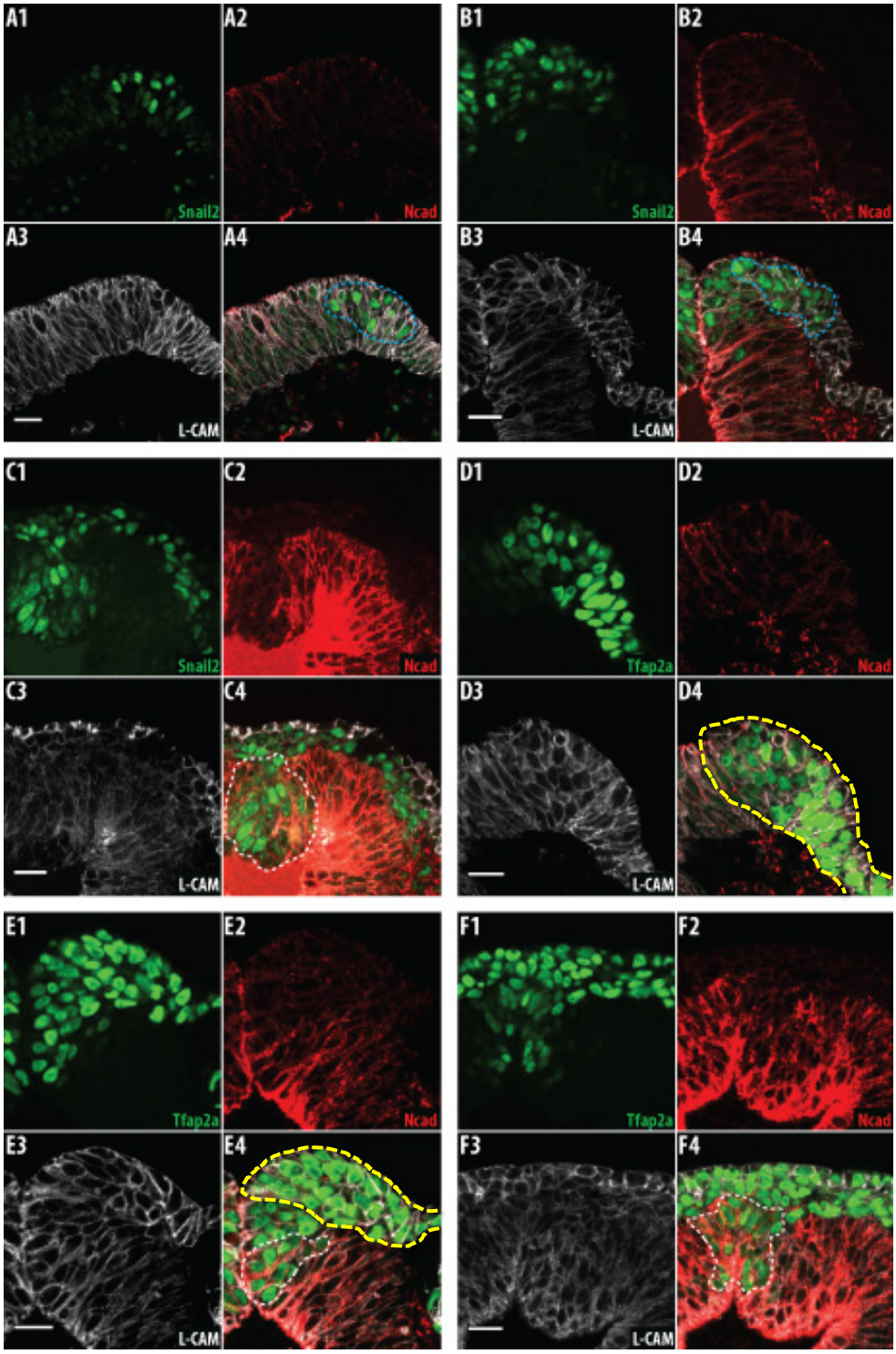


**Fig. 4.2: Expression of Snail2 and Tfap2a in the neural fold of the chicken.**

Cells co-expressing Snail2 and L-CAM, blue dotted line (A-B). Snail2 expressing cells in the neural ectoderm, white dotted line (C). Yellow dotted line; L-CAM expressing region (D and E). Tfap2a expressing cells in the neural ectoderm (E and F). (A), 1-2 somites. (B), 5 somites. (C), 7-8 somites. (D), 2 somites. (E), 5 somites. (F), 7 somites. Scale bar is 20μm.

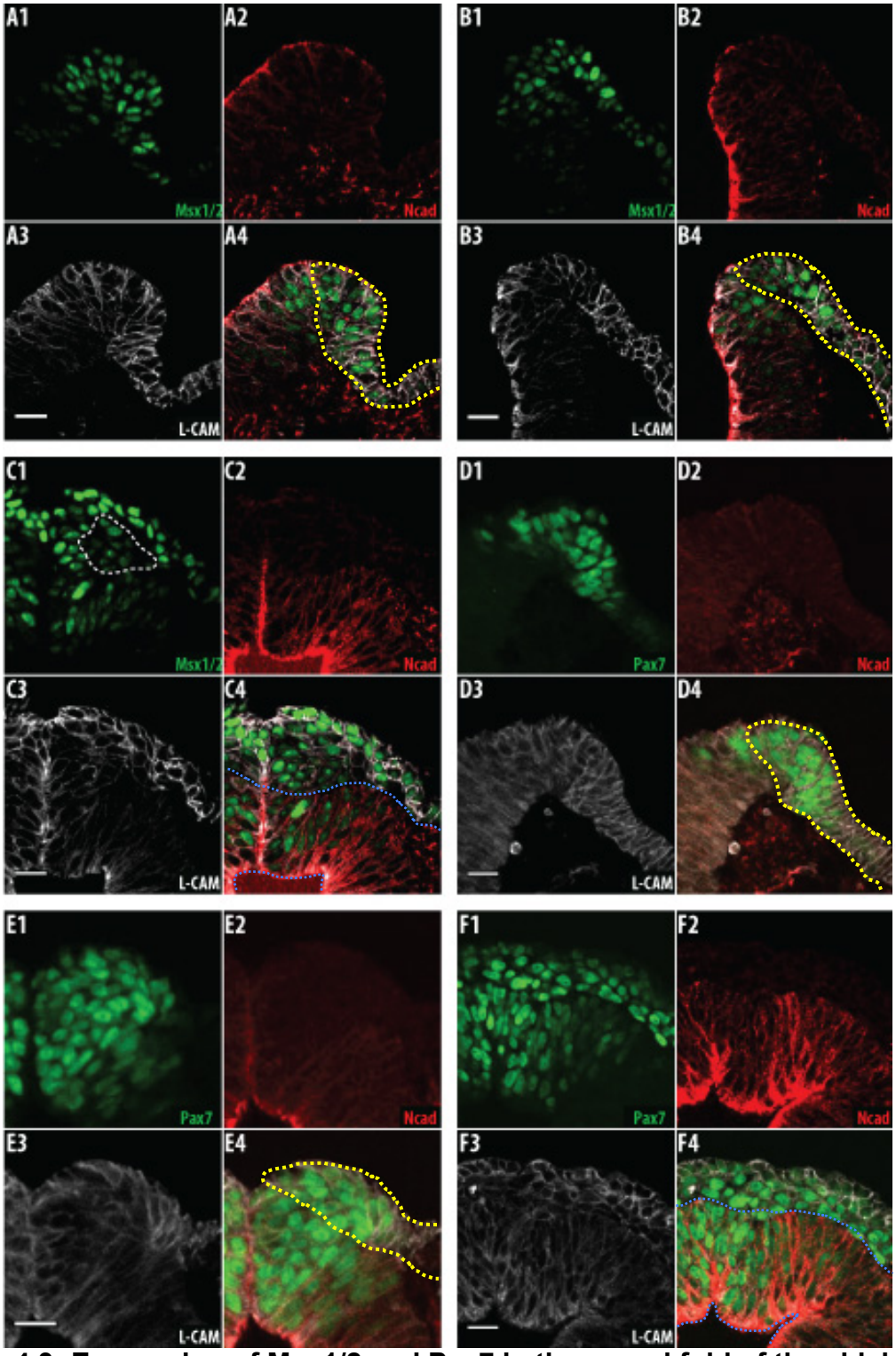


**Fig. 4.3: Expression of Msx1/2 and Pax7 in the neural fold of the chicken.** White dotted line; cells that expresses lower levels of Msx1/2 (C). (A), 2 somites. (B), 4 somites. (C), 7 somites. (D), 2 somites. (E), 5 somites. (F), 7 somites. Yellow dotted line; L-CAM expressing non-neural ectoderm. Blue dotted line; N-cadherin expressing neural ectoderm. Scale bar is 20μm

As mentioned before, the mode of delamination of cells in chicken is different from mouse. We looked at the expression pattern of Snail2 in the midbrain region of chicken embryos at various stages to try to determine the dynamics of cell delamination and whether there are two populations of Snail2 expressing cells. Fig. 4.1 shows the formation of the midbrain neural tube and the expression of Snail2, N-cadherin and L-CAM at various intermediate stages. The neural fold first elevates at about 2-3 somites, apposition of the neural fold occurs at about 5 somites and fusion of the neural tube occurs at about 7 somites. During this process of neural tube formation (fig 4.1), Snail2 is expressed in the neural fold and cells can be seen migrating away at about 7 somites a time at which fusion of the neural tube has occurred (fig. 4.1D), when there are still Snail2 expressing cells within the neural tube(Newgreen and Erickson, 1986).

From this series of images it is difficult to determine the exact time when EMT occurs, unlike in the mouse where cells delaminate directly into the mesenchyme. The morphology of the neural fold undergoes dramatic changes due to fusion and delamination of cells. In order to better define the delaminating cells, expression of Snail2 and other neural crest specifiers like Tfap2a, Msx1/2, Pax7 and Sox9 in the neural fold at different stages of neural tube formation was looked at (fig. 4.2 to 4.4). This will also determine whether all delaminating cells express the same markers.

At HH7+ (1-2 somites), the neural plate is beginning to form and at this stage N-cadherin is mainly localized to the apical surface of the central part of the neural plate, whereas L-CAM is expressed at the lateral edges of the neural

fold. Snail2 (expressed very early during the formation of neural crest (Nieto et al., 1994)) is expressed in the L-CAM expressing non-neural ectoderm (fig. 4.2A).

The morphology of cells that are expressing Snail2 is also more cuboidal and resembles the cells that are in the non-neural ectoderm rather than the spindle shaped cells of the neural ectoderm. Most of the neural crest markers like Tfap2a, Msx1/2 and Pax7 are also expressed in the L-CAM expressing non-neural ectoderm at this stage (fig. 4.2D, 4.3A and 4.3D).

At HH8 (3-5 somites), the neural groove is beginning to close and the neural folds are apposed to each other and the neural fold thickens compared to earlier stages (fig. 4.2A and B). N-cadherin in addition to being localized to the apical surface is also localized to lateral region of neural ectodermal cells, which are found in more medial regions of the neural tube beginning at 3 somites (fig. 4.1B). Snail2 is expressed in the non-neural ectoderm at this stage, on the outer surface of the neural fold (fig. 4.2B). This is also consistent with the fact that these cells express lower levels of L-CAM compared with more lateral cells since it is known that Snail2 down-regulates the expression of E-cadherin (Nieto et al., 1994). Tfap2a is also expressed in the non-neural ectoderm (fig. 4.2E) and some cells in the N-cadherin expressing neural ectoderm.

Msx1/2 is mainly expressed in the non-neural ectoderm (fig. 3.3B, very little Msx1/2 expressing cells co-express N-cadherin). Whereas Pax7 (fig. 4.3E) is expressed in more ventral regions of the neural ectoderm compared to

Msx1/2. At this stage, both Msx1/2 and Pax7 are also expressed in the L-CAM expressing non-neural ectoderm. In the case of Msx1/2 (fig. 4.3B), it seems to be expressed broadly in the non-neural ectoderm but is expressed more strongly in the neural fold which express higher levels of L-CAM. In the spinal cord, both Msx1/2 and Pax7 have been shown to be important for specification of different types of neurons in the dorsal and intermediate neural tube (Timmer et al., 2002).

At HH9 (7-8 somites), the neural groove has completely fused and cells have started migrating out. The neural and non-neural ectoderm has fully separated (fig. 4.2C, complementary expression of L-CAM in non-neural ectoderm and N-cadherin in neural ectoderm). Snail2 is only expressed in the neural ectoderm (fig. 4.2C). Tfap2a is expressed in both the neural and non-neural ectoderm, very similar to the previous stage (fig. 4.2F). Both Snail2 and Tfap2a are also expressed in migratory cells.

In fig. 4.3C, Msx1/2 is still expressed in the non-neural ectoderm overlying the fusion point. Expression of Msx1/2 is lower in the core of the neural fold (these cells are presumably going to migrate soon); cells that are at the edges of the neural fold express Msx1/2 at higher levels. Pax7 is expressed in the migratory cells and is expressed at lower levels in the non-neural ectoderm (L-CAM expressing cells express lower levels of Pax7 compared with migrating cells, fig. 4.3F). It is also expressed in the dorsal neural ectoderm (fig 4.3F).

Sox9, a neural crest marker, is weakly expressed in the neural fold from HH7+ to HH8+ (fig. 4.4). At these stages expression of Sox9 is very restricted; it is only expressed in the non-neural ectoderm of the neural fold that is going to fuse. Comparing the expression pattern of Sox9 with Msx1/2, Msx1/2 expression is much broader and earlier than Sox9. At HH9, Sox9 is expressed in the dorsal neural ectoderm (fig. 4.4C).

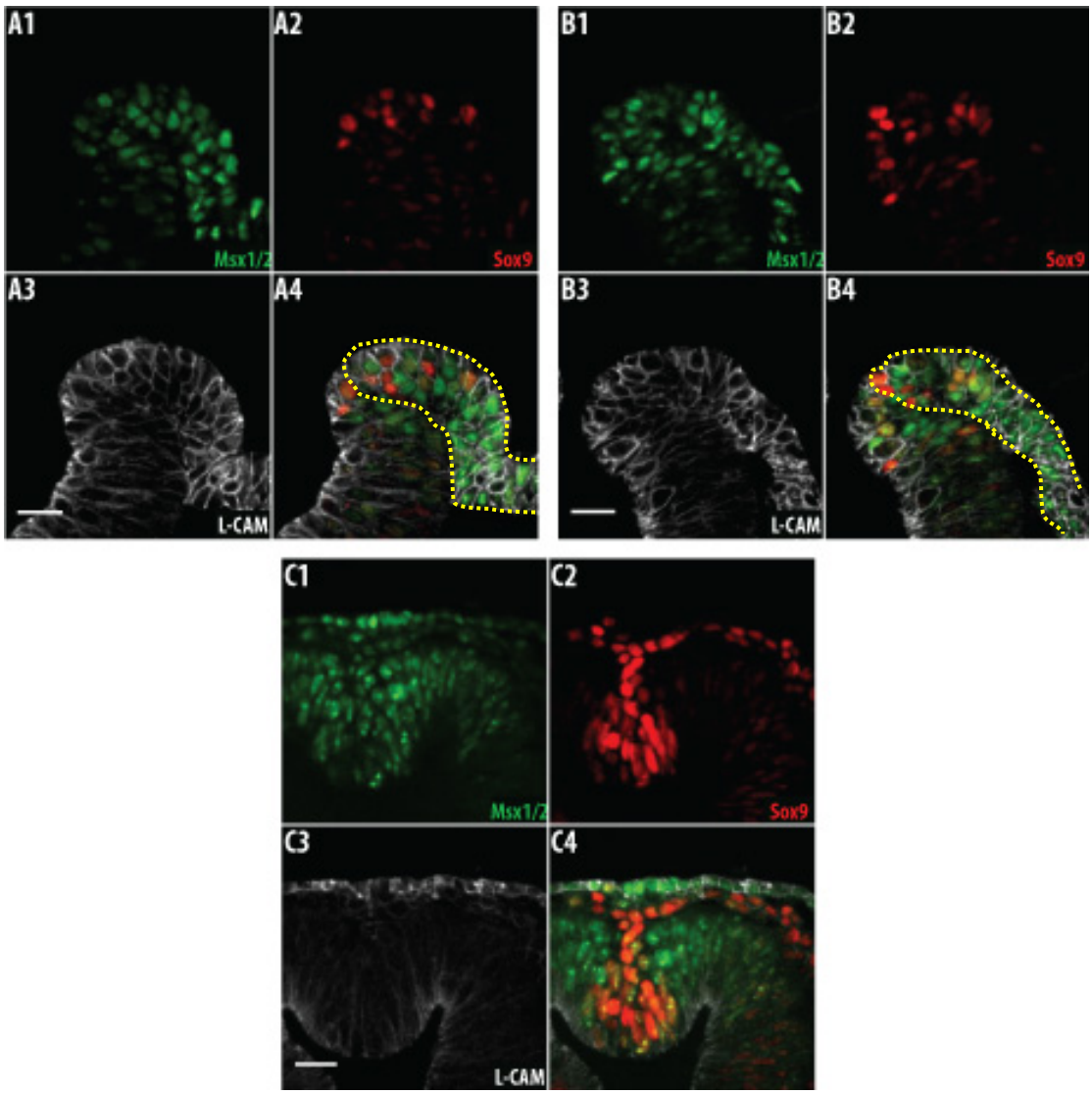


**Fig. 4.4: Expression of Msx1/2 and Sox9 at various stages in the neural fold.**

Sox9 is not expressed by all Msx1/2 expressing cells. (A), 2-3 somites. (B), 4 somites. (C), 7 somites. Scale bar is 20μm

These images show that the neural fold is actually made up of two regions: a non-neural ectoderm and a neural ectoderm domain and the expression pattern of neural crest markers within the neural fold is highly dynamic (differences in their levels and location of expression). These images also show that there is two populations of cells within the neural fold namely cells that originate from the non-neural and neural ectoderm. It is unclear whether all cells within the neural fold express the same markers in the same temporal sequence. For example, only a very small subset of cells in the neural fold express Sox9 compared to Msx1/2 (fig. 4.4A and B) however at later stages Sox9 is expressed by cells emerging from the neural ectoderm (fig. 4.4C). Furthermore, the topology of the neural fold makes it difficult to determine which population of cells delaminate and migrate out first.

## 4.2 Cells found in the non-neural ectoderm delaminate first

It has been shown that cells that delaminate first give rise to ventral derivatives whereas cells that delaminate later give rise to dorsal derivatives. In the head, the mesectoderm in the BA is found more ventrally compared to neuronal derivatives in the cranial ganglion. In cranial regions, this means that cells that delaminate first give rise to mesectodermal derivatives in the BA whereas cells that delaminate later give rise to neuronal derivatives in the cranial ganglia. One of the characteristics of an epithelium is the presence of a basement membrane on the basal side. In order to undergo EMT, epithelial cells have to break down the basement membrane before migration starts.

We tried to determine whether the first cells to delaminate from the neural fold originate from the non-neural ectoderm by looking at the integrity of the basement membrane but at this stage there is no intact basement membrane between neural and non-neural ectoderm (fig. 4.5), hence based on the morphology of the basement membrane, we were unable to determine the sequence in which cells delaminated from the neural fold. This is similar to electron microscope studies by Tosney and immunostaining of basement membrane by Lawson *et. al.* (Tosney, 1982; Lawson et al., 2001). In fig 4.5C, the neural crest cells are probably starting to migrate out.

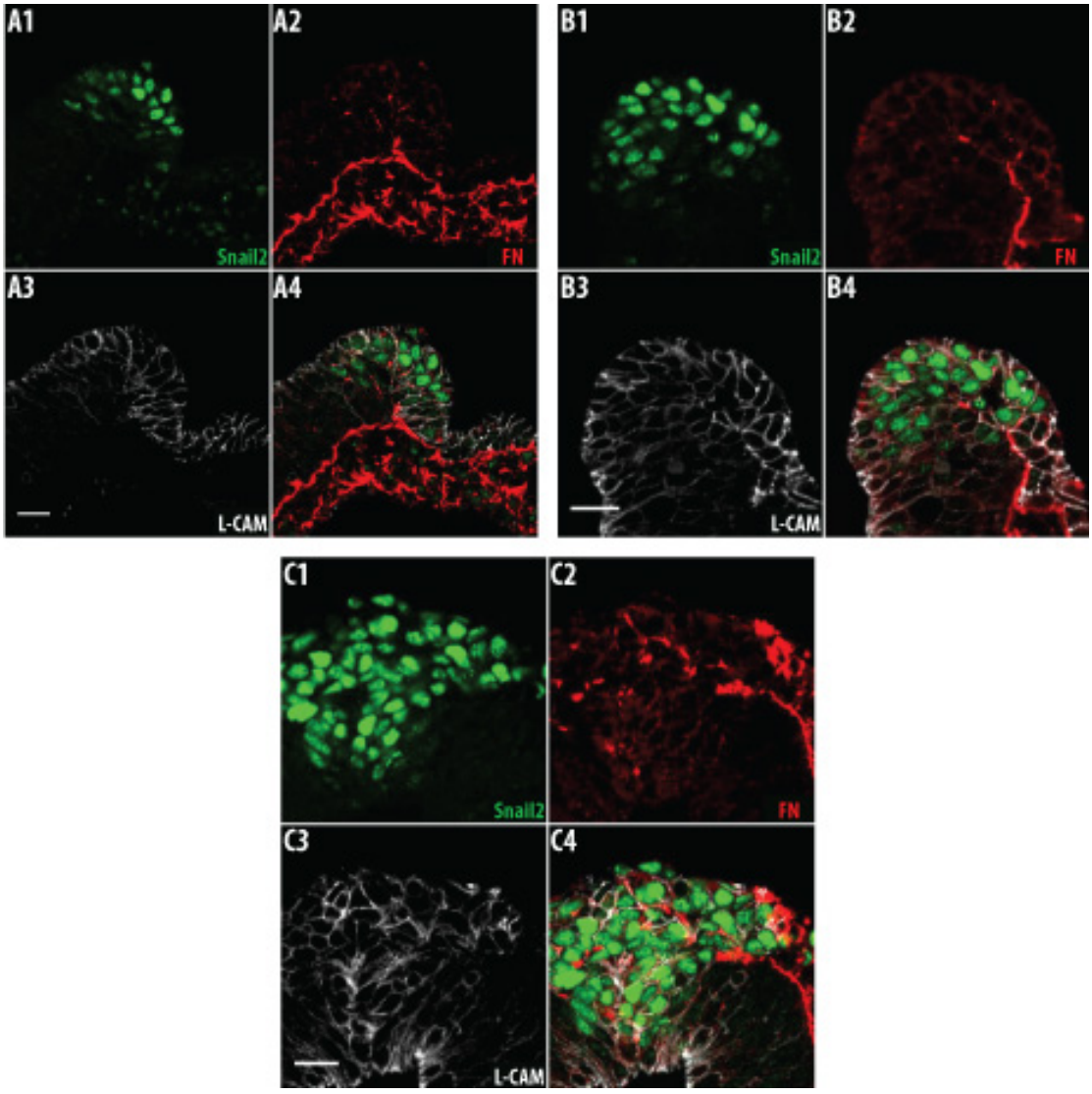


**Fig. 4.5: Morphology of the basement membrane at various stages during neural crest formation/migration.**

Fibronectin is a component of the basement membrane. An intact basement membrane is present under the Snail2 expressing cells only at 2 somites (A), at later stages the Snail2 expressing cells are not contacting the basement membrane (B and C). (A), 2 somites. (B), 3-4 somites. (C), 5-6 somites. Scale bar is 20µm**.**

RhoB has been shown to be expressed in neural crest cells and is important for the remodeling of the actin cytoskeleton required for changes in cell shape as well as migration (Liu and Jessell, 1998; Del Barrio and Nieto, 2004). When the neural fold becomes apposed to each other at 5 somites, not all

Snail2 expressing cells express RhoB (fig. 4.6A). RhoB is only expressed in the Snail2 expressing cells that also express L-CAM. However when the neural tube becomes closed at 7 somites, most Snail2 expressing cells express RhoB including Snail2 expressing cells in the neural ectoderm (fig. 4.6B). This means that the first population of cells that is formed in the non-neural ectoderm is the first to express RhoB.

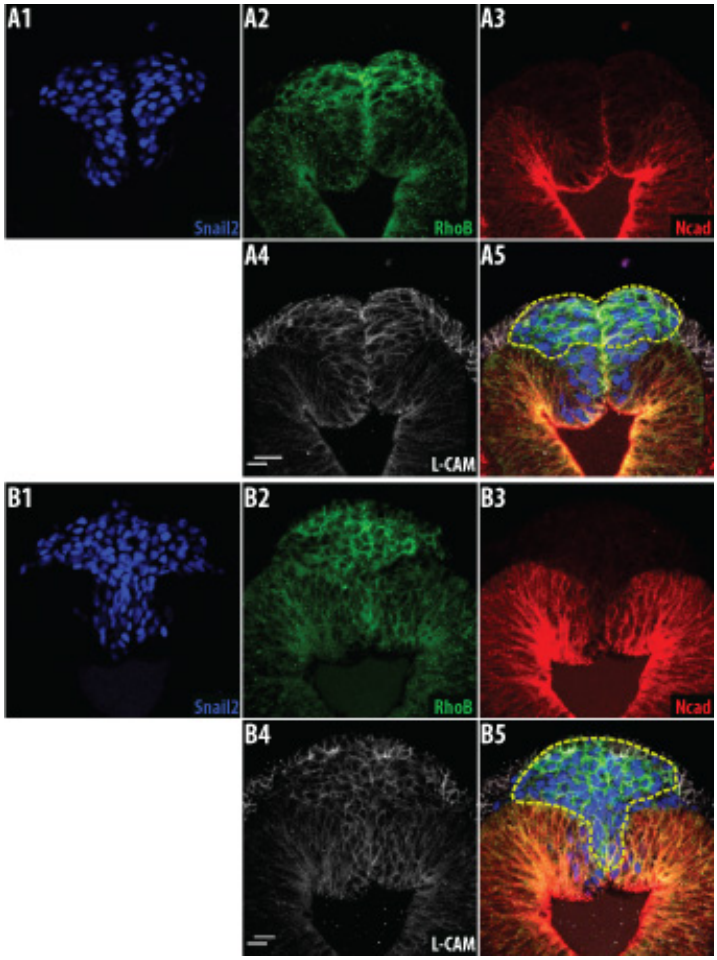


**Fig. 4.6: Snail2 expressing cells in the non-neural ectoderm expressing L-CAM are the first cells to express RhoB.**

Snail2 expressing cells found in the non-neural ectoderm are the first cells to express RhoB (A). Subsequently after fusion of the neural tube, Snail2 expressing cells found in the neural ectoderm also express RhoB (B). Yellow dotted lines show the region that is expressing RhoB. (A), 5 somites. (B), 7 somites. Scale bar is 20μm.

Cells within the epithelium exhibit apical/basal polarity, and during EMT, this apical/basal polarity is lost. **β**-dystroglycan is found in the basal compartment of epithelial cells (Nakaya et al., 2012). When the neural fold becomes apposed to each other at 5 somites, **β**-dystroglycan is not localized to the basal compartment in Snail2 expressing cells that also express L-CAM, instead **β**-dystroglycan is found in the entire cell cortex (fig. 4.7). **β**-dystroglycan in Snail2 expressing cells, which are found more ventrally in the neural ectoderm, is only localized to the basal compartment. At 5 somites, Snail2 expressing cells, which are found in the non-neural ectoderm, have lost their apical/basal polarity and undergone EMT.

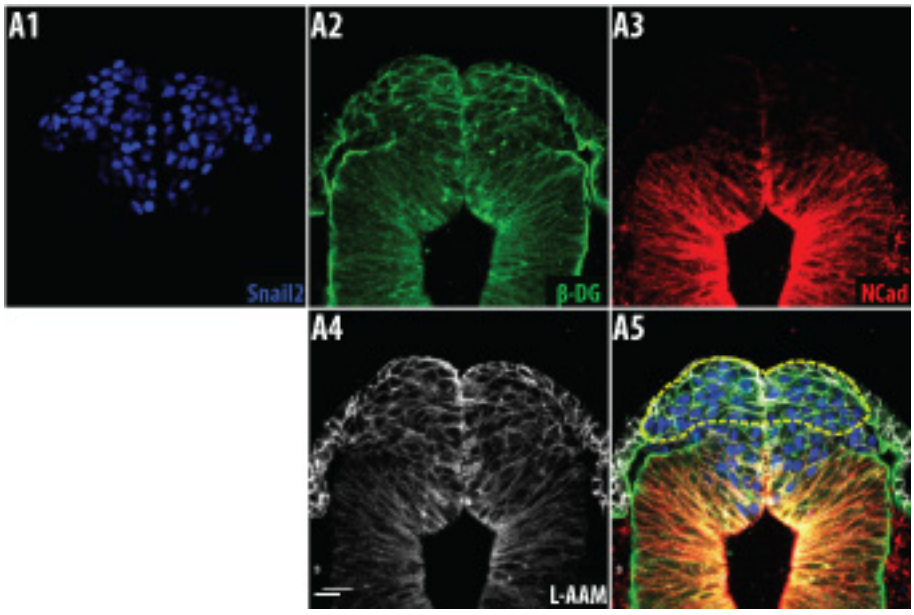


**Fig. 4.7: Snail2 expressing cells in the non-neural ectoderm expressing L-CAM are not polarized.**

At 5 somites when the neural tube is beginning to fuse, Snail2 expressing cells found in the non-neural ectoderm expressing L-CAM are not polarized as shown by the localization of **β**-dystroglycan (**β**-DG) in the entire cell cortex. Yellow dotted lines show the region of the neural fold that **β**-dystroglycan is found in the entire cell cortex. Scale bar is 20μm**.**

At 5 somites, only Snail2 expressing cells in the non-neural ectoderm express RhoB and are not polarized, as shown by a cortical localization of **β**-dystroglycan. This shows that these Snail2 expressing cells in the non-neural ectoderm have already undergone EMT and delaminate first.

It has been shown that during the fusion of the neural fold to form the neural tube, cells at the neural fold move medially towards the midline (Brouns et al., 2005). This might mean that the Snail2 expressing cells in the non-neural ectoderm moves towards the midline prior to delamination. To address this issue, we labeled the neural fold with DiI at 5 somites to follow the movement of these cells.

From fig. 4.8, cells that are initially located laterally move towards the midline and subsequently after fusion of the neural folds, migrate outwards. During closure of the neural tube, cells that express Snail2 in the non-neural ectoderm move towards the midline and subsequently delaminate after fusion of the neural folds.

Both morphogenetic movement as well as the organization of the neural fold supports the idea that the Snail2 expressing cells in the non-neural ectoderm delaminate first and give rise to the leading edge of the migratory cells. These cells would be expected to migrate further and give rise to more ventral derivatives whereas cells that delaminate later from the neural ectoderm would give rise to more dorsal derivatives.

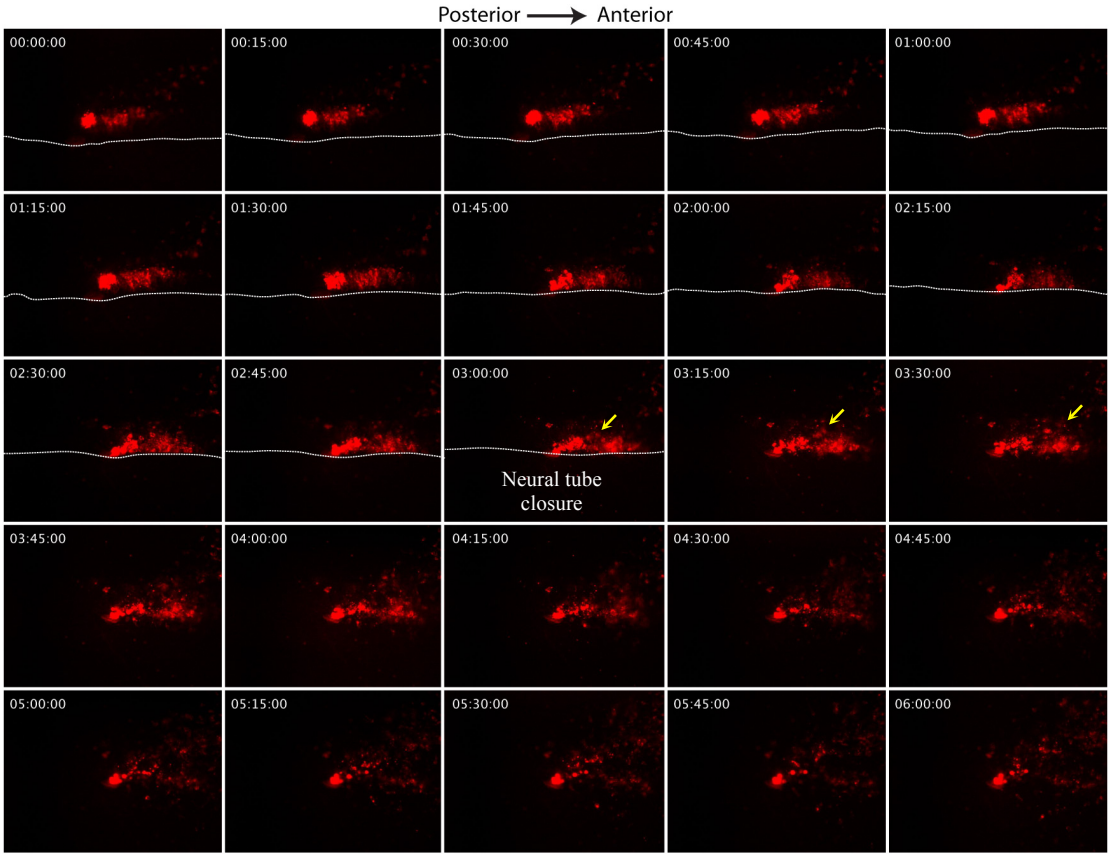


**Fig. 4.8: Lateral to medial movement of the cells in the neural fold prior to fusion of the neural tube and subsequent delamination of these cells.** DiI was used to label the lateral non-neural ectoderm at 5 somites (prior to fusion of the neural tube). A time-lapse of the movement of these DiI labeled cells is shown. Midline of the neural tube is shown by the dotted line, anterior of the embryo is on the right side of these images. After about 3hrs, the neural tube is fully closed and migration of the DiI labeled cells can be seen 15mins later (yellow arrow).

## 4.3 Lineage tracing in chicken embryo with DiI

We took advantage of the fact that chicken embryos could be cultured ex-ovo for approximately two days to differentially label different regions of the neural fold to determine whether they gave rise to cells with different cell fates. The non-neural ectoderm was labeled by applying a spot of DiI on the surface of the neural fold. We made use of the fact that at approximately 7 somites the neural tube is closed and injected DiI solution into the neural tube to label the cells delaminating from the neural ectoderm.

To determine whether only cells delaminating from the neural ectoderm are labeled when DiI is injected in the lumen of the neural tube at HH9 (7 somites), we fixed and sectioned the embryo after labeling. Fig. 4.9A shows the embryo after labeling; cross-sections of these embryos show that only cells within the neural ectoderm are labeled (fig. 4.9B and fig 4.9C).

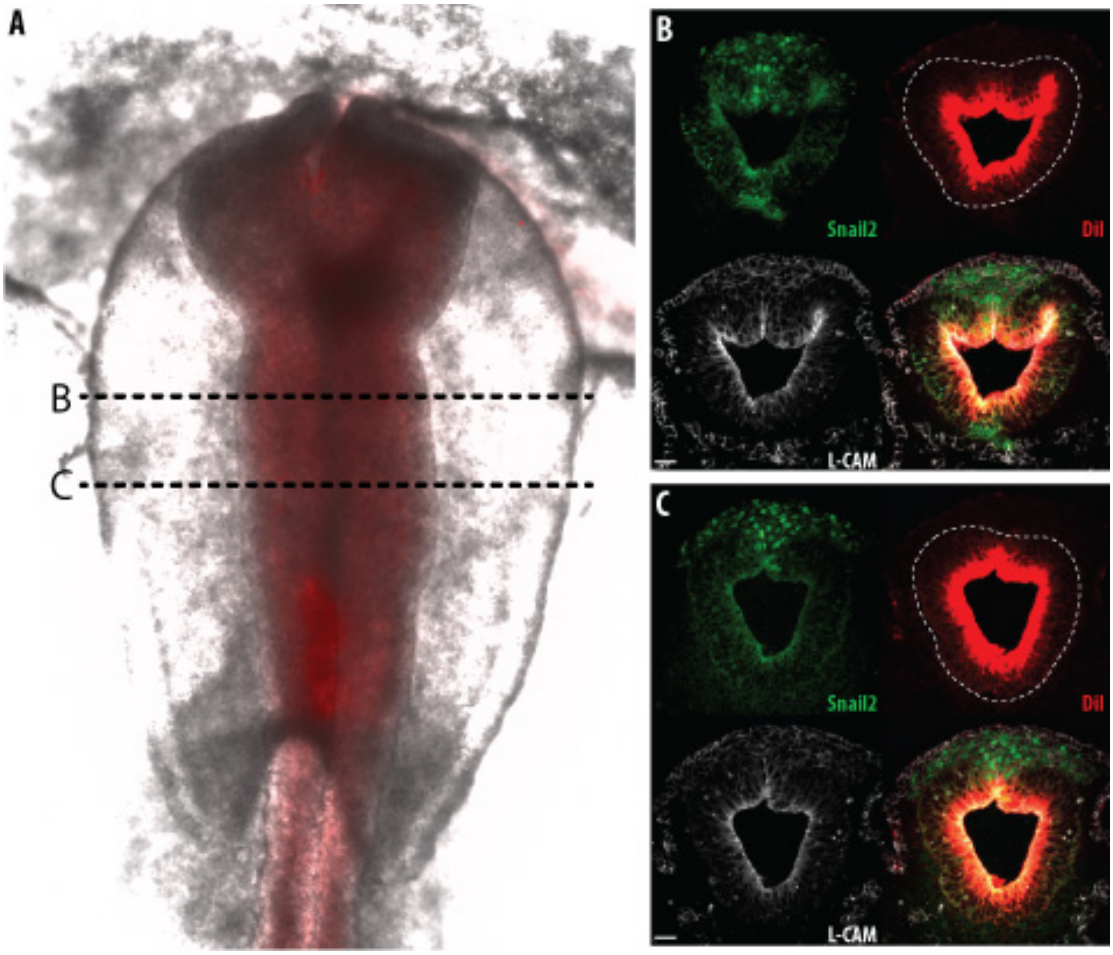


**Fig. 4.9: DiI labeling of cells delaminating from neural ectoderm.**

(A), DiI labeling of the neural tube of a 7 somite chicken embryo. (B) and (C) cross section at level indicated in (A). Dotted lines highlight the labeled cells within the neural ectoderm, in particular the Snail2 expressing cells are labeled. Note that in the images of L-CAM immunostaining the apical surface of the neural ectoderm seem to be L-CAM positive but this is probably due to bleed-through from the strong DiI signal. Scale bar is 20µm

When the non-neural ectoderm of the neural fold is labeled with DiI, mesectoderm within BA1 is labeled, and only some cells within the trigeminal ganglia are labeled (fig. 4.10). The majority of the cells end up in the branchial arch. Fig. 4.11 show a cross-section of the BA1 in the first embryo of fig 4.10. This section shows clearly that mesectoderm within BA1, which expresses Tfap2a at this stage, is labeled with DiI. Hence, the non-neural ectoderm within the neural fold gives rise to mesectoderm of the first branchial arch. From the embryos that survive to day two of culture, 10 out of 12 embryos show that when the non-neural ectoderm is labeled, cells in the branchial arch are preferentially labeled.

Although there are cells within the trigeminal ganglia that are labeled, at present we are unable to determine whether they are derived from the non-neural ectoderm component of the neural fold or from cranial placodes. Xu *et. al.* show that at the 4 somite stage, the cranial placodes are derived from the Pax3 negative ectoderm (Xu et al., 2008). We have stained the sections with Islet1/2, which is expressed by neurons derived from the cranial placode (Ericson et al., 1992; Xu et al., 2008), but due to the juxtaposition of the glia and neurons within the trigeminal ganglia as well as the dispositions of DiI within the cell, we are uncertain whether the labeled cells are neurons or glia and whether they are derived from cranial placode.

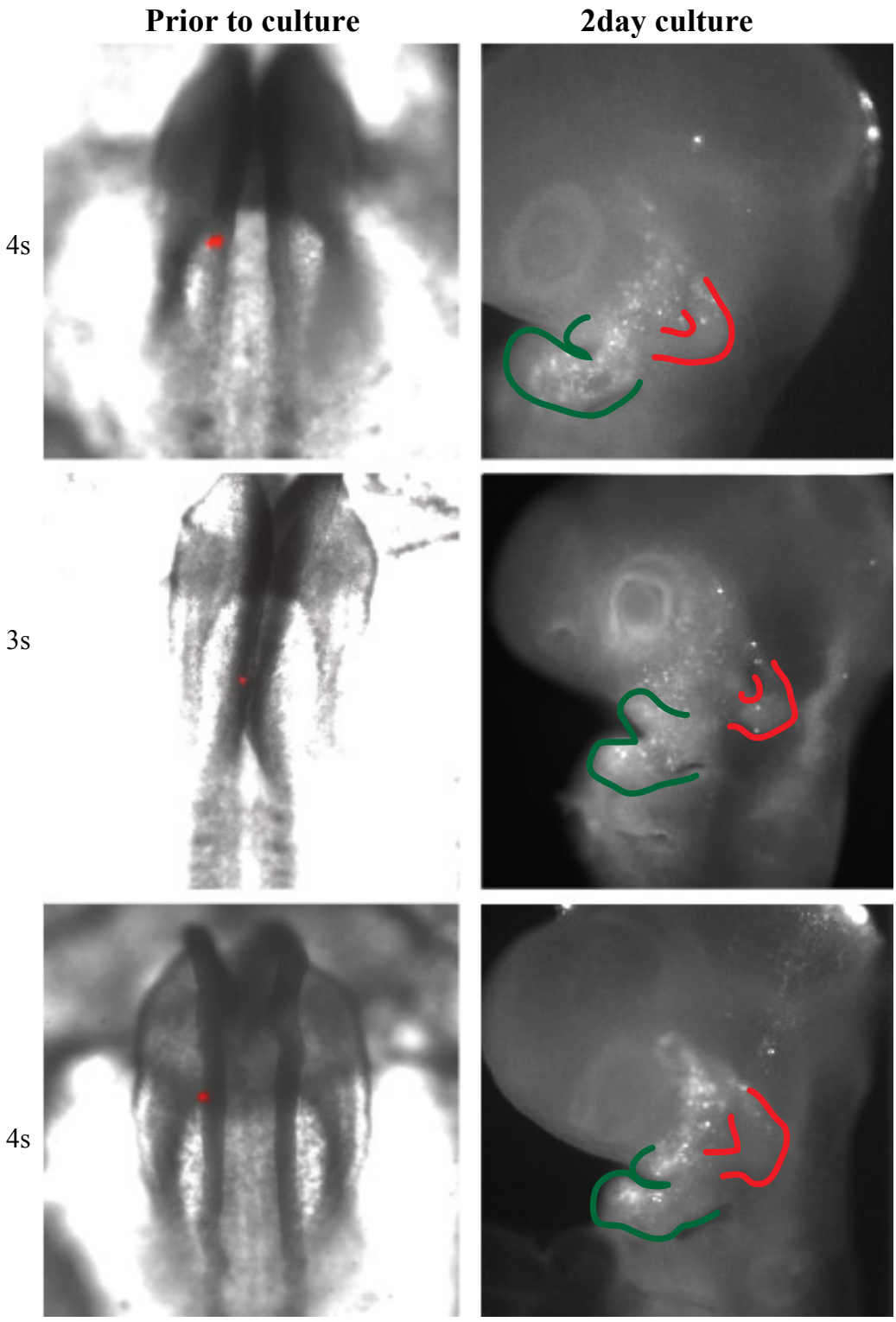


**Fig 4.10: DiI labeling of non-neural ectoderm.**

DiI was used to label the lateral non-neural ectoderm of the neural fold at the stages indicated on the left, and embryos were cultured for 2 days. Note that the mesectoderm in BA1 (green line) are labeled, whereas cells within the trigeminal ganglia (red line) are not well labeled.

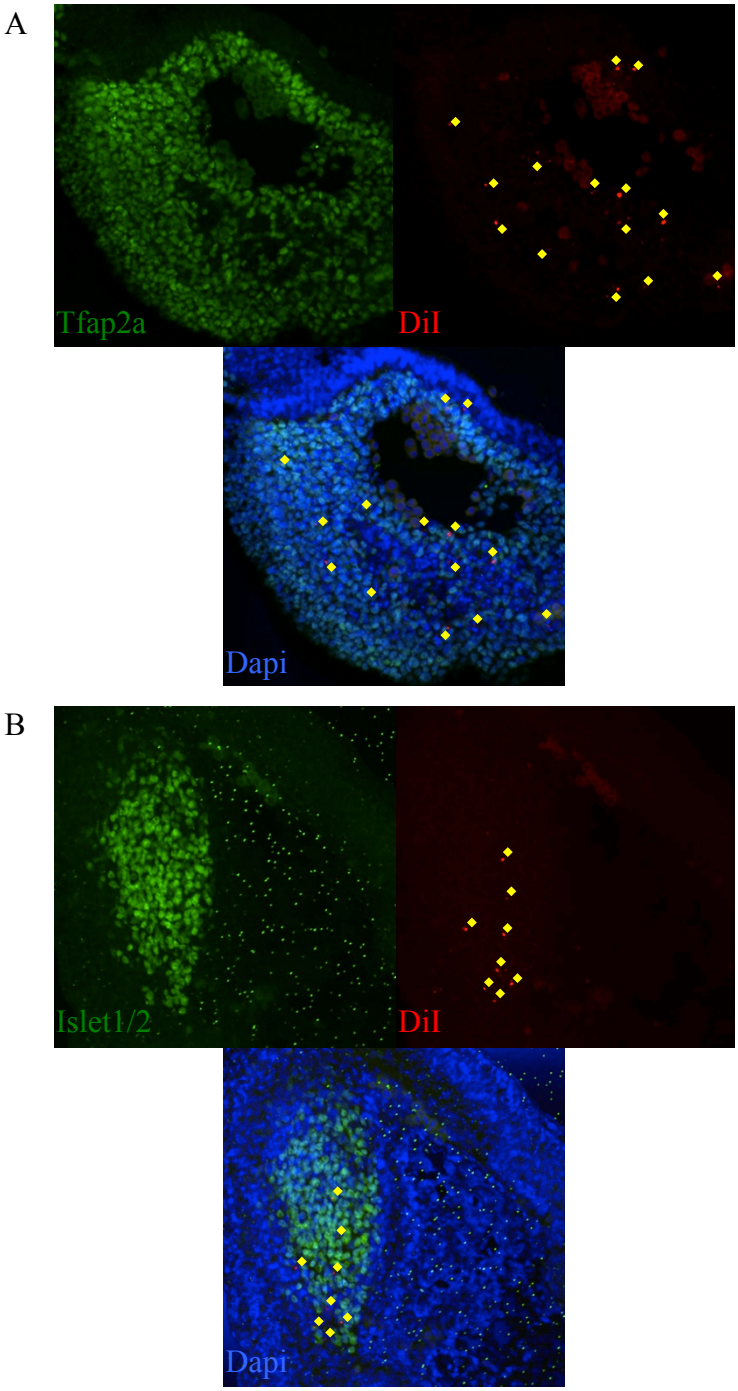


**Fig 4.11: Section of DiI labeled non-neural ectoderm of chicken embryo after 2 days of culture.**

Cross-section of the first embryo in fig. 4.7 after 2 days of culture. (A), section through BA1 immunostained with Tfap2a. (B), section through the trigeminal ganglia immunostained with Islet1/2. Yellow diamonds; DiI label.

When the neural ectoderm was labeled with DiI, it did not give rise to mesectoderm within BA1 but the trigeminal ganglion was well labeled (fig. 4.12). Sections through the trigeminal ganglion show that there are many DiI labeled cells here. In this case these labeled cells are neural crest derived and not derived from cranial placode because the non-neural ectoderm could not have been labeled in this case (fig. 4.9). When the neural ectoderm was labeled, eight out of ten embryos that survived to day two of culture showed absence of labeled cells within the branchial arches and labeled cells within the trigeminal ganglia.

From the expression pattern of the neural crest markers as well as labeling experiments in the chicken, we conclude that similarly to the mouse, there seems to be two populations of cells within the neural fold that have distinct cell fates. In the chicken, the expression profile of neural crest markers seems to be more drawn out compared to the mouse and it seems to reveal that the cells within the neural fold are highly heterogeneous, and this heterogeneity might reflect differences in induction.

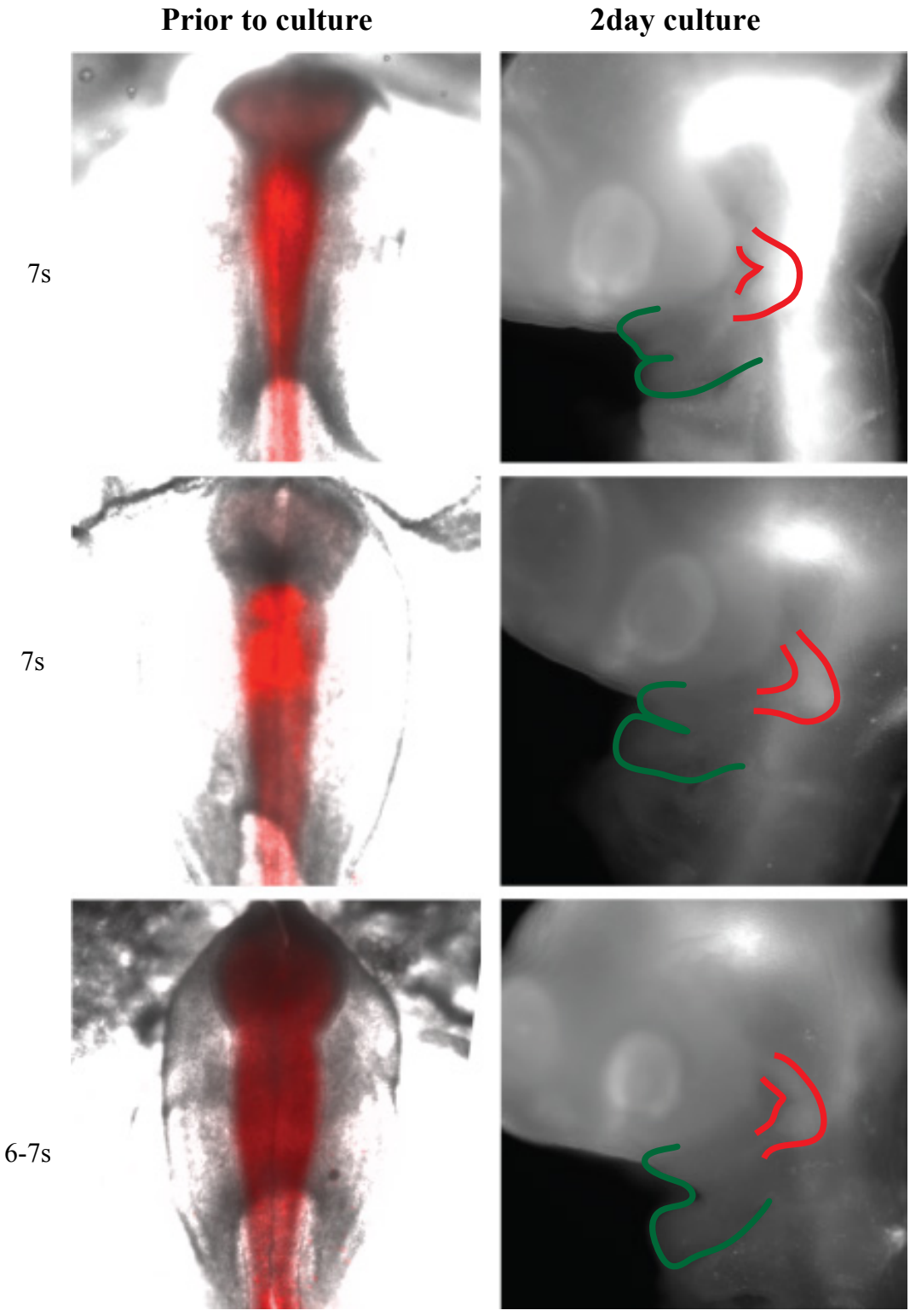


**Fig 4.12: DiI labeling of neural ectoderm.**

DiI was injected into the neural tube at the stages indicated on the left, and embryos cultured for 2 days. Note that the mesectoderm in BA1 (green line) is not labeled. Trigeminal ganglion is shown by the red line.

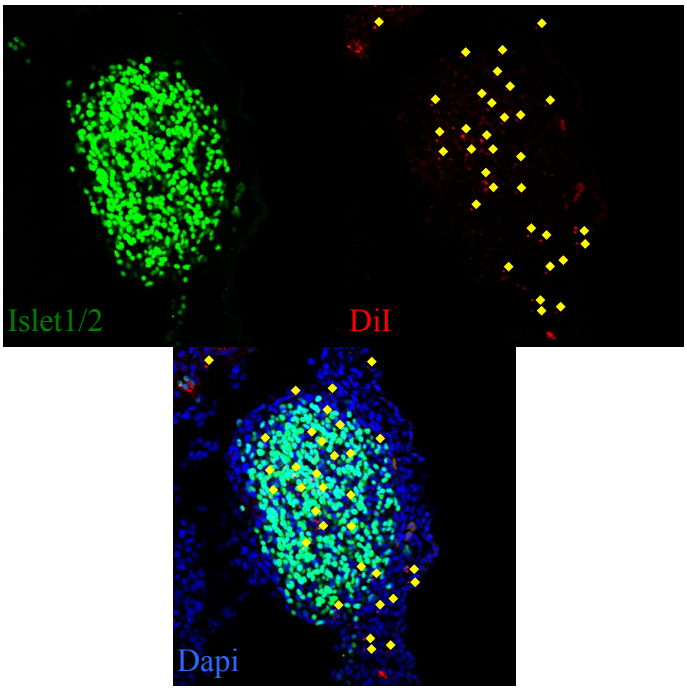


**Fig 4.13: Section of DiI labeled neural ectoderm of chicken embryo after 2 days of culture.**

Cross-section of the trigeminal ganglion (first embryo in fig. 4.9), with Islet1/2 expression shown in green. Yellow diamond; DiI label.

# Chapter 5: Discussion

## 5.1 Characteristics of the two populations of cells within the cranial neural fold

In the current study, we focused on neural crest cells that originate from the midbrain and give rise to both the mesectoderm of BA1 as well as the trigeminal ganglion (Lumsden et al., 1991; Osumi-Yamashita et al., 1994).

As mentioned in the introduction, neural crest markers are broadly categorized based on their expression pattern (Nieto, 2001; Morales et al., 2005; Sauka-Spengler and Bronner-Fraser, 2006; Betancur et al., 2010a). Pax3, Pax7, Msx1 and Tfap2a are categorized as neural plate border specifiers, whereas Sox9 and Snail2 are categorized as neural crest specifiers (as mentioned before, it should be noted that Snail2 is not strictly a neural crest specifier but the expression of Snail2 within the neural fold represent cells which are going to undergo EMT and it is also for this reason that Snail2 is categorized as a neural crest specifier). We have shown that at early stages (HH7+, TS12), in both mouse and chicken, these molecules are all expressed in the non-neural ectoderm whereas at later stages they are expressed in the dorsal neural ectoderm.

We find that there are two populations of delaminating cells within the neural fold of cranial regions. Both population of cells express neural crest markers however the first population originates from the non-neural ectoderm expressing E-cadherin or L-CAM whereas the second population originates from the neural ectoderm expressing Sox1 or N-cadherin, in mouse and

chicken respectively. This means that the neural fold as well as cells that are leaving are phenotypically heterogeneous. The second population of cells originating from the neural ectoderm in the head seems to be more similar to the cells in the trunk in terms of origin and perhaps fate.

In fig. 5.1, we show a summary of the expression pattern of these neural crest markers during early stages of embryogenesis. There are differences in the expression pattern of the dorsal markers in mouse and chicken, for example Msx1/2 and Pax7 are still expressed in the non-neural ectoderm after extensive migration of neural crest in chicken but not in mouse. In mouse embryos the region that cells are delaminating from does not seem to show as much differences in the expression pattern of the neural crest markers as it does in chicken. This is especially the case for Sox9 which in mouse is expressed much earlier and in all cells that delaminate whereas in chicken Sox9 seems to be only expressed in a subset of cells and also expressed at a later time. From our observation, we propose that in the head, neural crest formation first occurs in non-neural ectoderm and subsequently occurs in the neural ectoderm. This is in contrast to the trunk where neural crest is formed exclusively in the neural ectoderm.

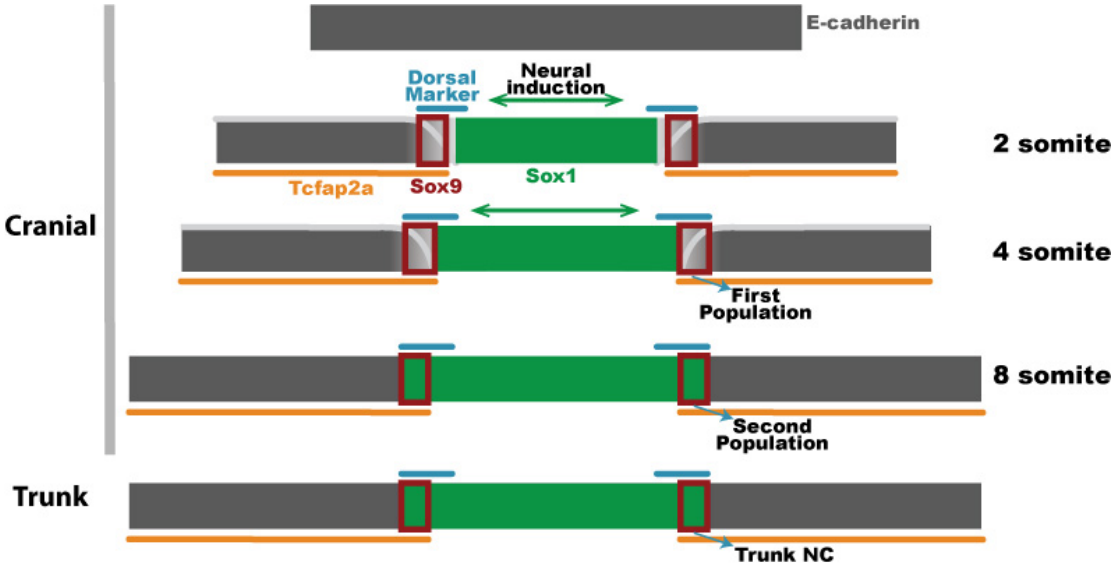


**Fig. 5.1: Summary of neural crest marker expression at different times in the cranial and trunk region.**

Dorsal markers in the figure refer to Pax3, Pax7 and Msx1/2. Snail2 is expressed in a similar area as Sox9.

The period of formation and delamination of neural crest cells in chicken is much longer compared with mouse and perhaps it is for this reason that there seems to be greater temporal resolution of the expression profiles of the neural crest markers in avian. The expression profiles of neural crest markers in chicken reveal that there is a lot of heterogeneity, i.e. it is unclear whether all cells express the same markers. From this study it is unclear whether during the process of induction/formation of neural crest cells, all the neural crest markers are expressed in the same sequence or at the same time during induction/formation.

In the mouse where the neural crest cells delaminate into the underlying mesenchyme, it is obvious that the first population of cells does not get incorporated into the dorsal neural tube, therefore this first population of cells that delaminate never express Sox1 and are distinct from the second

population of cells. This finding is also supported by the histological studies by Nichols (Nichols, 1981; 1986).

However in the chicken, because of the topology of the neural fold, it is much more difficult to conclude which cells are delaminating. At 2-3 somites (fig. 4.2A), Snail2 is mainly expressed in the non-neural ectoderm (L-CAM expressing) in the midbrain. Apposition of the neural fold occurs at around 4-5 somites (fig. 4.2B), and at this stage Snail2 is expressed in the entire neural fold, in particular Snail2 expressing cells also express L-CAM. Fusion of the neural fold occurs around 7-8 somites (fig. 4.2C), when most of the Snail2 expressing cells are seen migrating out between the neural and non-neural ectoderm but there are still Snail2 expressing cells within the median dorsal neural ectoderm (N-cadherin expressing) that are still delaminating (cells stop migrating out of the midbrain after ~10 somites).

In chicken embryos, during the period spanning neural crest formation to their emigration out of the ectoderm, the entire cranial ectoderm is undergoing morphological rearrangement, from a relatively flat ectoderm to formation of the neural tube. Numerous studies on neural tube closure in the chicken have shown that there is a medial movement of the neural fold in the midbrain region (van Straaten et al., 2002; Brouns et al., 2005; Fleury, 2011). When DiI was used to label the non-neural ectoderm of the neural fold (fig. 4.8), we observed that these cells move towards the midline and subsequently delaminate. What this means for neural crest cells is that the L-CAM expressing cells at 2-3 somites, which are initially lateral of the neural ectoderm, moves to the top of the closing neural tube at 4-5 somites. At about

7-8 somites, when the cells start to migrate out of this region, the definitive ectoderm moves over this region and because there is still a population of cells that are still delaminating from the neural ectoderm, this may create a false impression that all the cells that delaminate from this region are derived from the neural ectoderm.

We also show that Snail2 expressing cells found in the non-neural ectoderm undergoes EMT first based on the expression of RhoB and loss of apical/basal polarity. This means that Snail2 expressing cells in the non-neural ectoderm expressing L-CAM do not convert into neural ectoderm expressing N-cadherin before delaminating, instead they are delaminating first and forming the lead edge of the migratory mass of cells to form mesectodermal derivatives that are located more ventrally.

Furthermore, numerous studies have shown that melanocytes that are the last to depart, and melanocyte progenitors differentially express melanocyte markers prior to departure from the neural tube (DuShane, 1935; Hayashi, 1993; Erickson and Goins, 1995; Wehrle-Haller and Weston, 1995; Wilson et al., 2004). This provides very convincing proof that the premigratory neural crest cells are a heterogenous population of fate-restricted cells.

In Sox1 Cre YFP E9.5 mouse embryos, neuronal derivatives like the cranial ganglia in the head are preferentially labeled. In these embryos the second population of delaminating cells is labeled whereas the first population is not labeled. This means that the second population preferentially gives rise to the neuronal derivatives.

Our lineage tracing experiments in chicken embryo show that labeling the cells in the non-neural ectoderm in the neural fold labels mesectoderm of BA1, whereas labeling the neural ectoderm in the neural fold labels neuronal derivatives. This shows convincingly that these two populations of cells give rise to distinct population of cells with different cell fates. The results obtained from our study is also similar to what Lumsden *et. al*. obtained in 1991 (Lumsden et al., 1991), although in their study they did not try to label specific regions within the neural fold, but they found that cells gave rise to ventral derivatives (mesectoderm) before giving rise to more dorsal ones (neuronal derivatives). Interestingly, they found that labeling the neural fold at early stages only gave rise to mesectodermal derivatives in BA1 whereas at later stages both neuronal and mesectoderm labeling was obtained. This shows that cells in the neural fold are not pluripotent and also reconfirms the results from the lineage tracing data from the Sox1 Cre embryos.

In the studies that concluded that the neural crest cells are pluripotent, the authors propose that these pluripotent neural crest cells self-renew and subsequently generate progenies that have more restricted developmental potentials (Anderson, 1989; Le Douarin et al., 2004). Furthermore, they found that they could isolate these pluripotent neural crest cells during later stages of embryonic development even when the cells had already differentiated (Morrison et al., 1999; White and Anderson, 1999).

If all neural crest cells are pluripotent prior to delamination, a mosaic pattern in all derivatives (both mesectodermal and neuronal derivatives) would be expected. Cells were labeled prior to delamination in all our labeling

experiments and we show that the region where cells are originating from and their time of delamination makes a difference in the ultimate cell fate that they adopt. Based on this, we conclude that the neural fold consists of at least two populations of cells and therefore it is unlikely that all the cells are pluripotent. Studies by Krispin *et. al.* have also shown that the trunk neural crest is fate restricted (Krispin et al., 2010) similar to our results.

## 5.2 Definition of neural crest and origin of cranial mesenchyme

As mentioned in the introduction in the original studies that looked at the origin of the cranial mesenchyme, there was disagreement over whether mesectoderm originated from neural crest or lateral ectoderm. Platt JB attributed the lateral ectoderm as the origin of mesectoderm (Platt, 1893; 1894). However Landacre attributed the origin of the mesectoderm to neural crest cells (Landacre, 1921). A critical reading of these articles reveals that the disagreement was over how the neural fold and neural crest were defined.

Platt in her 1894 paper describes the formation of the neural fold and contribution to cranial mesenchyme and cranial ganglia as follows (original German text):

“In Fig. 5 sieht man, dass, noch vor dem Schlusse der Neuralfalten, das Ektoderm Zellen zur Bildung der Trigeminus-Anlage zu liefern angefangen hat. Sowie sich die Neuralfalten in dieser Gegend schliessen, vereinigt sich das Ektoderm auf einer ansehnlichen Strecke in vertikaler Richtung (Fig. 8). Von dem oberen Theil der Vereinigung spaltet sich die Oberhaut ab, aus dem unteren bildet sich die dorsale Wandung des Gehirns, während Zellen aus dem mittleren Theile der Vereinigung in die Trigeminus-Anlage auswandern, welche auch noch weiter durch Zellen verstärkt wird, die vom ektoderm zu beiden Seiten der vereinigungslinie fortdauernd abgegeben werden, wie es in Fig 14 abgebildet ist.”

“Durch Vergleich der Schnitte 5, 8 and 14 wird man überzeugt, dass die Behauptung Beard's (2), die äussere Schicht des Epiblasts sei an der Bildung der Ganglienanlage unbetheiligt, bei Necturus nicht zu vertheidigen ist, denn aus der ektodermalen Vereinigung wandern viele mittlere Zellen, die einst in der äusseren ektodermschicht gelegen, direct in die Trigeminus-Anlage über. An der Bildung der Trigeminus-Anlage nehmen wenige Zellen aus der Wandung des Neuralrohrs Theil. Für den Facialis gilt aber das Gegenteil, da durch die Auswanderung der Zellen aus der oberen Wandung des Neuralrohres die mittlere dorsale Decke des Gehirns eine Zeitlang verloren wird, was eine secundäre Schliessung nöthig macht.”

English translation, in this article we interpret the Trigeminus-Anlage and Facialis as the primordium of cartilage/bone and cranial ganglia respectively:

“In Fig 5 you see that, before the neuronal fold fuses, the ectoderm have already started to proliferate to form the Trigeminus anlage. As soon as the neuronal fold fuses, the ectoderm forms a vertical contact for a considerable length (Fig 8). From the upper part of this vertical contact derives the epidermis, and from the lower part of this vertical contact the dorsal wall of the brain, while the cells from the middle part of that vertical contact migrate into the Trigeminus anlage. This Trigeminus anlage gets further reinforcement from ectodermal cells which are constantly generated on both sides of this vertical contact.”

“Comparing the sections 5, 8, 15, one gains the conviction that the claim of Beards (2), that the outer layer of the epiblast would not be involved in the

formation of the ganglion anlage, cannot be defended in the case of Necturus. This is because many middle cells, which once belonged to the outer layer of the ectoderm, migrate from the ectodermal contact directly into the Trigeminal Anlage. Only few cells from the neuronal tube wall take part in the formation of the Trigeminus Anlage. Yet, the opposite is the case for the facialis. This is because cells migrate away from the dorsal neural tube, and by doing this the middle dorsal roof of the brain gets lost for some time. This necessitates a secondary fusion."

In the paper by Landacre (Landacre, 1921), the author describes the neural crest as follows:

"The neural crest cells - Selachians and other types - represent the dorsal portion of the lateral walls of the neural tube which is at first continuous with the ectoderm. The neural crest is incorporated in the neural tube, forming a wedge-shaped mass in its dorsal portion. This wedge-shaped mass later becomes detached from the tube and migrates laterally and ventrally."

Furthermore although Landacre acknowledges that there are ectodermal cells overlying the neural fold, strangely he fails to account for these cells after the fusion of the neural fold, we think that these cells actually contribute to the formation of the neural crest in this case. He describes the fusion of the neural fold as follows:

"In the closure of the neural groove (fig. 2) the superficial pigmented cells lining the dorsal two-thirds of the neural tube come into contact and obliterate that portion of the canal lined by flat cells."

From the authors' description of the neural fold fusion and subsequent delamination of cells, the disagreement between these two authors stem from their definition of the neural fold. Platt describes the neural fold as consisting of two separate components, a neural and non-neural component, whereas Landacre interpreted the neural fold as a single entity, which was derived from the neural ectoderm. Based on this, Platt concludes that there are two populations derived from the neural fold, the neural crest originating from the neural ectoderm which gives rise to the cranial ganglia and the lateral ectoderm, which gives rise to mesectoderm. In contrast Landacre interpreted that the entire neural fold gives rise to neural crest, which subsequently gives rise to mesectoderm as well as cranial ganglia (fig. 5.2).

Both authors relied on morphological features to distinguish origins so what might have led both authors to have different views of the same anatomical structure? We think that the probable reason for this discrepancy is maybe due to the preparation of the sample as well as species analyzed (Landacre was using *Plethodon glutinosus* whereas Platt was using Necturus – both are urodeles). Neural induction and neural crest formation in avian and amphibians (urodeles) are quite similar. This observation coupled with the fact the papers published by Platt were sometimes confusing and difficult to follow, as commented by Landacre (Landacre, 1921), led subsequent authors also to conclude that it was the neural crest that gave rise to cranial mesenchyme.

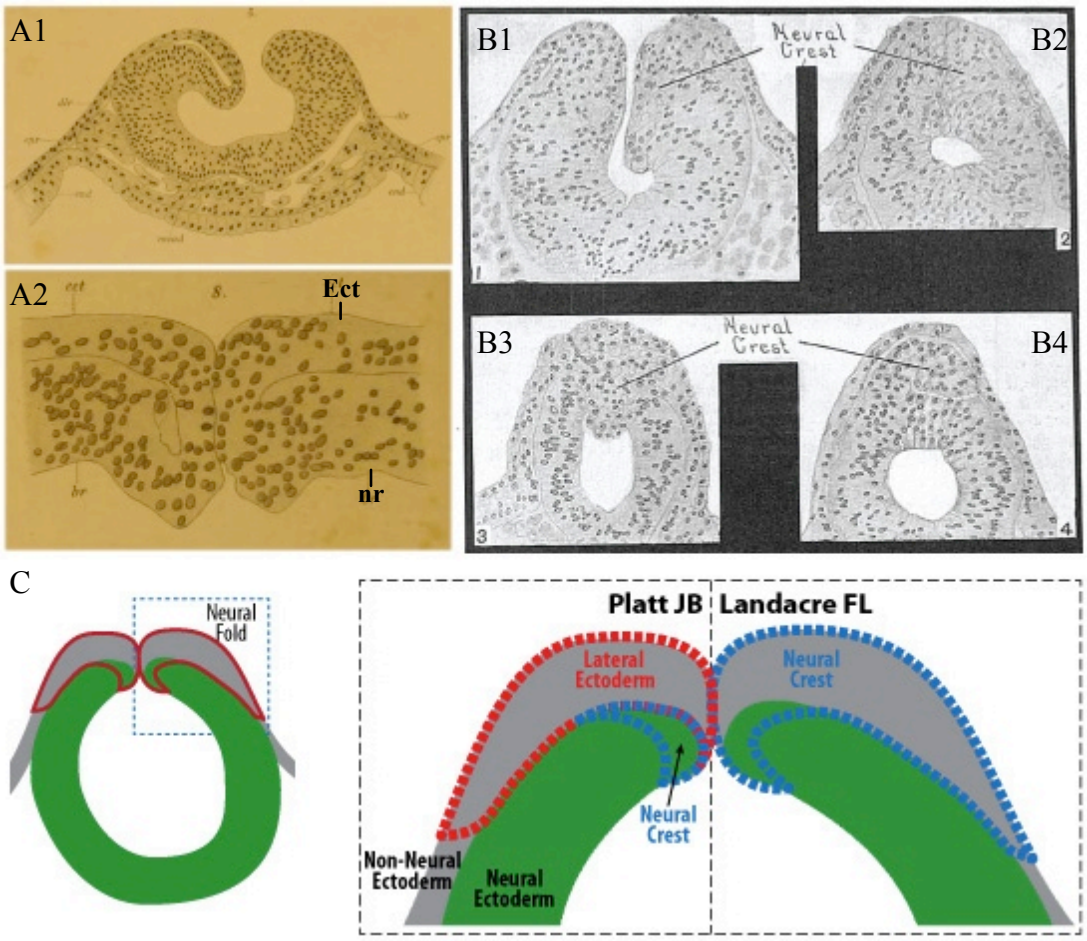


**Fig. 5.2: Discrepancy in the definition of neural fold territories between Platt and Landacre.**

Open neural tube of Necturus (A1) and fusion of the neural tube (A2), figure 5 and 8 taken from Platt respectively (Platt, 1894). (A), the neural fold is illustrated as 2 layers, Ect, non-neural ectoderm and nr, neural ectoderm. Image courtesy of Biodiversity Heritage Library. (B1-4), figures 1 to 4 taken from Landacre (Landacre, 1921). This series of pictures illustrate the fusion of the neural fold. It should be noted the neural crest is depicted as a separate region in these images. (C), definition of neural fold (left), lateral non-neural ectoderm and neural crest according to Platt JB and Landacre FL (right).

Newgreen and Thiery in 1980, showed that cranial and sacral neural crest cells in *in-vitro* culture are able to secrete Fibronectin (Newgreen and Thiery,

1980). Furthermore they show that the neural crest cells at the peripheral of the culture, delaminating first from the explant, secrete much more Fibronectin and have a distinct morphology to other cells that are more stellate. In this study the authors propose that Fibronectin might have a role in guiding the migration of neural crest cells. Furthermore, in the studies by Nichols, it was found that the early and late delaminating cells originate from sites stained differentially with toluidine blue (toluidine blue stains carbohydrates found in glycoproteins and proteoglycans) (Nichols, 1981; 1986). These studies further indicate that the neural fold is heterogeneous (at least made up of two populations of cells) and fate restriction probably happens prior to cell delamination.

The first cells to delaminate from the neural fold express E-cadherin whereas the second population of cells express N-cadherin, this difference in adhesion system might also mean that they delaminate in different ways since delamination requires the downregulation of the adherens junctions. De Calisto *et. al.* showed that the first neural crest cells to delaminate in *Xenopus* express Frizzled7 (De Calisto et al., 2005). They also show that migration of the neural crest requires the activation of the non-canonical Wnt signaling. This is another demonstration that the cells that are at the leading edge are distinct from cells that delaminate slightly later.

In summary, we find that cells in cranial regions are delaminating from different ectodermal regions within the neural fold at different times, first from non-neural ectoderm and subsequently from neural ectoderm, we conclude that it is more probable that the neural crest is already heterogeneous in the

neural fold (Fig. 1.4C) it is also possible that fate restriction happens shortly before delamination from neural fold (fig. 1.4B). As alluded in the introduction, the original problem lies in the definition of the neural fold and based on the heterogeneity of the cranial neural fold, we would like to propose that there are two distinct regions within the neural fold and to separately define these two populations of cells; ectodermal crest for cells delaminating from the E-cadherin expressing region in the neural fold; and neural crest for cells originating from the Sox1/N-cadherin expressing region of the neural fold (Fig. 5.3). The ectodermal crest and neural crest would give rise to different derivatives namely mesectodermal and neuronal/pigment derivatives respectively.

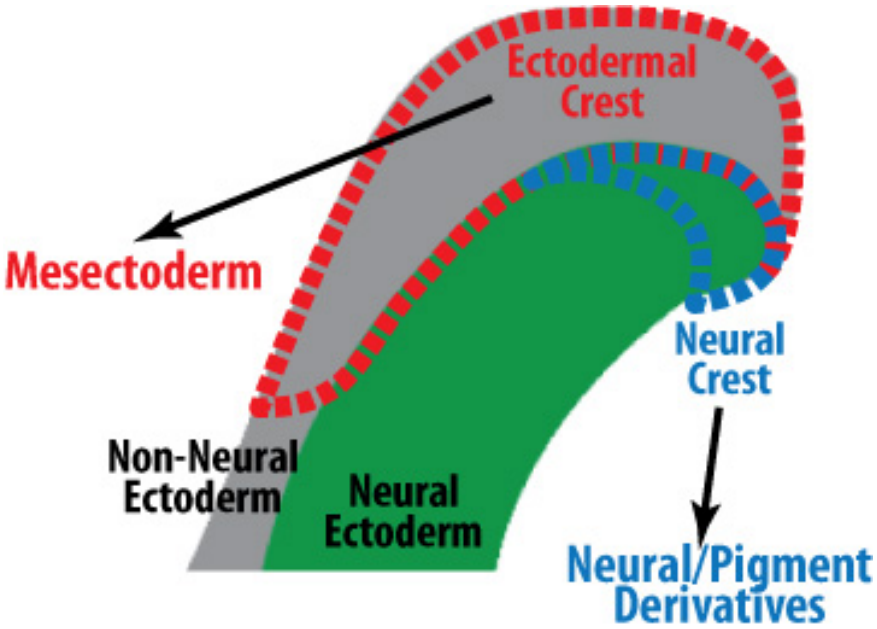


**Fig. 5.3: Revisiting the neural crest.**

Ectodermal crest originates from E-cadherin positive region and gives rise to mesectodermal derivatives whereas neural crest originates from Sox1/N-cadherin positive region and gives rise to neural/pigment derivatives.

## 5.3 Fate restriction and cell fate commitment

It must be emphasized that although we propose that these cells are fate restricted, it does not mean that these cells are committed to a particular cell fate meaning that the ectodermal crest will only adopt a mesectodermal fate and the neural crest will only adopt neuronal cell fate. As explained before these two processes are distinct. Challenging single cells with new environments and determining whether they retain their original cell fate is required to determine whether these cells are committed.

Initial studies have shown that the cranial neural crest cells that give rise to BA1 forms ectopic mesectoderm when transplanted into the trunk, this provides some evidence that perhaps the ectodermal crest is committed to a mesectodermal fate. However orthotopic and heterochronic grafting have also shown that the early and late neural crest cells can compensate for each other (Baker et al., 1997) but the study by McGonnell *et. al.* and Abzhanov *et. al.* have shown that trunk neural crest cells can produce mesectoderm (particularly cartilage and bone) but do this via first dedifferentiating to a cranial state (McGonnell and Graham, 2002; Abzhanov et al., 2003). These studies provide somewhat conflicting evidence for whether cells are committed. Under normal circumstances the place as well as time of delamination determines the particular cell fate whereas ablation or transplantation into a new environment might trigger some form of reprograming to induce adoption of new characteristics.

## 5.4 Mesectodermal derivatives from other axial levels

In other areas of the LacZ labeled Sox1 Cre skull, there seems to be a rather large amount of mesectoderm that is labeled in BA2 (fig. 3.16 and fig. 3.17). Mesectoderm in BA2 originates from neural crest cells that are derived from the posterior forebrain and hindbrain. Neural crest from different axial levels migrates in segmental streams to their target sites. For example neural crest cells from the midbrain and anterior hindbrain migrate to BA1 whereas neural crest cells from rhombomere 3-5 migrate into BA2 (Lumsden et al., 1991; Osumi-Yamashita et al., 1994; Santagati and Rijli, 2003). It should be noted that rhombomere 3 makes a minor contribution to BA1 (Sechrist et al., 1993).

In our characterization of the cranial neural crest of the midbrain, we have found that the second population of cells originates from the neural ectoderm and give rise to neuronal derivatives. This second population of cells has very similar characteristics to trunk neural crest cells, in terms of origin as well as differentiation potential. In this way, origin or perhaps segregation of the primitive ectoderm might have a direct effect on cell fate.

The organization of the ectoderm is very different along the anterior posterior axis. Interestingly segregation of neural and non-neural ectoderm seems to correlate with the loss of mesectoderm derivatives as well as the type of mesectodermal derivatives from different axial levels.

In the anterior cranial region, the neural fold adopts a very open configuration; neural and non-neural ectoderm is continuous, whereas in the posterior trunk the neural and non-neural ectoderm is separated very early on. However in

hindbrain regions the segregation of the neural and non-neural ectoderm seems to be an intermediate between the cranial and trunk region. If the segregation of neural and non-neural ectoderm has an effect on neural crest formation and fate then it is possible that at these intermediate levels, mesectoderm might be derived from cells, which have an intermediate ectodermal phenotype. Alternatively other factors may be responsible for the ability of neural ectoderm derived cells to give rise to mesectoderm or neural ectoderm derived cells may have a reduced capacity to give rise to mesectoderm.

There was a recent study investigating the migration of the vagal neural crest which reports that neural crest cells that populate the gut migrate ventrally whereas neural crest cells that migrate to the pharyngeal arches and heart migrate dorsolaterally (Kuo and Erickson, 2011). They show that these cells are fate restricted, i.e. early emigrating cells give rise predominantly to smooth muscle cells (these cells migrate dorsolaterally). In the head, neural crest cells only migrate dorsolaterally whereas in the trunk neural crest cells migrate both dorsolaterally as well as ventrally. It is unclear why vagal neural crest cells behave this way. Furthermore anterior vagal neural crest in contrast to posterior vagal neural crest show a difference in their migratory plasticity and the fate that they adopt. For example, when anterior vagal neural crest cells that normally migrate dorsolaterally (these cells give rise to smooth muscle cells in the pharyngeal arches) migrate ventrally they still go on to form smooth muscle cells in the pharyngeal arches. Maybe these neural crest cells from intermediate levels are coming from a region that has characteristics of both neural and non-neural ectoderm.

It must be emphasized because of the clearer segregation of neural and non-neural ectoderm in the midbrain as well as the unique ability of this region to give rise to cartilage and bone, we chose to focus on the midbrain.

## 5.5 Future work

### 5.5.1 What accounts for the difference between the two populations?

One thing that the current work does not address is how the two populations of neural crest cells within the head are different. This is especially disturbing when you consider that both populations of cranial neural crest cells express a very similar repertoire of transcription factors except Sox1/N-cadherin or E-cadherin/L-CAM in mouse and chicken respectively. But based on fate mapping experiments, these two populations of cell have very distinct cell fates. Therefore there are two scenarios, which might explain why the two populations of cells have distinct cell fates:

1. Neuralization of the ectoderm endows the cells with a more neural fate.
2. Differential activity of each transcription factor

#### 5.5.1.1 Neuralization of the ectoderm endows the cells with a more neural cell fate

In cranial regions, there is a gradient of Sox1 expression within the neural fold, higher expression in more ventral regions and lower expression in more dorsal region. There is also an opposite gradient of E-cadherin expression, higher expression in more lateral regions. Sox1 is a member of the SoxB1 family of transcription with known roles in the induction of neural ectoderm.

This expression gradient of Sox1 and E-cadherin might mean that the neural fold region is in a state of deciding between neural and non-neural ectoderm fate. This idea is supported by the study by Streit and Stern, showing that this region, unlike the lateral ectoderm, can be induced to form neural ectoderm (Streit and Stern, 1999; Linker et al., 2009). They also show that BMP, FGFs and their inhibitors in the surrounding region are important for the establishment and maintenance of this region.

What this might mean for neural crest formation is that these cells are generated in a region where there is a gradient of different inducers and these inducers might have different effects on cell fate. In the studies on the developmental potential of neural crest *in-vitro* by Le Douarin *et. al.* and Anderson *et. al.*, they show that various growth factors have different effects on the types of derivatives that are obtained (Baroffio et al., 1988; 1991; Shah et al., 1994; 1996; Shah and Anderson, 1997; Lahav et al., 1998; Trentin et al., 2004). It has been proposed that these factors act when the cells have reached their destinations.

It has been shown that there are a number of growth factors that are involved in the specification of neural crest, but as we have shown that fate restriction happens relatively early (prior to delamination), it is unclear when and what are the specific effects of these growth factors. Are the two populations of cells induced in the same way or are they induced in a different way?

The study by Lee *et. al.* shows that constitutive Wnt signaling induces neural crest cells to adopt a sensory fate at the expense of other cell types (Lee et

al., 2004). Another study by Blentic *et. al.*, show that FGF has an important role in specification of mesectoderm, although the authors attribute it to FGF8 that is secreted by the pharyngeal endoderm and not responding to FGF from the isthmus (Blentic et al., 2008). From these studies, it is clear that growth factors have very important roles for the specification of cell fate however it is unclear what are the specific effects of these growth factors and when they act. These studies also highlight the possibility that these factors might be inducing the formation of cells with distinct cell fates rather than inducing "pluripotent" neural crest progenitors.

There have been a number of studies on the activation of signaling pathways during embryonic development (Corson et al., 2003; Lunn et al., 2007; Mani et al., 2010). A systemic temporal analysis of pathway activation during embryonic development in relation to expression of neural crest markers might reveal what are the effects these growth factors have and perhaps it might reveal that the two populations of cells are induced differently.

#### 5.5.1.2 Differential activity of each transcription factor

From the expression studies of transcription factors involved in neural crest formation in chicken embryos, it is unclear whether all neural crest cells express the same repertoire of transcription factors and in the same sequence. In such a scenario, there might be dramatic differences in the transcriptional output of the neural crest cells, since the activity (either repressor or activator) of each transcription factor might be affected by the

activation or absence of another transcription factor. Alternatively these transcription factors might form different complexes with each other and this will also affect the transcription output.

Even if the cells within the neural fold might express the same transcription factors, it is possible that the duration of expression of each transcription factor is different in the different regions of the neural fold. For example, if a transcription factor has a role in promoting a neuronal cell fate and it is expressed for a longer period in the cells that are more medial, it would promote adoption of a neuronal cell fate in these medial cells.

From the inactivation of transcription factors that are involved in neural crest formation, there has not been a single mutant so far, which totally lacks all neural crest cells and most of these mutants have very specific defects, either neuronal or mesectodermal defect. This seems to indicate that the neural crest cells are a fate restricted population of cells and that the transcription factors involved in inducing neural crest cells may also have roles in specifying cell fate.

So far there has only been a few studies, which have indicated that the transcription factors involved in the induction of neural crest cells also have roles in specifying cell fate (Drerup et al., 2009; Mundell and Labosky, 2011). If these transcription factors besides acting on inducing the formation of the neural crest, also have later roles in specification of cell fate it would be reasonable to expect that other populations of cells are expanded when these

factors are lost. Based on this it might be worthwhile to re-analyze mutants to determine whether there is re-specification of cell fate.

These two scenarios may not be mutually exclusive and both might be acting together. For example in the development of the spinal cord, generation of specific neurons is a very orderly process. There are progenitors within the early spinal cord that respond to gradients of growth factors (Jessell, 2000). This growth factor gradient is translated into a differential expression of transcription factors, which have distinct roles in the formation of different types of neurons, by the progenitor cell. Deletion of these transcription factors leads to the absence of certain neurons but an expansion of other neurons. Perhaps for the neural crest specification of cell fate might operate in a similar way.

### 5.5.2 Analysis of promoter elements

As pointed out in the previous section, the knockout phenotypes of transcription factors within the genetic network of neural crest seems to suggest that these transcription factors also have roles in the specification of cell fate. This means that these transcription factors like the growth factors have multiple roles at different times and it would be of interest to determine whether there are distinct regulatory elements within the promoter that might reveal spatial and temporal roles.

For example if these transcription factors also have roles in determining cell fate, then it is possible that they might be differentially activated in the two populations. Sox10 is expressed in the otic placode and neural crest cells at different times, this reflects the complex regulation of this gene and there are a number of studies that have looked for enhancers that lie within this gene (Deal et al., 2006; Dutton et al., 2008; Betancur et al., 2010b; 2011). The studies by Betancur *et. al.*, demonstrate that the same enhancer is being regulated by different transcription factors. Perhaps a more extensive study of the different genes that have been implicated for the formation of neural crest would allow more insights on what determines the cell fate of individual cells and when cell fate is established. It might also yield a regulatory element that is specific for the ectodermal and neural crest.

## 5.6 Conclusion

In the current study we have focused on analysis of cell delaminating from midbrain regions and we have shown that there seems to be a link between ultimate cell fate and origin. We show that the neural fold is heterogeneous and consist of two regions, one that is E-cadherin/L-CAM positive (non-neural ectoderm) and the other is Sox1/N-cadherin positive (neural ectoderm). In the mouse, delamination of cells from the neural fold follows a lateral to medial sequence, from the non-neural ectoderm first followed by the neural ectoderm. These two populations of cells have distinct cell fates. Based on the differences in origins as well as cell fates, we propose to define the cranial neural crest as two distinct populations of cells: ectodermal crest for the cells originating from the non-neural ectoderm and giving rise to mesectoderm; and neural crest which originate from the neural ectoderm giving rise to neuronal derivatives. Fate analysis in the mouse has shown that mesectodermal derivatives in more caudal regions are also labeled; at present we cannot provide a definitive explanation for this finding. We acknowledge the possibility that neural ectoderm might also be able to give rise to mesectoderm in this region.

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