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Molecular signaling directing neural plate border formation

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ABSTRACT During embryonic development, the vertebrate embryonic epiblast is divided into two parts including neural and superficial ectoderm. The neural plate border (NPB) is a narrow transitional area which locates between these parts and contains multipotent progenitor cells. Despite its small size, the cellular heterogeneity in this region produces specific differentiated cells. Signaling pathways, transcription factors, and the expression/repression of certain genes are directly involved in these differentiation processes. Different factors such as the Wnt signaling cascade, fibroblast growth factor (FGF), bone morphogenetic protein (BMP) signaling, and Notch, which are involved in various stages of the growth, proliferation, and differentiation of embryonic cells, are also involved in the determination and differentiation of neural plate border stem cells. Therefore, it is essential to consider the interactions and temporospatial coordination related to cells, tissues, and adjacent structures. This review examines our present knowledge of the formation of the neural plate border and emphasizes the requirement for interaction between different signaling pathways, including the BMP and Wnt cascades, the expression of its special target genes and their regulations, and the precise tissue crosstalk which defines the neural crest fate in the ectoderm at the early human embryonic stages.

KEYWORDS: neural plate, neural plate border, neural crest cell, signaling pathway

Introduction

The human embryonic period, which occurs during the first 8 weeks after fertilization, is categorized using a morphological system. This system consists of 23 distinct Carnegie stages, with each stage representing a span of 2 to 3 days. The purpose of this staging system is to facilitate an understanding of the timing and sequence of embryonic development. The fetal period begins at the 9th week after fertilization and continues until birth. Fetal age is primarily determined through measurements as there is no equivalent morphological staging system available (Flierman *et al*., 2023). Gastrulation and formation of the three main germ layers are the major events during the 3rd week of embryo development (Sauka-Spengler and Bronner-Fraser, 2008a, Sauka-Spengler and Bronner-Fraser, 2008b). One key structure that develops during this period is the neural tube, which eventually forms the brain and spinal cord. In initial neurulation process, the neural tube is formed from the neural plate (Ravi *et al*., 2021). At the beginning of the third week, the central part of the ectoderm that is located on the developing notochord thickens and forms the neural plate or neuroectoderm. While the rest of the ectoderms forms surface ectoderm. The formation of neural crest cells determines the border between these two areas. This border has a very high sensitivity in terms of evolution because it organizes and forms various structures such as the formation of placodal derivatives, the development of skull bones, the formation of nerve tissues, and other structures (Grocott *et al*., 2012). The complete development and closure of the neural tube takes place between days 17 and 30 after gestation, equivalent Carnegie stages 8 to 12 in the embryonic period. The caudal eminence, an extension of the primitive streak, gives rise to various structures including the notochord, somites, vertebrae, and hindgut. From the caudal eminence, the neural cord emerges and forms the caudal part of the spinal cord. This process is known as secondary neurulation (Catala, 2020). What is involved in this process is the presence of huge gene regulatory networks (GRNs) that drive cells toward differentiation (Williams *et al*., 2022). However, the only available evidence to determine this border is the time of neurulation and their separation during neural tube formation (Williams *et al*., 2022, Yardley and García-Castro, 2012). In many studies, it has been reported that any defect in the formation, migration, and lack of regulation of cell division of these border cells leads to birth defects in the fetus (Siismets and Hatch, 2020, Gandhi *et al*., 2020). On the other hand, these border cells are

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affected by various factors in each region along the neural plate, and this causes their differentiation into specialized cells, and the absence of these factors in a specific region causes the formation of specialized cells in that region (Schille and Schambony, 2017).

Here, this study aims to investigate the factors affecting the border of the neural plate and determine the role of the factors affecting its formation. Several studies related to the examination of the neural plate border (NPB) have been published recently, and therefore, in addition to focusing on presenting their important and useful content, our attention has been on finding answers to the questions in the minds of researchers about the NPB.

An overview of genes involved in determining the boundary between neural and surface neural ectoderm

Delving into the molecular mechanisms that drive neurulation process is vital for deciphering the intricacies of embryonic development and organogenesis in vertebrates (Mirdass *et al.*, 2023). According to the evidence and data available from previous studies, the development of neural crest cells from the beginning to the formation of specialized cells can be divided into 5 stages. These stages include initial induction, establishment of neural plate (NP) border, maintenance of multipotency; control of cell cycle and epi-

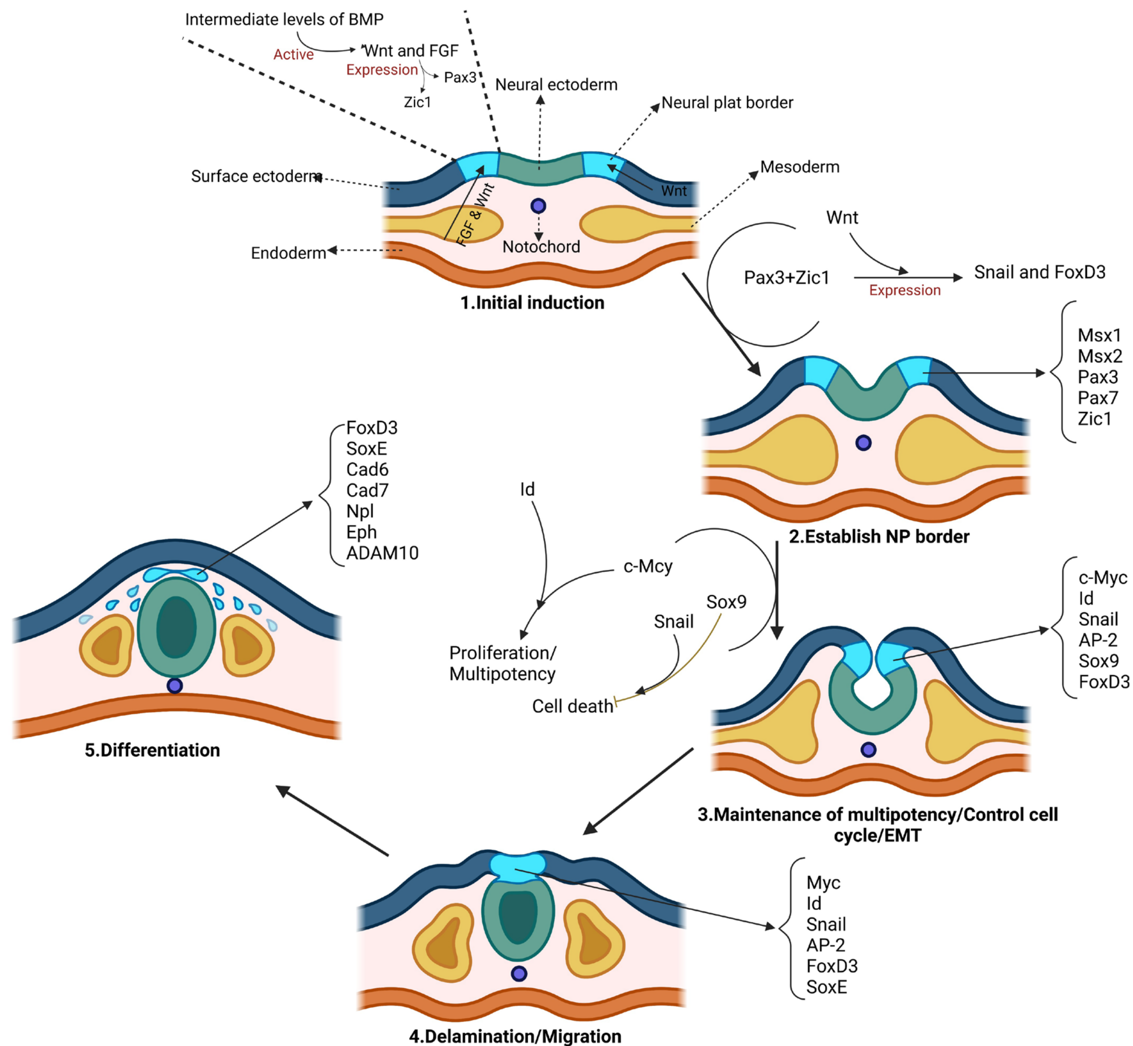

Fig. 1. Regulatory steps in the formation of the border between neural ectoderm and surface ectoderm. Induction is initiated by signaling molecules such as fibroblast growth factor (FGF) from the underlying mesoderm, as well as Wnts from the mesoderm and the adjacent non-neural ectoderm at the border between the neural ectoderm and the surface ectoderm. Each of these molecules, if the bone morphogenetic protein (BMP) level is moderate, separately, or in interaction with each other, causes the expression of *Pax3* and *Zic1* molecules, which are responsible for determining the border between neural ectoderm and surface ectoderm. *Pax3* and *Zic1*, dependent on Wnt, synergistically activate *Snai* and *FoxD3* which are neural crest (NC) specifiers. These molecular interactions are the result of studies on *Xenopus laevis*, so these events may not be true for other organisms. The c-Myc-Id cassette is one of the molecules involved in the cell cycle that may play an important role in determining and maintaining the multipotent state. *Sox9* can maintain trunk NC precursors by affecting anti-apoptotic factors such as *Snai*. The specific expression of early NC markers in the progenitor NC population causes their separation from the dorsal neuroepithelium. Moreover, these factors are involved in the regulation of many processes such as cell proliferation, stratification, and the initiation of epithelial-to-mesenchymal transition (EMT). *FoxD3* and *Sox10* play a role in the migration and delaminating of neural crest cells and also regulation of other factors such as Cad7, MMPs, ADAM10, Npl, and Eph. Abbreviations: NP, neural plate; Cad7, cadherin-7; MMPs, matrix metalloproteases; ADAM10, a disintegrin and metalloprotease-10; Npl, neuropilins; Eph, ephrin type-A receptor 1.

thelial to mesenchymal transition (EMT); delamination; migration, and differentiation (Fig. 1). Although these stages are serial, the noteworthy point is that each stage is under the control of special regulators (Prasad *et al*., 2019).

These steps are involved in the fate determination of neural crest cells, along with several signaling factors. In the initial stage, with the secretion of factors such as Delta, Wnt, FGF, and BMP in the third week, it leads to the determination of neural crest cells (Tang *et al*., 2023, Roure *et al*., 2023). The effects of these factors in the next stage led to the expression of *Msx*, *Pax3/7*, *Zic1*, and *Dlx3/5* factors at the border between neural ectoderm and superficial ectoderm. These signaling molecules in this border region led to the formation and specialization of neural crest cells. Neural plate boundary markers lead to the expression of signaling molecules and neural crest-determining genes such as Snail/Slug, AP-2, *FoxD3*, *Twist*, *Id*, *cMyc*, and *Sox9/10*. In the developmental process, a sequence of events occurs that enables neural crest cells to sustain their population through cell division and react to their surroundings by producing different cell receptor molecules, junctions, and metalloproteases (Sauka-Spengler and Bronner-Fraser, 2008a). Finally, the neural crest cells also migrate and differentiate into different cell types under the influence of this set of signaling factors. *SoxE* genes are one of gene specifier that play a significant role both in the early stages of neural crest formation and in the stages of differentiation into cartilage, neurons, and glia (Kelsh, 2006). It is not yet known precisely how the molecules that affect the growth, specialization, and movement of neural crest cells interact with each other. What is important to note is that there is no specific gene responsible for neural crest formation. Instead, it is a combination of these signaling molecules and other factors at specific times and places that directs this complex process (Méndez-Maldonado *et al*., 2020).

The most important signaling factors in the determination of neural crest cells between neural and surface ectoderm

During embryonic development in many vertebrates, the main time for the formation of neural and surface ectoderm cells is before the gastrulation initiation. The dorsal ectoderm plays a crucial role in inducing the formation of the neural plate through the expression of genes such as *Otx2*, *Sox3*, ERNI, and Geminin. These genes are considered the most important signaling factors in determining neural crest cells between neural and surface ectoderm. (Bally-Cuif *et al*., 1995, Rex *et al*., 1997). In addition to the previously mentioned genes, other molecules such as FGFs, Wnt, and BMP are crucial in inducing the formation of the neural plate. These molecules play a significant role in the expression of pre-neural genes. (Stern and Downs, 2012, Rogers *et al*., 2011, Albazerchi and Stern, 2007). In regions of the epiblast where Wnt and BMP signals are active, they induce the expression of non-neuronal markers, including genes from *Ap2*, *Dlx*, *Foxi*, *Gata2/3*, and *Msx* transcription factor families (Fig. 2) (Pieper *et al*., 2012, Li and Cornell, 2007, Hoffman *et al*., 2007, Hans *et al*., 2007, Phillips *et al*., 2006). In *Xenopus*, it is proposed that the dorsolateral marginal zone (DLMZ) of the gastrula, which is located beneath the prospective neural crest, serves as the source of neural crest-inducing signals. The DLMZ expresses various Wnt and FGF ligands, as well as the BMP antagonist Chordin, all of which are known to play a role in neural crest induction. Additionally, the DLMZ expresses several other regulators of Wnt and BMP signaling, including *Noggin*, *Cerberus*, *Frzb1*, *Dkk1*, *Sfrp2*, and *Crescent* (Alasaadi *et al*., 2024). A recent study has also highlighted the importance of Snai2 in mesoderm formation and its involvement in regulating the signals originating from the DLMZ, thus making Snai2 a crucial factor in early neural crest development. As development progresses to the neurula stage, the DLMZ gives rise to the paraxial or intermediate mesoderm, which underlies the proper formation of the neural crest. Recombination experiments involving the DLMZ and animal caps, as well as grafts of the paraxial mesoderm into ventral epidermis, have shown the expression of neural crest markers (Li *et al*., 2019).

It is hypothesized that the interaction between the neural and surface ectoderm is necessary for the formation of the neural border (Groves and LaBonne, 2014). Experimental studies have shown that the grafting of neural plate cells on the surface ectoderm causes the formation of neural crest and placode cells (Selleck and Bronner-Fraser, 1995). In addition, Wnt, BMP, and FGF signals are necessary for the formation of the NPB (Yardley and García-Castro, 2012, Endo *et al*., 2002). *In vitro* observations have shown that mesodermal cells play an important role in the induction of neural ectoderm (Brugmann *et al*., 2004). Reports have shown that the hypoblast alone is not responsible for the formation of the neural border, but the inductive role of the underlying mesoderm cannot be ignored (Albazerchi and Stern, 2007, Richard *et al*., 2016). In the third week of embryonic development, the neural and surface ectoderm domains are specified (Meulemans and Bronner-Fraser, 2002, Sauka-Spengler and Bronner-Fraser, 2008b). As a result of inducing responses caused by the presence of factors such as Wnts and BMPs, it causes the expression of *Tfap2*, *Pax3/7*, *Dlx3/5*, and *Msx1/2* factors at the NPB (Fig. 2) (Moody and LaMantia, 2015).

NPB progenitor cells are created in response to FGF, BMP, Wnt, and retinoic acid (RA) and finally differentiate into the neural crest and placodal ectodermal cells (Grocott *et al*., 2012, Sauka-Spengler and Bronner-Fraser, 2008a). Reports have shown that NPB expresses moderate levels of BMP signaling that along with levels of BMP agonists, cause their differentiation into specialized cells (de Crozé *et al*., 2011, Garnett *et al*., 2012). For example, in animal studies, it was found that high levels of the BMP antagonist, Noggin, cause the cells to differentiate towards the placode, while medium and high levels of Noggin cause the cells to differentiate towards the neural crest and neural plate, respectively (Hong and Saint-Jeannet, 2007, Park and Saint-Jeannet, 2008). Research on neural crest development has been conducted in various species, with *Xenopus* and chick studies providing valuable insights into the earliest inductive signaling events. Recent findings from these model organisms propose a two-step process for neural crest induction, involving FGF and Wnt signaling during gastrulation followed by Wnt and BMP signaling during neurulation to maintain the neural crest population. Despite the conservation of signaling pathways across species, differences exist in the source, timing, and regulation of neural crest development (Prasad *et al*., 2019, Rodrigues-Da-Silva *et al*., 2022). Understanding the diverse mechanisms underlying neural crest development is crucial for the advancement of clinical diagnostic and therapeutic strategies. because disruptions in neural crest development can lead to a range of severe human health conditions known as neurocristopathies, including malignant tumors like melanomas and neuroblastomas, rare syndromes such as Hirschsprung and Waardenburg syndromes, and structural abnormalities like cleft lip/palate and aganglionic megacolon.

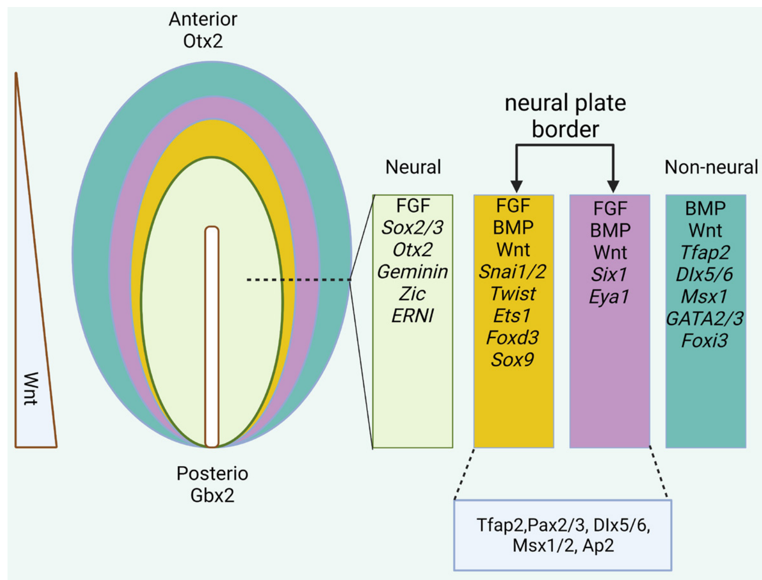

Fig. 2. Epiblast differentiation in the anterior region. The expression of different genes and factors in different parts of the ectodermal layer. In the final stages of neurulation, the neural plate border is divided into two medial (yellow) and lateral (purple) parts.

The expression patterns of neural crest markers at the beginning varies among different species. In *Xenopus*, most neural crest genes (*Snai2*, *FoxD3*, and *Sox8/9*) are first expressed shortly after the appearance of the NPB and before gastrulation is complete.

However, in the chick, Snai2 is first observed at a later stage (stage 6) and not strongly expressed until several hours after the NPB has formed (stage 8 also named as 4-somite stage) (Ponzoni *et al*., 2022, Ben Amar *et al*., 2022). Distinctive neural crest cells expressing a full complement of neural crest markers are not apparent until just before migration at stage 9/10. Despite these differences, the avian neural crest seems to be specified before gastrulation, while *Xenopus* neural folds do not maintain expression of neural crest markers without additional signals. In zebrafish, *FoxD3* is expressed first and has a unique role early in gastrulation, while other neural crest markers label the neural crest towards the end of gastrulation. In mice, neural crest markers such as *Sox9/10* and *FoxD3* label the neural folds soon after the expression of NPB markers and before cranial neural crest migration. Other experiments, on the other hand, proposed a different mechanism for the induction of neural crest cells involving the interaction between neural and non-neural ectoderm (Rocha *et al*., 2020). Stuhlmiller conducted experiments using two different species of amphibians and demonstrated that when neural and epidermal tissues were placed in close proximity, they were able to generate neural crest cells (Stuhlmiller and García-Castro, 2012a). This finding was later supported by Pieper, who used pigmented and non-pigmented axolotl embryos in donor/host combinations and showed that both neural and epidermal tissues could give rise to neural crest cells (Pieper *et al*., 2012). Similar experiments conducted in *Xenopus* and chick, where neural tissue was grafted into lateral epidermis, also confirmed that both tissues were capable of producing neural crest cells in these species. Additionally, a recent study in *Xenopus* suggested that the neural non-neural ectoderm (NNE) loses its ability to generate neural crest cells towards the end of gastrulation, while the neural plate (NP) retains its competence until neurulation. These findings collectively supported a model in which neural crest induction occurs through interactions between the NP, NNE, and underlying mesoderm (Gouignard *et al*., 2021). *Six1* and *Eya1* play an important role in fate determining of the region referred to as the preplacodal region (PPR). On the other hand, the expression level of *Six1* has a direct effect on determining the cell fate towards the neural crest or placode. PPR contains undifferentiated placodal progenitors. Initially, certain genes like *Foxi3* and Gata3 are broadly expressed in the non-neural ectoderm but eventually refine to the PPR (Sullivan *et al*., 2019). On the other hand, genes belonging to the *Six* and *Eya* family are newly expressed in the anterior NPB region, which extends from the first pair of somites to the most anterior regions of the neural plate. Subsequently, genes specific to sub- populations of placodes emerge in this region in response to local inducing signals. For instance, *Pax2/8* genes appear in the otic and epibranchial placode region, Pax3 in the ophthalmic trigeminal ganglion, and Pax6 in the future lens and olfactory placodes. *Otx2* and *Gbx2* are expressed in the anterior and posterior epiblast, respectively, and their expression patterns are maintained through mutual repression during the induction and patterning of the neural plate. Ultimately, this process helps establish the boundary between the midbrain and hindbrain. As development progresses, additional changes occur in this region (Thawani and Groves, 2020, Seal and Monsoro-Burq, 2020).

The interaction of *Eya* and *Six* proteins causes a change in the function of the *Six* transcription factor with DNA (Patrick *et al*., 2013). FGF signaling induces PPR formation, while deletion of FGF signaling can increase or decrease PPR through the presence of Wnt and BMP signaling. In addition, the presence of FGF signaling is necessary for the expression of genes such as *Six* and *Eya* (Fig. 2) (Hintze *et al*., 2017, Litsiou *et al*., 2005).

A set of gene expressions including *FoxD3*, *Snai1/2*, *Twist*, *Sox10*, and *Ets1* in the inner part of NPB causes the formation of neural crest cells (Fig. 2) (Barembaum and Bronner, 2013, Simões-Costa *et al*., 2012). Factors such as *FoxD3*, *Pax3/7*, and *Msx1* determine the identity of neural crest cells in the head and trunk region (Simões-Costa *et al*., 2012). *Pax3/7* interacts directly with *Zic1*, which, depending on the species, eventually leads to the activation of *Snai1/2* and the expression of *Ets1* (Plouhinec *et al*., 2014, Simões-Costa *et al*., 2014, Milet *et al*., 2013).

Pattern of the neural plate border formation

As soon as the neural tube is closed, the neural crest cells, which are located on the dorsal midline of the neural tube, begin to migrate. Therefore, under the EMT influence, the neural crest cells begin to separate and spread throughout the body of the embryo and differentiate. At this stage, neural crest cells begin to express genes involved in migration such as *Sox10*, *Sox9*, *FoxD3*, and *Ets1*, and on the contrary, they reduce or silence the expression of mesenchymal to epithelial state markers such as *N-cadherin*, *E-cadherin*, *Cadherin11* and *Cadherin7* (Simões-Costa and Bronner, 2016, Kashef *et al*., 2009).

During gastrulation, molecular asymmetry occurs along the rostrocaudal axis. In the epiblast region, *Otx2* and *Gbx2* genes are expressed in the anterior and posterior axes, respectively. Maintenance of this pattern occurs through bilateral suppression as the neural plate is induced and formed. At this stage, the border between the midbrain and the hindbrain was determined (Fig. 2). *Pax6* and *Pax2* gene expression occur in the anterior region of the forebrain and midbrain, respectively, while *Six1* and *Irx3* are exclusively expressed in the forebrain. The formation pattern of anterior-posterior PPR also occurs similarly. Hence, the expression of *Otx2* and *Gbx2* genes in these areas is necessary for developing the trigeminal and otic placode, respectively (Steventon *et al*., 2012). *Pax* family genes such as *Pax6*, *Pax3*, and *Pax2/8* are expressed along the anterior-posterior axis which is involved in the developmental stages of placodal derivatives (Saint-Jeannet and Moody, 2014, Koontz *et al*., 2023).

On the contrary, *Hox* family genes are expressed in the neural plate region posterior to rhombomere 1 of the hindbrain, while no expression of them is seen in the pre-placodal ectoderm region. Since the neural crest cells located in the hindbrain begin to migrate and enter the pharyngeal arches, they do not necessarily express *Hox* family genes in the pharyngeal arches. For example, neural crest cells located in the first pharyngeal arch do not express any *Hox* family genes, while cells located in the second pharyngeal arch express *Hoxa2* and *Hoxb2* genes (Parker *et al*., 2018). Reports have shown that cultured migrating neural crest cells have the ability to express *Hox* family genes, while the factors and signals that caused their non-expression in the pre-placodal ectoderm region have not yet been identified (Trainor and Krumlauf, 2000). However, the investigation of factors affecting the expression of *Hox* genes in neural crest cells continues, and understanding how the expression of these genes is silenced in the placodal region is welcomed by researchers (Trainor and Krumlauf, 2000).

Researchers have been trying to understand why neural crest cells are not formed in the more anterior regions of the neural plate. Studies have shown that olfactory placode cells derived from the anterior neural fold can differentiate into the epidermis, olfactory placode, olfactory bulb, and forebrain, but not into neural crest cells (Parker *et al*., 2018). Furthermore, studies have shown that the anterior neural fold can differentiate into some neural crest cells when transplanted to the rostral hindbrain (Torres-Paz *et al*., 2021, Ezin *et al*., 2014). However, the induction signals for placodal formation and the lack of neural crest cells in the anterior region of the neural plate have not been fully described. Researchers suggest that signals such as Wnt, BMP, and retinoic acid may play an essential role in this pathway (Villanueva *et al*., 2002). Some studies have also shown that inactivating the Wnt antagonist gene expression, Dkk, and some members of Tcf/Lef in the anterior region of the epiblast can induce neural crest cells (Mašek *et al*., 2016). The visualization of the NPB formation can be achieved through techniques such as immunostaining or *in situ* hybridization, which utilize markers specific to different border derivatives. These techniques reveal that at initial stages the boundaries between cells differentiating into various derivatives are imprecise. However, over time, these boundaries become more distinct and form clear domains. This process of self-organization and refinement has been observed not only in the NPB but also in micropatterned cultures of embryonic stem cells (Pla and Monsoro-Burq, 2018). With the help of Hybridization Chain Reaction and *in vivo* transcriptomic techniques, such as Multiplexed Error-Robust Fluorescence *in situ* Hybridization (MERFISH), visualization of border genes in response to high concentrations of activin is becoming more feasible (Pajanoja *et al*., 2023, Choi *et al*., 2018).

With the advent of single cell technologies, it is now possible to compare the epigenetic and transcriptional states of stem cells as they transition into neural crest cells. This will help to determine which GRNs governing pluripotency are present in pre-migratory and migratory neural crest cells, and even neural crest-like peripheral glia stem cells (Erickson *et al*., 2023). The NPB region of the chordates has only a few cellular thickness, and techniques like Slide-seq and MERFISH may provide higher resolution spatial information on the cellular transcriptome during border development. The former technique transfers the single cell thick tissue onto a sequencing grid to analyze single cell RNA while the latter method uses a multichannel *in situ* hybridization (ISH) technique to probe the same sections for thousands of RNA transcripts. Both methods provide a spatial context to the transcriptomic profiles. These techniques can be used to identify the cellular identities at the NPB region where the four ectodermal lineages are intermingled over just a few cell diameters, depending on their cellular resolution (Thawani and Groves, 2020, Yao *et al*., 2023). Single-cell RNA sequencing techniques can provide more detailed information on the levels of signaling and expression of downstream effectors, allowing for a deeper understanding of the interactions in signaling pathways at the NPB. Techniques such as multiple annealing and dC-tailing-based quantitative single-cell RNA-seq (MATQ-seq) and SMARTer single cell total RNA sequencing (SMARTer-seq) are highly sensitive but have lower throughput, making them ideal for a more detailed analysis of individual cells. For example, recent studies have used SMART-seq2 to understand the fate programs of neural crest cells and detected over 7,000 genes per cell, providing a better understanding of the transcriptomic decisions made by premigratory/migratory crest cells as they proceed towards sensory, glial, or mesenchymal fates. In addition to the transcriptomic status of the cells in developing NPB region it remains to be elucidated when the cells are fully committed to a lineage are fully committed to a particular lineage (Jovic *et al*., 2022). Epigenomic sequencing analysis, such as Assay for transposase-accessible chromatin with high-throughput sequencing (ATAC-seq), can identify accessible genomic loci that are available for transcriptional activity (Buenrostro *et al*., 2015). Single cell ATAC-seq is now available and can be combined with scRNA-seq to identify relevant enhancers for lineage-specific transcription factors, evaluate plasticity of the cells and determine whether trans-differentiation is feasible from one ectodermal path to another (Jiang *et al*., 2023). For instance, Lukoseviciute *et al.,* used single cell RNA-seq, ATAC-seq, and ChIP-seq data to identify a bimodal function for *FoxD3*, a key transcription factor that plays a crucial role in neural crest specification and differentiation. Their results revealed that *FoxD3* activates cis-regulatory elements for neural crest specifier genes as an activator and represses mesenchymal and migratory programs at later stages to prevent premature differentiation (Lukoseviciute *et al*., 2018).

Over-expression and knockdown studies in various species have demonstrated cross-repressive interactions between transcription factors expressed in the early non-neural ectoderm and those expressed in the definitive neural plate. For instance, the over-expression of *Dlx*, *Gata*, *Msx*, *Foxi*, and *Ap2* factors repress neural markers like *Sox2*, while knockdown of these genes expands the

neural plate at the expense of non-neural ectoderm. Conversely, positive auto-regulatory interactions between non-neural genes can sharpen the boundary between neural and non-neural domains. Examples of such genes include *Ap2c*, *Foxi1*, and *Gata2* (Lau *et al*., 2023). In experiments conducted on amphibian blastula, it was observed that knockdown or over-expression of certain neural crest transcriptional effectors, such as *Snail* or *Sox5*, led to a depletion of pluripotency-associated genes and reduced the cells' ability to form mesoderm. On the other hand, over-expression of transcription factors that induced a neural crest or NPB state, extended the cells' competence to form mesoderm and endoderm (Schock *et al*., 2023). It was also observed that NPB tissue could be induced to form endoderm. The potential for development of cells is regulated by FGF/MAPK signaling and a transition to PI3K/Akt signaling, along with the replacement of *SoxB* effectors with *SoxE* effectors leads to a more restricted developmental state (Pla and Monsoro-Burq, 2018).

Signaling pathways related to neural plate border formation

It seems that the differentiation of NPB cells is influenced by various signals which affect several transcription factors. According to research, the *Tfap2a* and *Gbx2* genes are crucial in inducing and differentiating neural crest cells by triggering the expression of genes like *msx1*, *pax3*, and *hes4* (de Crozé *et al*., 2011). Additionally, *Tfap2a* is essential for the formation of placode progenitors through *Six1/Eya1 induction* and neural crest via *FoxD3 gene*, while *Gbx2* enables differentiation towards neural crest by inhibiting the expression of *Six1* (Maharana and Schlosser, 2018). On the other hand, *Gata2/3* and *Foxi* TFs induce differentiation towards placode progenitors by regulating the expression of *Dlx3/5* and affecting the *Six1* gene (Li *et al*., 2009). Studies have shown that *Dlx3* and *Dlx5* play a direct role in inducing the formation of placode progenitors by affecting Six1 (Hintze *et al*., 2017). *In vivo* studies have shown that *Dlx3* and *Dlx5* play a direct role in inducing the formation of placode progenitors by affecting *Six1*. Interestingly, *Msx1* represses *Six1* expression, thus inhibiting placode progenitor's formation and inducing neural crest formation (Maharana and Schlosser, 2018). However, a recent study has reported that the expression of *Six1/Eya1* genes requires the expression of *Msx1*, indicating a direct relationship between them, while the lack of control of *Msx1* expression causes aberrant expression of *Six1/Eya1* (Rothstein and Simões-Costa, 2020). Reports have also shown that the expression of genes such as *Tfap2a* and *msx1* is necessary for the subsequent stages of neural crest development. Although these results may seem contradictory, they can be attributed to different stages of differentiation. In confirmation of this, reports have shown that the expression of genes such as *Tfap2a* and *Msx1* is necessary in the next stages of neural crest development (Sato *et al*., 2010).

Pax3 and the more anteriorly localized *Zic1* factor are considered to be the most important factors involved in the induction of neural crest and placode progenitors (Plouhinec *et al*., 2014, Bae *et al*., 2014). It has been reported in several studies that high levels of *Pax3* and *Zic1* expression cause the differentiation of ectodermal cells into glandular and placode progenitors, respectively, while the expression of both of them leads to differentiation into the neural crest. *Zic1* interacts with *Dlx3* to differentiate ectodermal cells into placode progenitors, while *Pax3* expression directly results in the lack of expression of *six1/eya1* (Maharana and Schlosser, 2018). *Pax3/Zic1* in interaction with each other directly affects the expression of specific genes of neural crest cells (Plouhinec *et al*., 2014, Simões-Costa *et al*., 2014). Various studies have reported that in the gastrulation stage in the anterior neural border region, the expression of *Zic1*-positive/*Pax3*-negative causes the formation of placode progenitors, while the overlapping of *Pax3* and *Zic1* causes the formation of neural crest cells. In addition, there is a slight overlap between *pax3/7*-negative and *Six1/Eya1*-positive, which raises the question of how cells in these regions differentiate into different cell types (Roellig *et al*., 2017). Such questions can be solved with more studies in the future and taking into account temporal, morphogenetic, and species differences in the neurulation stage.

Several transcription factors that are involved in cell differentiation into neural crest/placode progenitors have been identified. Studies conducted on *Xenopus* have shown that the expression of *Hes4* (hairy2b) and *znf703* genes in the neural border region is necessary for differentiation into the neural crest. *Hes4* controls the increased expression level of *FoxD3*, which induces pluripotency in neural crest cells. This mode of action activates the Notch/Delta signaling pathway and triggers *Id3*, which finally leads to the differentiation of neural crest cells (de Crozé *et al*., 2011, Nichane *et al*., 2008). *Znf703 is a gene necessary for neural crest differentiation, targeting Pax3 and Zic1 genes* (Hong and Saint-Jeannet, 2017, Janesick *et al*., 2019). Several *in vivo* studies have demonstrated that the expression of *Axud1*, *Pax7*, and *Msx1* is crucial for the formation of neural crest (Simões-Costa and Bronner, 2015, Azambuja and Simões-Costa, 2021). On the other hand, *Znf462* and *Pdlim4* have a direct impact on the expression of *Foxi3* and *Dlx5*, respectively, which leads to the development of placode progenitors (Mohammadparast and Chang, 2022).

According to a recent study, it was found that *Ash2L* and *Dpy30* are crucial in the development of neural crest. The study also revealed that *Dpy30/Ash2L* has a direct correlation with NPB transcription factors such as *Msx1* and *Tfap2a*. These transcription factors activate/deactivate a series of molecular signals for their differentiation through the induction of *Dpy30* and *Ash2L* in the defining regions of the neural crest (Mohammadparast and Chang, 2022). Another research team has reported that the transcription factor zinc finger of the cerebellum 1 (*ZIC1*) determines the fate of neural crest cells in the NPB. *ZIC2*, *ZIC3*, and *ZIC5* transcription factors work together with *FoxD3* to increase conventional Wnt activity at the border of the rodent neural plate. The function of *ZIC* proteins is greatly improved by SUMOylation. Conversely, the presence of basal *ZIC* proteins in the lateral regions of the neuroectoderm (a region of low canonical Wnt activity) represses Wnt/TCF-mediated transcription factors. Therefore, Wnt signaling and SUMOylation play an important role in the induction and differentiation of neural crest cells in the NPB (Bellchambers *et al*., 2021).

Cranial neural crest cells are another derivative of NPB. The development and differentiation of these cells depend on the crucial role played by *Twist1* and *Irf6*. *β/δ-catenins* interaction with *Twist1* leads to neural tube closure, while *Irf6* determines the boundary of neural folds by limiting *AP2α* expression. *Twist1* is also involved in the EMT and migration of cranial neural crest cells by repressing *Irf6* and other factors. If *Twist1* is suppressed, it prevents the migration of these cells and increases cell adhesion (Bertol *et al*., 2022).

According to a recent report, the cell population in the NPB is uniform during the gastrulation stage. However, this uniformity is disrupted during the neurulation stage, resulting in heterogeneity on the outer side of the border. This heterogeneity is caused by the expression of *Pax7*, which initiates cell differentiation (Williams *et al*., 2022). The report also suggests that knocking out *Pax3/7* can lead to defects in neural tube closure and damage to Motor Ganglion neuron specification (Kim *et al*., 2022).

Folate, which is the precursor of S-adenosylmethionine, plays a crucial role in determining the fate of NPB cells. Since this type of folate has a significant impact on DNA methylation. The methylation changes that occur in specific genomic regions due to Folate Carrier 1 (*RFC1*) deficiency can disrupt early developmental pathways such as Notch1 and BMP4 signaling according to recent reports in this field. These interactions have a direct effect on the establishment and connection of progenitor cells in the neural plate and NPB (Alata Jimenez and Strobl-Mazzulla, 2022). In confirmation of this result, a recent report has demonstrated that BMP signaling and its antagonists, Noggin and Chordin, play a crucial role in the development of cranial neural placodes in the anterior NPB. This is because the expression of *Foxg*, *Six1/2*, and *Zf220* requires BMP signaling in this region (Liu *et al*., 2023).

It has been reported that different molecules are involved in the signaling pathways related to neural tube closure, proliferation, and migration of cranial neural crest cells. One such molecule is *Adam11*, which is a non-proteolytic *ADAM* and a possible tumor suppressor. *Adam11* has the ability to bind to proteins involved in both the Wnt and BMP4 signaling pathways. It acts by upregulating BMP4 signaling and downregulating *β-catenin*. Through its modulation of these signaling pathways, *Adam11* plays a critical role in regulating neural tube closure time, as well as the proliferation and migration of cranial neural crest cells (Pandey *et al*., 2023).

Recent advances in transgenic animal models, CRISPR technology, and high-resolution live imaging of fluorescent reporters have provided us with new tools to visualize signaling dynamics in space and time. For instance, we can use these techniques to investigate whether the developing epiblast contains intermediate cell types that have the potential to give rise to both neural crest cells and placodes. This can help us test the "binary competence model" of ectodermal patterning. Despite the limitations of these technologies, we can integrate data from published studies to trace cell lineage along the developmental timeline (Huang *et al*., 2023).

The most important signaling factors in the formation of the neural plate border

Wnt signaling pathway

The NPB is initially induced by external signaling molecules such as Wnts, FGFs, BMPs, and Notch. These molecules activate specific pathways that lead to the expression of genes that specify the NPB (Stuhlmiller and García-Castro, 2012a). After activation of these signaling pathways, a group of molecular inducers and downstream signaling pathways cause neural crest cell formation, differentiation, and migration (Simões-Costa and Bronner, 2015).

The Wnt signaling pathway is a group of secreted molecules that bind to receptors on the cell membrane. This binding triggers a variety of responses within the cell. Wnt signaling has been shown to play a critical role in various developmental stages, diseases, and the progression of cancer (Akoumianakis *et al*., 2022, Zhang and Wang, 2020). In the process of forming the NPB, the Wnt pathway plays an important role in two ways: canonical and non-canonical. The canonical pathway is more commonly observed in the forma-

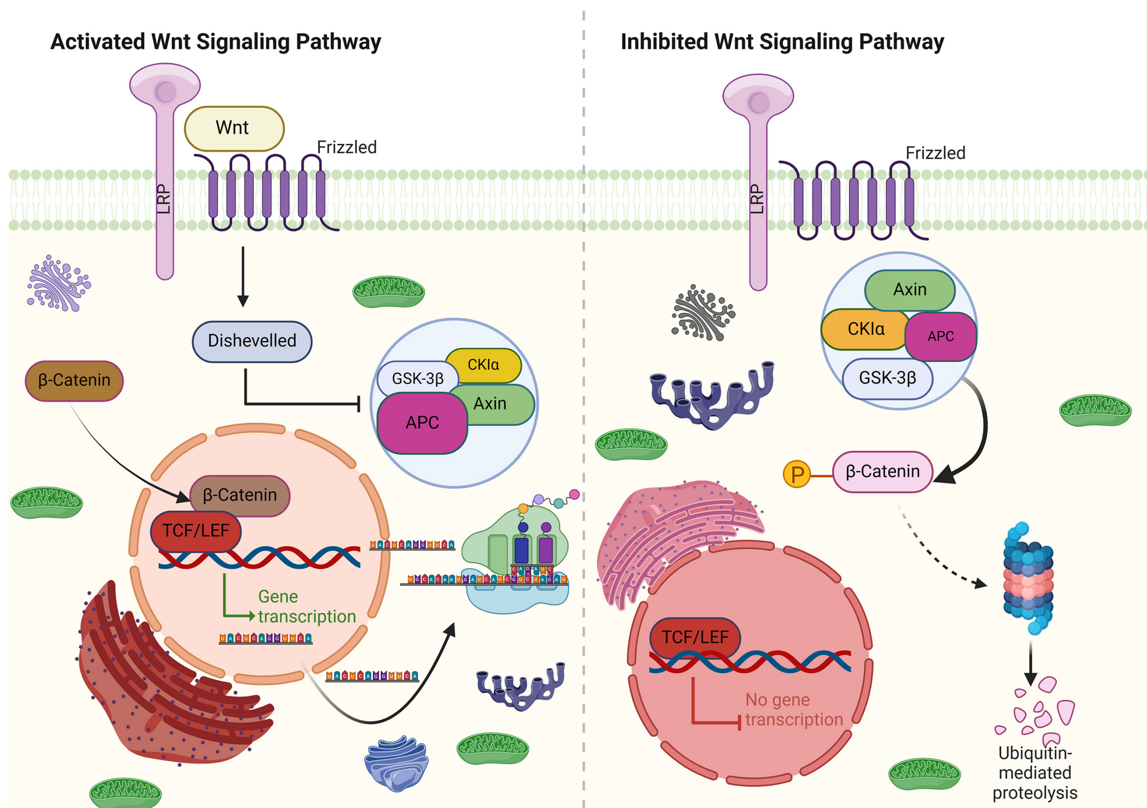

Fig. 3. Canonical Wnt signaling. In the absence of a Wnt ligand, β-catenin phosphorylation by forming a degradation complex (consisting of Auxin, APC, CK1, and GSK3β) causes its ubiquitination and is ready for degradation by the proteasome. The absence of β-catenin in the nucleus leads to the binding of the repressor complex containing TCF/LEF to the target gene, thereby suppressing its activity. As a result of the Wnt ligand binding to the Frizzled receptor and co-receptor LRP (right), the β-catenin degradation complex is deactivated, which leads to the accumulation of β-catenin in the cytoplasm and its transport to the nucleus, where it forms a complex with TCF/LEF and transcribes the target genes.

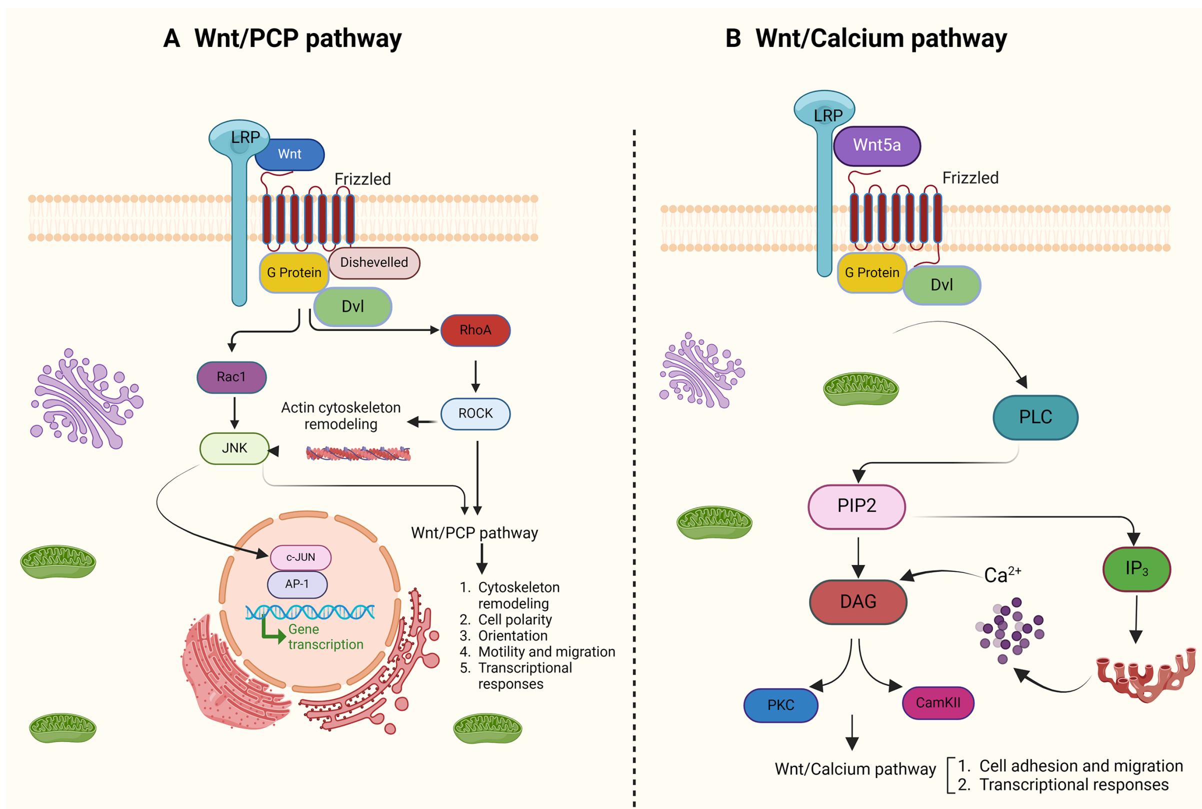

Fig. 4. Non-canonical Wnt pathways. (A) In the Wnt/PCP pathway, distribution of FZD receptors causes cell polarity and activation of RhoA/Rock GTPases and JNK through Dsh and DAAM1. This mechanism affects cytoskeleton arrangement and cell migration. **(B)** In the the Wnt/calcium pathway, as a result of Wnt-Fz binding, PLC is activated, which then hydrolyzes PIP2 and produces IP3 and DAG. IP3 induces the release of calcium from the endoplasmic reticulum, which is a stimulus for the activation of calcium/calmodulin-dependent protein kinase II (CamKII) and PKC.

tion of the NPB and it acts on molecules involved in cytoskeletal organization and transcriptional regulation, such as β-catenin. In the absence of ligand, β-catenin is phosphorylated and marked for degradation by glycogen synthase kinase 3 (GSK3). However, in the presence of the Wnt ligand, it binds to the Frizzled (Fzd) / Lrp receptor, which activates the Disheveled (DSH) protein and prevents the formation of the degradation complex. This leads to the stabilization of β-catenin, which is then transferred to the nucleus. Together with T-cell factor/lymphoid enhancer factor (TCF/LEF) proteins, β-catenin activates genes transcription involved in the NPB formation (Fig. 3) (Steinhart and Angers, 2018, Ali *et al.*, 2021).

One of the canonical Wnt signaling pathways is the planar cell polarity (PCP) pathway, which triggers asymmetric cytoskeletal organization (Humphries and Mlodzik, 2018). Therefore, the Wnt/PCP pathway is crucial in regulating tissue patterns and cell migration. When Wnt binds to Fzd receptors, it can recruit DSH to the cell membrane, resulting in the formation of a complex with Dvl-associated activator of morphogenesis 1 (Daam1) (Mayor and Theveneau, 2014). Following the formation of the complex, Rho GTPases become activated and subsequently lead to the activation of Rho-associated kinase (Rock) in this pathway. This, in turn, affects the arrangement of the cytoskeleton and cell migration (Ridley, 2011). The non-canonical Wnt-cGMP/Ca2+ signaling pathway is another pathway that can affect the amount of Ca2+ inside the cell. When Wnt binds to Fzd, this mechanism leads to the production of IP3 and DAG inside the cell. IP3 triggers the endoplasmic reticulum (ER) to release Ca2+. This increased Ca2+ secretion results in the release of DAG, which then activates protein kinase C (PKC) and calcium/calmodulin-dependent protein kinase II (CamKII). Ultimately, these factors stimulate transcription factors inside the nucleus (De, 2011).

Several *in vivo* studies have shown that the expression of canonical ligand genes Wnt1 and Wnt3a occurs in the NPB and the dorsal neural tube (as depicted in Fig. 5A). Elimination of the expression of these genes results in the lack of NPB formation and defects in the differentiation and formation of neural crest cells. Furthermore, these studies have reported that retinoic acid (RA) plays a significant role in the expression of Wnt3a (Ribes *et al.*, 2009). Similar results have also shown that deleting enzymes of the RA synthesis pathway has a direct impact on the expression of Wnt3a and reduces the expression of NPB determinants, such as Msx1 and Pax3 (Duester, 2008, Ribes *et al.*, 2009). Unlike Wnt1 and Wnt3a, Wnt8 is expressed in the paraxial mesoderm, as opposed to the NPB (Fig. 5A).

In *Xenopus*, *Fgf8* is the most important inducing factor for Wnt8 expression and NPB formation in paraxial mesoderm (Fig. 5B) (Hong *et al.*, 2008). When the Wnt/β-catenin signaling pathway is activated, it triggers the activation of a transcription factor called *Tcf7l1*. If *Tcf7l1* fails to bind with β-catenin, then the NPB formation (Msx1) is not formed, and instead neural crest induction (*Snai2*, and *Sox9*) occurs (Heeg-Truesdell and LaBonne, 2006). Gbx2 is a transcription factor involved in Wnt/β-catenin pathway. expression and functional defect in *Tcf7l1* negatively activates Gbx2 (Li *et al.*, 2009). Gbx2 plays a crucial role in the development of neural

folds and NPB by affecting the expression of *Zic1*, *Pax3*, and *Msx1*. The activity level of *Gbx2* determines whether neural crest cells will differentiate into pre-placodal cells or not, by influencing the expression of *Zic1* (Li *et al*., 2009).

Studies conducted *in vivo* have shown that conventional Wnt/β-catenin signaling is involved in the expression of *Apoc1*, which plays a role in the formation and translocation of lipids (Bolanos-Garcia and Miguel, 2003). Also, another recent result showed that *Apoc1* protein levels were affected by the expression of genes such as *Msx1*, *Pax3*, and *Zic1*, which caused impairment in the formation of the NPB. Additionally, genes like *c-Myc*, *Sox9*, *Snai2*, *Twist1*, and *Id3* affected the formation of neural crest cells, resulting in its disruption (Yokota *et al*., 2017). In addition, Wnt signaling also affects *Sp5* (a transcription factor). *Fgf8a* or Wnt8 signals can induce neural crest formation through *Sp5* (Park *et al*., 2013). *Sp5* controls the progression of NPB formation by directly affecting *Msx1* and *Pax3*, and induces neural crest cells by affecting *Zic1* (Park *et al*., 2013). In addition to its other functions, Wnt signals have an impact on various kinases, including *Awp1*. Furthermore, they regulate the expression of several genes, such as *Msx1*, *Pax3*, *Sox10*, and *Snai2* that play a crucial role in the formation and induction of the NPB and neural crest (Seo *et al*., 2013).

Research in the field of NPB formation has demonstrated the significant role played by non-conventional Wnt signaling. *In vivo* experiments have revealed that the expression of genes related to the formation of NPB and neural crest is facilitated by the involvement of *Dvl* and *Ror2* in conjunction with Wnt5a and Wnt11. The function of *Par1* in the expression of genes *Sox8*, *Foxd3*, and

Fig. 5. Neural plate border formation under the influence of Wnt signaling. During gastrulation, the formation of the neural plate border is under the control of canonical (*Wnt1* and *Wnt3a*, *Wnt8*) and non-canonical (*Wnt5a* and *Wnt11*) Wnts secreted from the neural plate border and paraxial mesoderm.

Snai2, which are involved in neural crest development, has been established. The effect of Wnt5/Wnt11 signaling on *Par1* triggers a series of enzymatic reactions that ultimately control *Pax3* expression (Ossipova and Sokol, 2011). *Ror2* plays a crucial role in determining the polarity of neuroectodermal cells and forming the NPB. If *Ror2* is not functioning properly, it can lead to a decrease in the expression of genes involved in the formation of NPB and neural crest (Schille *et al*., 2016).

BMP signaling pathway

BMP molecules play a crucial role in various developmental processes and belong to TGF-β family. When BMP secretory proteins bind to their receptors, they cause the activation of transcription factors like *Smad1/5/8* through phosphorylation. It has been proven that BMP signaling is involved in the development and its defective function leads to several abnormalities and diseases (Liu *et al*., 2023). The formation of neural crest cells has been shown to be influenced by BMP signaling. Several hypotheses have been put forth to explain how BMP works, including the gradient model. In this model, different levels of BMP concentration lead to the formation of different cells. Specifically, high concentrations of BMP lead to the formation of the epidermis, medium concentrations lead to the formation of the neural crest, and low concentrations lead to the formation of the neural plate (as shown in Fig. 5A). Another hypothesis suggests that the attenuation of BMP signaling can trigger neural crest formation by creating a competence zone with the help of Wnt and FGF signaling (Steventon *et al*., 2009). In the next stage of development, BMP signaling is responsible for the expression of NPB and neural crest genes in the corresponding region. Recent studies have revealed that molecules such as *SNW1* are responsible for maintaining BMP levels. This molecule acts upstream of BMP signaling in the NPB and restricts the scope of BMP activity in this area (Wu *et al*., 2011). In addition, a recent report has shown that *CKIP-1*/*Smurf1* modulates the precise level of phospho-*Smad1/5/8* to induce neural crest cells at the NPB (Piacentino and Bronner, 2018).

Special NPB genes, namely *Msx1*, *Msx2*, *Dlx5*, and Dlx6 genes are expressed under the control of BMP. It's interesting to note that BMP concentration level for induction the expression of these genes is also different, which means that on the lateral side of the NPB, its high concentration causes the expression of dlx3 compared to other genes in this region (Tríbulo *et al*., 2003). In other investigations, it has been proven that the expression of *tfap2a* in the NPB is dependent on BMP concentration. (Nordin and LaBonne, 2014). Several *in vitro* studies have also demonstrated that BMP is required for the formation of the NPB (Wu *et al*., 2011).

The role of fibroblast growth factor in the formation of neural plate border

The FGF signaling pathway has a significant impact on various differentiation, migration, and patterning processes (Dorey and Amaya, 2010). Studies have shown that mesoderm is the main target of the FGF signaling pathway in the induction of neural crest cells (Monsoro-Burq *et al*., 2003). *In vivo* studies have revealed the occurrence of BMPs, FGF and Wnts signaling pathways in the lateral epiblast, while the FGF signaling pathway is present only in the medial epiblast (Wilson *et al*., 2001). In the early stages of gastrulation, FGFs, and Chordin are expressed in the primitive node, while Wnts and FGFs are expressed in the primitive streak (Chapman *et al*., 2004). During gastrulation, NPB is determined by FGFs which originate from paraxial mesoderm (Streit and Stern, 1999). It has been reported that disrupting FGF (dnFgfr1/Mkp3) signaling in the NPB region during gastrulation inhibits *Pax7* and *Snai2* expression (Stuhlmiller and García-Castro, 2012b). The results obtained suggest that FGF/MAPK signaling plays a direct role in the development of NPB cells. However, it is important to note that the independent role of mesoderm in FGF signaling cannot be overlooked, as *FGFR1/4* is only expressed in the NPB and not in the mesoderm (Stuhlmiller and García-Castro, 2012b). In confirmation of these findings, a recently published report indicates that the expression of specific neural crest genes (*Pax7* and *Sox10*) does not occur by FGF inhibition (Betters *et al*., 2018). In another recently published report, it has been demonstrated that FGF affects the transcription factor *Foxg* in the anterior NPB regions, leading to the development of placodes and telencephalon (Liu and Satou, 2019).

The role of Notch signaling in the formation of neural plate border

As a result of the Notch receptor binding to its ligand, a series of proteolytic reactions takes place within the cell. Once this proteolytic cascade is activated, the Notch intracellular domain (NICD) is released. Ultimately, NICD is transported to the nucleus where it functions as a transcription factor (Kopan and Ilagan, 2009). *In vivo* studies have shown that Notch signaling in the BMP4 upstream induces neural crest cells during gastrulation (Endo *et al*., 2002). In addition, recent studies have revealed that the activation of Notch signaling pathway is critical in the formation of neural crest cells, but is only limited to certain regions along the neural tube (Hernandez-Lagunas *et al*., 2011). However, the role of Notch signaling in the formation of the NPB remains unclear in many species. Nonetheless, it is known that Notch signaling influences various processes such as growth, differentiation, and migration of nerve cells. During the early stages of NPB formation, Notch signaling affects the expression of hairy2. It is required for the development of neural crest cells in the initial and final stages of gastrulation. However, for the formation of the NPB, hairy2 expression depends on BMP, FGF, and Wnt factors but independent of Notch (Cornell and Eisen, 2005).

Conclusion

Despite numerous studies over the past few decades, there is still a need for further research to uncover convincing answers to questions regarding the formation of the NPB and provide a clear understanding of how progenitor cells differentiate, form and migrate from this region. The lack of detailed descriptions of the processes involved in this phenomenon could be attributed to the one-dimensional nature of studies on transcription, gene regulation, and molecular pathways at the cellular level. Comprehensive analysis of all the factors that affect different parts of the NPB and the interactions between cells and adjacent tissues is essential to answer these questions. It is important to note that there is a close relationship between Wnt signals, FGF, BMP signaling, Notch, and many other factors. These factors affect

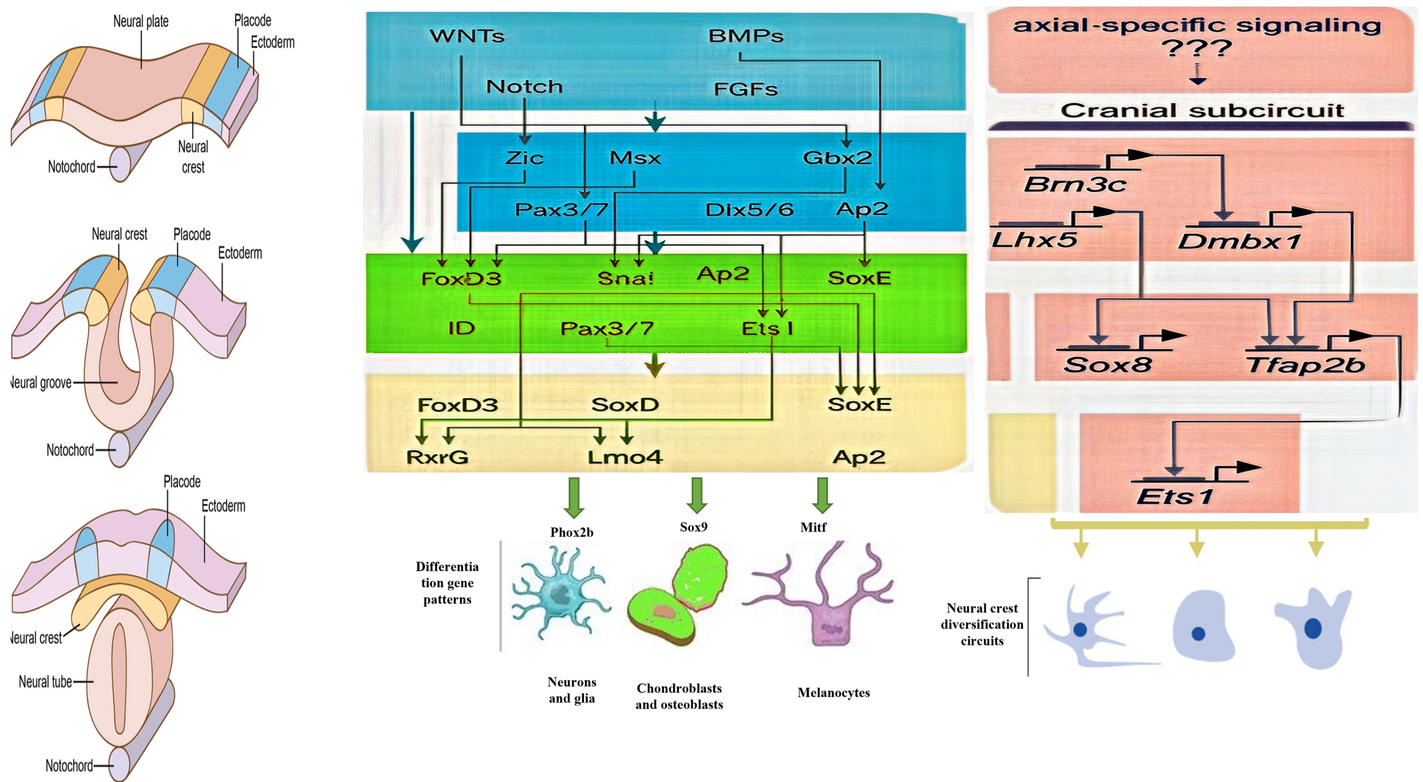

Fig. 6. The outline of a gene regulatory network (GRN) that controls the formation of neural crest cells. During embryonic development, various inductive signals pattern the embryonic ectoderm and induce the expression of neural plate border specifier genes. These genes work together to define the neural plate border territory, and also drive neural crest specification by activating the neural crest specifier genes. This neural crest specification program leads to the activation of the EMT machinery, which allows the neural crest cells to become migratory. The migratory neural crest cells express specific regulators that provide them with motility and the ability to initiate various differentiation programs.

the activation or repression of genes and molecular cascades in this field (Fig. 6). Additionally, time, place, and concentration of these factors play an important role in the nearby structures. By studying these pathways and factors and their interactions with adjacent cells and structures, we can improve our understanding and potentially develop better treatments for diseases originated from defect in NPB development.

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Conflicts of interest

The authors declare no conflict of interest.

Competing interests

The authors declare that no competing interests exist.

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