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INSIGHTS INTO VERTEBRATE HEAD DEVELOPMENT: FROM CRANIAL NEURAL CREST TO THE MODELLING OF NEUROCRISTOPATHIES

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Abstract

Although the vertebrate head has evolved to a wide collection of adaptive shapes, the fundamental signalling pathways and cellular events that outline the head skeleton have proven to be highly conserved. This conservation suggests that major morphological differences are due to changes in differentiation and morphogenetic programs downstream of a well-maintained developmental prepattern. Here we provide a brief examination of the mechanisms and pathways responsible for vertebrate head development, as well as an overview of the animal models suitable for studying face development. Besides, we describe the criteria for neurocristopathies classification, highlighting the contribution of zebrafish to the modelling of Treacher Collins/Franceschetti Syndrome, an emblematic neurocristopathy. The contributions from our laboratory reveal that proper zebrafish head development depends on the fine-tuning of developmental-gene expression mediated by nucleic acid binding proteins able to regulate the DNA conformation and / or neuroepithelium redox state.

1. Introduction

Vertebrates are the most species-rich and geographically dispersed deuterostomes on the Earth. This is likely due to the advantages provided by the evolution of key innovations such as a bony skull and jaws, vitals for a predatory lifestyle. In the most primitive vertebrates, the presence of pharyngeal pumping favored early development success by simply increasing the rates of respiration and filter feeding (Gans and Northcutt, 1983). Then, the evolution of the specialized structures facilitated the shift from passive to active feeding behaviors, thus enabling the extraordinary radiation of the vertebrate lineage (Hall, 2000). Although the vertebrate head has evolved to a wide collection of adaptive structures for respiration, feeding, communication, and sensing the environment, the fundamental signaling pathways and cellular events that shape the head skeleton in the embryo have proven to be highly conserved. This conservation suggests that major morphological differences are due to changes in differentiation and morphogenetic programs downstream of a well-maintained developmental patterning.

Much of the skull and the entire pharyngeal skeleton derive from the cranial neural crest (cNC). Cranial neural crest cells (cNCCs) delaminate from the dorsal neural tube and migrate ventrolaterally to form the ectomesenchyme of facial primordia known as the frontonasal prominence (FNP) and pharyngeal arches (PAs; also referred to as branchial arches in aquatic species). Induction of the NC occurs at the neural plate border (NPB) via a signaling interaction between neural and non-neural ectoderm. After their specification, NC precursors reside within the elevating neural folds and dorsal neural tube until its closure. NCCs then undergo an epithelial to

mesenchymal transition (EMT) and migrate ventrally from the neuroepithelium to distant sites throughout the embryo, often traveling great distances before reaching their destination and differentiating into a variety of derivatives (Martik and Bronner, 2017; Mayor and Theveneau, 2013). cNCC migration occurs as three topographically conserved streams as proceed towards the pharynx: pre-oral and PA1 cells in the first stream, PA2 cells in the second stream, and pharyngeal arch (PAs 3+) cells in the third stream (Figure 1). The cNCC-negative regions (between the streams) are consistently situated beneath rhombomeres 3 and 5 of the brain (Theveneau and Mayor, 2012). Each uniquely stream is molecularly defined by *hox* gene expression (see below). In addition, studies from multiple model organisms have revealed that cNCC migration is regulated by a variety of repulsive signals and chemo-attractants (Halloran and Berndt, 2003; Mayor and Theveneau, 2013).

Subsequently, the FNP becomes the mid- and upper face, while the first PA (PA1) develops into most of the jaw, the lateral skull, palate, and the middle ear. PA1 is further divided into maxillary arch (prospective upper jaw) on the proximal half and mandibular arch (prospective lower jaw) on the distal half. The second PA (PA2) mainly contributes to the ear and neck skeleton (reviewed in Parada and Chai, 2015). Alterations in the establishment and/or maintenance of specific developmental domains of cNCC leads to craniofacial pathologies collectively classified into neurocristopathies (NCP) (Bolande, 1997).

Focusing on the cNC, the aim of the present article is to review our current understanding of the vertebrate face development.

2. Gene expression control during rostral head development

2.1. Gene regulatory network (GRN) governing cNCC differentiation

Comparative analyses performed on numerous animal models and using various experimental methodologies (tissue transplants, *in situ* hybridizations, gain - and loss-of-function) allowed identifying the GRNs governing the progressive and conserved steps that NCCs undergo during development – specification, EMT/delamination, migration, and differentiation. Data enabled to build a classical sequential model consisting of a hierarchical series of circuits contributing to the various stages of NC development. According to this model, NCCs activate only one of many alternative cell fate programs. Indeed, the action of *Fgf*, *Wnt, Notch*, and *Bmp* specify the NPB between non-neural and neural ectoderm, and leads to the expression of NPB-specifier genes, such as *Pax3/7, Tfap2, Msx,* among others. The expression of NPB-specifier genes induces the expression of a set of NC-specifier genes (*Snail1/2*, *Foxd3*, *Twist, Sox5/6, Pax3/7, Ets1, Myc, Myb, Id, Tfap2,* and *Sox9/10*) in the NPB, which then promote EMT and migration (*Ebf1* and *RxrG* expression start to be detected) (reviewed in Martik and Bronner, 2017). Cranial-specific regulators acting in a

hierarchical pathway has been recently involved in establishing cNC identity in avian embryos. At the top of this pathway is *Brn3c*, which is necessary for the activation of *Dmbx1* in the anterior NPB. Subsequently, *Lhx5* and *Dmbx1* drive the expression of *Tfap2b* and *Sox8* in the dorsal neural folds. Finally, *Tfap2b* activates the expression of *Ets1* as the NC becomes specified (Simoes-Costa and Bronner, 2016). In zebrafish, *lhx5* and *dmbx1* are expressed in the early cNCC. In addition, *sox8b*, *sox10*, *tfap2a*, and *ets1*, but not *pou4f3* (the ortholog of *brn3c* in zebrafish) are expressed in premigratory and migratory cNCC at all axial levels. Noteworthy, this cranial-specific GRN is absent from other NC subpopulations and sufficient to provide to the cNCC with its unique potential to differentiate into the craniofacial skeleton of vertebrates (Martik and Bronner, 2017). Lastly, cNCC diversifies in chondrocytes by the expression of *Sox9*, *Sox5/6* and *Col2a1*. *Sox9* regulates cartilage formation by binding and activating the chondrocyte specific enhancer of the collagen type II (*Col2a1*) gene (Lefebvre *et al.*, 1997), thus promoting differentiation of the undifferentiated mesenchymal cells into chondrocytes (Akiyama and Lefebvre, 2011).

More recently, single-cell analysis combined with spatial transcriptomics of murine NCCs enabled the identification of substages of EMT during trunk NCC delamination (Soldatov et al. 2019). Data showed that pre-EMT NCCs express genes associated with NPB and neural tube identity, which are then down-regulated in more advanced cells allowing the up-regulation of NC specific genes. Besides, Soldatov et al. (2019) showed that migrating NCCs undergo a series of sequential binary fate restrictions and spatiotemporal segregation that involves initial coactivation of bipotential properties followed by gradual shifts toward commitment. The first bifurcation separates progenitors of the sensory lineage from those of autonomic and mesenchymal fates. Then, additional binary decisions separate autonomic neuronal fate from mesenchymal differentiation. Therefore, cellular fate is defined by the internal (autonomous activation of genes) and external (signals from neighboring cells) events that progenitors have experienced. According to this new model, progenitor cells may initially co-activate more than one gene expression program depending on their own history, thus leading to mutually exclusive and competitive cellular fates. Soldatov et al. (2019) also showed that, after delamination, a neuronal program is activated in the trunk, whereas cNCCs acquire ectomesenchyme potential upon activation of the transcription factor Twist1. Indeed, sustained overexpression of *Twist1*, normally activated upon delamination only in the cranial compartment, is sufficient to define the mesenchymal potential of migrating NCC and the subsequent cNCC differentiation (Soldatov *et al.*, 2019).

2.2. Transcription factors expressed in the pharynx and oral regions

cNCCs entering the pharynx and oral region are exposed to a range of intercellular signals, including Endothelin 1 (End1), Sonic Hedgehog (Shh), BMPs, and Fgfs, which regulate proliferation,

differentiation, and morphogenesis (Figure 1). Edn1-induced signaling through the Endothelin type-A receptor (Ednra) is crucial for cNCC patterning within the mandibular portion of the PA1, from which the lower jaw arises. cNCCs express Ednra whereas Edn1 expression is limited to the overlying ectoderm, core paraxial mesoderm, and endoderm of the mandibular arch. Deletion of Edn1, Ednra or endothelin-converting enzyme in mice causes the homeotic transformation of mandibular arch-derived structures into more maxillary-like structures. End1/Ednra signaling induces the expression of *Dlx5* and *Dlx6* (see below) and the consequent dorso/ventral identity of the PA1 (Clouthier *et al.*, 2010). NCCs themselves do not express *Shh;* however*, Shh* signal from the pharyngeal endoderm provides the cNCC with information about the size, shape, and orientation of the skeletal elements that will eventually form from the PAs. Shh signaling from the craniofacial ectoderm is also involved in patterning the outgrowth and development of the facial primordia. At early stages, *Shh* expression from the forebrain acts on the cNCC, which then induce *Shh* expression in the frontonasal ectoderm zone, regulating proximodistal and dorsoventral patterning in the craniofacial complex (Abramyan, 2019). The BMP signaling pathway is an important regulator in the shaping of the skeletal system, patterning the NC and craniofacial development. BMP2/BMP4, which can be secreted from cNCCs, binds to BMP receptor types I and II. This binding further activates the intracellular Smads phosphorylation and translocation into the nucleus, thus triggering bone-related gene expression, such as *Msx2* (Chen *et al.*, 2020). When early Fgf signals are lost, the endodermal pouches of the PA fail to form, and then, the pharyngeal cartilages are reduced or absent. Later, Fgf signals from the pharyngeal endoderm are required for induction and survival of chondrogenic precursors. cNCCs differentiate into collagen-containing cellular cartilage and related skeletal tissues by activating the expression of a core set of transcription factors that appear to drive skeletal differentiation in all cNCCs (Figure 1), including *SoxE*, *Twist*, and *Ets* (Meulemans and Bronner-Fraser, 2004). The conserved expression of these factors in all modern vertebrates suggests they mark evolutionarily conserved subpopulations of skeletal precursors present in their most recent common ancestor. However, how these genes confer regional shape and morphology still remains unclear (see Square *et al.*, 2017 for details).

During craniofacial development, *Dlx* family genes are regionally expressed within PAs conferring dorsal-ventral positional identity (Figure 1). At mid-pharyngula stages, *dlx* genes appear in nested PA expression domains (Depew *et al.*, 2002; Square *et al.*, 2017), which are established immediately after cNCCs stop their migration. Thus, *Dlx1/2* are expressed in both prospective upper and lower jaw territories, whereas *Dlx5/6* are expressed in prospective lower jaw only. *Dlx3/4* expression is further restricted to a narrow domain within the prospective lower jaw territory. *Dlx1/2* and *5*/*6* act partially redundantly and antagonistically, depending on the context, to achieve differential expression of their downstream genes in prospective upper and lower domains (Jeong

et al., 2008). *Dlx5/6* are not only required for lower jaw patterning, but also for the dorsal nasal capsule (Gitton *et al.*, 2011).

2.3. *Hox expression in the PAs confers specific positional identities to cNCCs*

Differential *Hox* expression confers NCCs antero-posterior axis identity. cNCCs migrating in stream 2 (hyoid) are the most anterior NCC to express *Hox* genes; they arise from the *Hox-2* expressing region of the hindbrain. In contrast, cNCC in stream 1 (mandibular) arise from *Hox*-negative regions of the anterior hindbrain and midbrain (Figure 1). Loss of *Hox* group 2 gene function and overexpression of *Hox* genes in cNC of stream 1 result in homeotic transformations suggesting that the *Hox* expression status of cNC confers a subsequent positional identity, which is given by the cNC origin in the hindbrain (reviewed in Parker *et al.*, 2018). However, molecular regulation of *Hox*-2 gene expression in migrating cNCC is independent to that in the hindbrain (Maconochie *et al.*, 1999), indicating that cNCC final fate is not dictated simply by its hindbrain origin, but requires signals from adjacent tissues. An extensive and detailed review describing the role of *Hox*-genes in cNCC development has been recently published (Parker *et al.*, 2018). Authors have addressed outstanding questions relating the interactions between *Hox* regulatory pathways and the cNCC-GRN. Collected data led the authors to propose an auto-/cross-regulation between both the Hox-GRN and the cNCC-GRN (Parker *et al.*, 2018).

3. Epigenetic regulation of cNCC

Evidences of epigenetic control in cNCC development have been formerly well-reviewed (Hu *et al.*, 2014; Strobl-Mazzulla and Bronner, 2014). Therefore, we summarize the more relevant evidences reported beyond the publication of the mentioned reviews.

3.1. DNA methylation

Most of the evidences showing a role of DNA methylation during NC development came from the analysis of the consequences generated by varying the levels of DNA methyl-transferases (Dnmt), demethylases, or folate (the precursor of *S*-adenosylmethionine; Beaudin and Stover, 2007).

Mutations in human Dnmt3B was found linked to craniofacial defects (Jin *et al.*, 2008) and, zebrafish Dnmt3 was reported participating in a specific histone methyltransferase network responsible for the silencing of critical regulators of cNCC fate (Rai *et al.*, 2010). In chicken, Dnmt3A and B participate in NCC early determination and timing by methylating CpG located into regulatory regions of specific NC genes, such as *Sox2/3* (Hu *et al.*, 2012) and *Sox10* (Hu *et al.*, 2014).

FolR1 and *Rfc1*, two of the main folate transporters, are robustly expressed in the neural tube and NCC, and their knockdown results in profound orofacial defects. Abrogation of either folate uptake or metabolism affects DNA methylation on the *Sox2* locus in the dorsal neural tube at the expense of NC marker expression. This finding suggests that DNA methylation restricts *Sox2* expression in the dorsal neural tube, allowing the acquisition of NC identity and preventing neural fate on the dorsal neural tube (Alata Jimenez *et al.*, 2018).

3.2. Histone modifications

3.2.1 *Histone methylation-demethylation*

Different approaches (including comparative histone methylated marks studies, specific inhibition, knock-down or deletion of methylases, demethylases or folate transporters) have shown that dynamic histone methylation is critical for proper temporal control of gene expression in the cNC.

Early postmigratory NCC subpopulations contributing to distinct craniofacial structures display similar chromatin accessibility patterns, but present differential transcriptional activities. Accessible promoters and enhancers of differentially silenced genes carry H3K27me3/H3K4me2 bivalent chromatin marks embedded in large Polycomb repressive domains. As H3K27me3 antagonizes H3K4me2 deposition at Polycomb domains, the regulatory elements and promoters of positional genes would switch from a poised to an active or inactive chromatin state, thus contributing to establish NCC subpopulation–specific transcriptional identities (Minoux *et al.*, 2017).

FolR1 and *Rfc1* knocked-down in chicken embryos also show a reduction of the level of H3K4me3, H3K9me3, H3K27me3, and H3K36me3 epigenetic marks in the neural tube and NC territory, indicating that folate-dependent H3 methylation is required for proper NC formation and the normal orofacial formation (Alata Jimenez *et al.*, 2018).

Mice carrying a conditional deletion of the H3K9 methyltransferase G9A in NCC display incomplete ossification and 20% shorter jaws. G9A inhibition up-regulates *Twist1* and *Twist2*, likely by removing repressive H3K9me2 marks catalyzed by G9A in regulatory regions of *Twist* genes (Higashihori *et al.*, 2017).

In zebrafish, depletion of *prdm3* or *prdm16,* two members of the Positive regulatory domain histone methyltransferase family, causes hypoplasia of the craniofacial cartilage elements, undefined posterior ceratobranchials, and decreased mineralization of the parasphenoid. In mice, while loss of *Prdm3* in the early embryo causes mid-gestation lethality, loss of *Prdm16* causes anterior mandibular hypoplasia, clefting in the secondary palate, and severe middle ear defects (Shull *et al.*, 2020).

Finally, histone demethylation was reported playing a role in craniofacial development. Indeed, in *X. laevis* developing embryos, depletion of *kdm3a,* which specifically demethylates mono and di methylated H3K9, produces head deformities, small‑sized eyes and abnormal pigmentation (Lee *et al.*, 2019). In chicken, loss of the histone demethylase JumonjiD2A (JmjD2A/KDM4A), which is expressed in the forming neural folds, causes dramatic downregulation of *Snail2* and *Sox10*, two typical NC-specifier genes (Strobl-Mazzulla *et al.*, 2010).

3.2.2. Histone acetylation

Chromatin modifications via modulating histone acetylation by means of histone deacetylases (HDACs) and histone acetyl transferases (HATs) activities have an essential role in several steps of NC development.

In zebrafish, *hdac4* knocked-down embryos exhibit loss of cNC derived palatal skeletal precursor cells, which results in defects in the developing palate (Delaurier *et al.*, 2012); besides, CRISPR/Cas9 *hdac4*-mutant shows a significant increase in pharyngeal ceratohyal cartilages ossification (DeLaurier *et al.*, 2019). Precocious cartilage ossification was also reported in *Hdac4*-mutant mice (DeLaurier *et al.*, 2019). In human, *HDAC4* haploinsufficiency was associated with brachydactyly mental retardation syndrome (Williams *et al.*, 2010), single nucleotide polymorphisms in the *HDAC4* gene were linked to nonsyndromic oral clefts (Park *et al.*, 2006), and inhibition of HDAC4 during pregnancy was shown to increase the chances of generating cleft lip and palate (Wyszynski *et al.*, 2005).

The HAT zebrafish *kat6a* rescues the aberrant *hox* patterning, histone hypoacetylation, and ectopic ceratobranchial formation caused by nitric oxide synthase inhibitor 1-(2-[trifluoromethyl] phenyl) imidazole (Kong *et al.*, 2014). Single and double *kat2a* and *kat2b* zebrafish mutants display an overall shortening of craniofacial cartilages and a disruption of the posterior ceratobranchial cartilage pattern. Similarly, *Kat2a* mutant mice show defects in the craniofacial skeleton, including hypoplastic bone and cartilage along with altered expression of typical cartilage marker genes (Sen *et al.*, 2018).

3.3. Chromatin structure

Chromatin architecture is regulated in NCC by several components of chromatin-remodeling complexes. The chromodomain helicase DNA-binding domain *CHD7* (Vissers *et al.*, 2004) and Williams syndrome transcription factor (*WSTF*) (Lu *et al.*, 1998) genes were associated with CHARGE and Williams syndromes, respectively, both characterized by typical craniofacial malformations. Haploinsufficiency in *Brg1*, one of the catalytic subunits of chromatin-remodeler SWI/SNF complex, affects neural tube closure and results in peri-natal mice lethality (Smith-Roe and Bultman, 2013).

Zebrafish *brg1* mutants display a cluster of NCC-related defects, including abnormal jaw skeleton differentiation.

Apart from the classical B-form, non-B (non-canonical) DNA structures may form depending on specific sequence motifs, DNA modification state, or interactions with proteins or RNAs. Among the non-B DNA structures, G-quadruplexes (G4s) outstand as a stable intramolecular secondary structure formed in G-rich single-stranded DNA. G4s are highly associated to regulatory and nucleosome-depleted chromatin regions and co-localize with active genes (Hänsel-Hertsch *et al.*, 2016). G4s may affect transcriptional activity through two different way of actions: i) altering *per se* the structure in nucleosome-depleted chromatin (Armas *et al.*, 2017); ii) anchoring different proteins involved in epigenetic processes (Varizhuk *et al.*, 2019). In zebrafish, the presence of conserved G4s in the proximal promoter regions of *col2a1* and *nog3* enhances transcription; in agreement, the abrogation of G4-folding leads to aberrant craniofacial phenotypes (Armas and Calcaterra, 2018; David *et al.*, 2016). *Nog3* expression is repressed by CNBP, a protein capable of binding and unfolding G4s structures (David *et al.*, 2019). Depletion of CNBP adversely affects craniofacial development in chicken, mice, and zebrafish (Calcaterra *et al.*, 2010; Sdrigotti *et al.*, 2017; Weiner *et al.*, 2011), suggesting that G4s are novel epigenetic elements involved in cNCC development.

3.4. *microRNAs (miRNAs)*

Many miRNAs have been identified as key players in different developmental stages of craniofacial structures. However, this information is still scattered and scarce, making it difficult to generalize the participation of particular miRNAs in specific GRNs. In many cases, although differentially expressed miRNAs have been identified, the molecular targets have not yet been found. Extensive studies regarding the role of miRNAs in NC development and facial morphogenesis have been recently published (Tavares *et al.*, 2015; Weiner, 2018); here we summarized the main evidences reported so far.

3.4.1. Data from depletion of Dicer

MiRNA biogenesis involves several stages catalyzed by different specific enzymes, among which the RNase III endonuclease Dicer plays a fundamental role. Therefore, the analysis of Dicer mutants and/or morphants has provided relevant evidence for miRNAs function in craniofacial morphogenesis. In mice, *Dicer*-deleted gene under the control of *Col2a1* promoter display severe skeletal defects and premature death due to progressive reduction in chondrocyte proliferation and precocious differentiation to hypertrophic chondrocytes (Kobayashi *et al.*, 2008). In *Dicer* conditional knockout through *Pax2*-Cre-driver line, secondary palatal development becomes

arrested prior to mineralization and apoptotic markers are overexpressed. MiR-101b, miR-140, and miR-145 are significantly downregulated in these *Dicer* mutant mice (Barritt *et al.*, 2012). In *Xenopus*, *Dicer* knock-down results in a severe cranial cartilage malformations (Gessert *et al.*, 2010). Significant shortening of ceratohyal, hyosymplectic-palatoquadrate, and Meckel cartilages was observed in zebrafish Dicer mutant and morphant larvae (Weiner *et al.*, 2019).

3.4.2. *Data collected from the study of specific miRNA families in mice*

Knockouts for the miR-17~92 family show expanded *Tbx1* and *Tbx3* expression in craniofacial structures (Wang *et al.*, 2013) and hypoplasia of most skull bones, including reduced ossification and cleft palate phenocopying abnormalities observed in Feingold syndrome patients (Tassano *et al.*, 2013). Aberrations in the Tgfb signaling by interactions between Tgfbr2 and miR-17~92 may explain the cleft-palate phenotype (Ries *et al.*, 2017).

Studies in pre-osteoblast cell culture evidence that miR-141 and miR-200a target *Dlx5* (Itoh *et al.*, 2009). The miR-452 targets *Wnt5a*, which down-regulates Shh signaling and indirectly promotes *Dlx2* expression in the neighboring cNCC-derived mesenchyme. The knockdown of miR-452, thus, down-regulates *Dlx2* expression in the PA1 (Sheehy *et al.*, 2010).

A high-throughput miRNA sequencing study carried out in developing-facial structures allows detecting hundreds of miRNAs differentially expressed. Among them, miR-23b and miR-133b were suggested as required for proper craniofacial development (Ding *et al.*, 2016).

The murine calvaria have several membrane bones with different tissue origins (NC derived frontal bone vs. mesoderm-derived parietal bone). In a recent small RNA deep sequencing study, a total of 83 differentially expressed miRNAs in frontal bones vs. parietal bones have been identified, which may count for the difference in osteogenic capacities of both tissues (Chen *et al.*, 2019).

3.4.3. Data from other experimental models

At least 170 differentially expressed miRNAs have been found by next-generation sequencing and computational annotation approaches, showing a remarkably dynamic regulation of miRNA expression during chicken, duck, and quail cNCC before and after species-specific facial distinctions take place. Data suggest that differential proliferation rates can influence the depth, width, and curvature of the beak, being miRNAs involved in the different cellular transitions (Powder *et al.*, 2012).

In *Xenopus*, the depletion of miR-96, miR-196a, and miR-200b results in abnormal cranial cartilage structures (Gessert *et al.*, 2010). miR-96 represses *Tbx1* expression and Tbx1 represses miR-96 (Gao *et al.*, 2015), probably working in a regulatory loop during cNCC differentiation.

In zebrafish, the BMP-miR-17-92 cluster pathway mentioned above also plays a role in cartilage differentiation. MiR-92a knock-down leads to disruption of cartilage morphogenesis by binding to the mRNA encoding the Bmp inhibitor *nog3* (Ning *et al.*, 2013). The knock-down of miR-27 causes severe defects in the neurocranium by impaired proliferation and differentiation of chondrogenic progenitors (Kara *et al.*, 2017). MiR-27 targets the focal adhesion kinase Ptk2aa, a key regulator in integrin-mediated extracellular matrix adhesion proposed to function as a negative regulator of chondrogenesis (Kara *et al.*, 2017). MiR-140 was found to directly downregulate the *pdfgra* expression, a gene required for cNCC migration and differentiation (Eberhart *et al.*, 2008). Targeted deletion of the miR-199/214 cluster leads to severe skeletal problems in axial and craniofacial structures (Watanabe *et al.*, 2008). Pri-miR-199-3a and pri-miR-214a are enriched in the mesenchyme surrounding the developing craniofacial structures during zebrafish development (Desvignes *et al.*, 2014).

Although great advances in the field of epigenetic regulation of NC development have been achieved, knowledge about how epigenetic mechanisms work individually and in groups to fine-tune the spatio-temporal expression of critical NC-specifier genes is still incomplete.

4. Animal experimental models for studying cNC development

Much of the evidence for gene functions in head skeletal development comes from gene molecular association underlying human craniofacial defects (Watt and Trainor, 2014). However, vertebrate animal models offer a vital platform for understanding key processes during craniofacial development, providing generally consistent genetic backgrounds, multiple replicates, and extensive information concerning their embryology. Moreover, advances in genomics and bioinformatics have accelerated the identification of genes controlling craniofacial development, as well as regulatory processes that go awry in disease in a broad spectrum of vertebrate species. Data generated in such studies, whether in a particular model or in combination of models, approach the understanding of the mechanisms involved in face morphogenesis and the etiology of associated diseases.

For many years, the avian and *Xenopus* models have been widely used mainly due to the size of the egg, the ease of handling the embryo, simplicity of live cell imaging, and the conserved genetic pathways with mammals. The avian model also has the possibility of performing graft/transplantation experiments (Le Douarin, 2012). However, both models display relatively long generation times and the inconvenience of performing genetic studies, transgenic, or even the no fusion of secondary palate in the case of chicken or absence of palate in the case of *Xenopus* (Van Otterloo *et al.*, 2016).

The mouse has been used for decades as an important model for studying gene function during face development. It is closely related to human and display similar morphogenesis, contains highly conserved cis-regulatory elements, is accessible to CRISPRs gene-editing and to powerful forward and reverse genetics (Van Otterloo *et al.*, 2016). For example, genetics enabled to address the stage-dependent fate mapping of NCC and their derivatives or fate mapping *in vivo* single cell tracing using inducible forms of Cre-recombinase in Cre-loxP-based conditional genetic recombination approaches (Baggiolini *et al.*, 2015; Kaucka *et al.*, 2016; Soldatov *et al.*, 2019). Apart from confirming in mice many of the findings obtained by fate mapping of avian NCC, genetic lineage tracing of murine NCC led to the identification of minor NC-derived cell populations present in tissues of non-NC origin, to the establishment of novel lineage trees, and to the demonstration of *in vivo* multipotency of single premigratory and migratory NCC (Debbache *et al.*, 2018). In addition, robust strategies for generating null or conditional mice knockouts have been developed. Many mutants exhibit aberrant craniofacial phenotypes, leading to important discoveries linked to human craniofacial malformations (Watt and Trainor, 2014). Nevertheless, the mouse model has the disadvantage of the in-utero development, which makes difficult assessing the earliest embryonic stages.

Despite the relatively short time that zebrafish has been used for the study of craniofacial morphogenesis, the combination of genetics and embryology afforded by the zebrafish embryo has led to many insights into the mechanisms that pattern the early craniofacial skeleton. The strengths of the zebrafish complement genetic studies in mice and embryological studies in chicken. Zebrafish form essentially all of the same skeletal and muscle tissue types as their higher vertebrate counterparts, but in much more simple spatial patterns composed of smaller cell numbers. Cartilage development is particularly rapid, and by five days post-fertilization most of the cartilages of the pharyngeal skeleton are well-formed (Schilling and Kimmel, 1994) and can be easily visualized by Alcian Blue staining (Schilling and Webb, 2007). A Plug-in for ImageJ has been developed to quantify the consequences of gene expression variations (Rosas *et al.*, 2019; Weiner *et al.*, 2019), drug treatments (Cedron *et al.*, 2020), or any other experimental approach designed for assessing changes in craniofacial cartilage pattern (Figure 2).

Nowadays, CRISPR-Cas9 gene edition is an efficient and robust technique used in zebrafish (Liu *et al.*, 2019). Zebrafish embryos are particularly well-suited for mutagenesis screenings based simply on visual inspection via a dissecting microscope, allowing for powerful *in vivo* analysis of gene function. Mutant screenings allowed the identification of new players in cell signaling during face morphogenesis (Jayasena and Bronner, 2012; Yelick and Schilling, 2002), leading to detailed GRN that subsequently informed human clinical data (Yelick and Schilling, 2002). Besides, various transgenic reporter lines allowed the visualization of NCC and craniofacial lineages at different

stages of differentiation in zebrafish embryos/larvae. Confocal microscopy images or time-lapse movies allow identifying the molecular and cellular basis of craniofacial morphogenesis and disease (McGurk *et al.*, 2014; Schilling *et al.*, 2010).

Of course, some of the assets of the zebrafish model can also be disadvantages. The small size of embryos and larvae that can be a benefit for some experiments (large number of individuals in small places) can make it difficult to collect adequate amounts of tissue for others. Similarly, the number of zebrafish-compatible biologicals (e.g. antibodies) and the utility of zebrafish cell culture and transplantation are limited compared with those for other animal models. While facilitating experiments involving water-soluble drug administration, the opposite is also true and water insoluble materials are difficult to introduce in fish media (Gut *et al.*, 2017).

The mouse has been typically considered the best model for both studying human development and modeling human diseases. However, it is not clear whether differences between mouse and other species reflect true generalities for mammals or are peculiarities of mouse development. Therefore, the use of different complementary models could broaden the knowledge of the molecular bases and processes governing the normal and pathological development of the vertebrate rostral head.

5. Neurocristopathies (NCP)

NCP are a class of pathologies occurring mainly in humans that result from the abnormal specification, migration, differentiation or death of NCC during embryonic development. The term was proposed by Robert P. Bolande in 1974 (Bolande, 1974), who highlighted aberrant NC development as a “common denominator” of a large set of human pathologies.

5.1. NCP classifications

Bolande initially divided NCP in two main categories: Simple and Complex and NCP syndromes (Bolande, 1974). This former classification was then further subdivided based on clinical assessments (Bolande, 1997). Besides, a classification according to NC affected process (Etchevers *et al.*, 2006) and, more recently, another based on the axial origin of the affected NC population (Vega-Lopez *et al.*, 2018) have been proposed (Figure 3A). Interestingly, Vega-Lopez et al. considered that many NCP are due to anomalous development not only of the NC, but also of the adjacent tissues. Authors also suggested that epigenetic mechanisms ruling NCCs development play a role in NCP establishment. Ciliopathies (pathologies affecting the assembly of the primary cilia; Vega-Lopez *et al*. 2018) and environmental factors (comprehensively discussed in Cerrizuela *et al.*, 2020) have been recently reported influencing NC development and, thus, contributing to NCP.

5.2. Modelling NCP in zebrafish: the TCS experience

TCS is a genetic condition characterized by bilateral facial features, such as malar and mandibular hypoplasia, downward-slanting palpebral fissures, coloboma of the lower lid, microtia, and it often is associated with conductive hearing loss (Watt and Trainor, 2014). The prevalence is estimated to be between 1 in 10,000-50,000 individuals in the general population. However, some mildly affected individuals may go undiagnosed, making it difficult to determine the disorder's true frequency in the population (https://rarediseases.org/rare-diseases/treacher-collins-syndrome/). A remarkable feature of TCS is the inter- and intra-family variation in phenotype severity, which reasons are not clear yet (Watt and Trainor, 2014).

Approximately 80% of TCS patients have a mutation in the *TCOF1* gene, with an autosomal dominant inheritance. Mutations in *POLR1B, POLR1C*, and *POLR1D* occur in 10-15% of patients (Ghesh *et al.*, 2019; Sanchez *et al.*, 2019). Around 4% of cases remain with an unidentified molecular defect. Different mutations such as deletions, insertions, splicing, missense and nonsense mutations have been detected in both *TCOF1* and *POLR1* genes (Splendore *et al.*, 2005; Teber *et al.*, 2004; Vincent *et al.*, 2016). Nonetheless, no clear correlation between a specific type of mutation and the resulting TCS phenotype has yet been described (Ghesh *et al.*, 2019; Splendore *et al.*, 2005; Vincent *et al.*, 2016). The overall information regarding TCS molecular features was gained by modelling the NCP in mice and zebrafish. In both cases, aberrant craniofacial phenotypes are due to a deficit of rRNAs synthesised by RNA pol I (Jones *et al.*, 2008; Lau *et al.*, 2016; Noack Watt *et al.*, 2016; Porcel De Peralta *et al.*, 2016; Sanchez *et al.*, 2019). The nucleolar stress triggered by deficient ribosomal biogenesis leads to extensive p53-mediated apoptosis in the neuroepithelium at the cNC formation stage (Jones *et al.*, 2008; Noack Watt *et al.*, 2016; Porcel De Peralta *et al.*, 2016; Sanchez *et al.*, 2019; Weiner *et al.*, 2012). It seems that molecular pathogenic mechanisms underlying TCS are similar and can be well-assessed in both zebrafish and mice. In addition, zebrafish has the advantage of allowing assessing non-invasive and investigational treatments in an easy and economic manner (Figure 3B).

5.3. TCS & zebrafish: looking for prevention

Zebrafish is not only an excellent model to study craniofacial genetics but, once a disease model is stablished, it also helps to assay therapies and strategies to ameliorate disease (Widrick *et al.*, 2019). Below we describe some approaches regarding TCS performed using zebrafish.

5.3.1. *P53 inhibitors.*

Researchers are exploring ways to inhibit p53 function or block the mechanisms leading to p53 activation as possible therapeutic treatments to prevent the development of TCS. Studies in the *Tcof1*$^{+/-}$ C57BL/6 mice indicate that intraperitoneal administration to pregnant females for three consecutive days (from E6.5 to E8.5) of pifithrin-alpha (PFT-α, a small synthetic p53 inhibitor that is used in neuroscience to block neuronal apoptotic cell death; Zhu *et al.*, 2002), reduces neuroepithelial cell–specific apoptosis (Jones *et al.*, 2008). Similar results were obtained by injecting specific p53-MO in TCS1-like zebrafish embryos and in TCS3-like fish in a *tp53*^{M241K} background (Lau *et al.*, 2016; Porcel De Peralta *et al.*, 2016). Evidence based on pharmacological and genetic experiments indicates that temporary suppression of p53 by PFT-α and genetic silencing does not increase the frequency of cancer (Gudkov and Komarova, 2010). However, the use of p53 inhibitors for the treatment of p53-related pathologies may raise a safety concern because an increased risk of tumour development is observed in mice and humans with p53 deficiency (Donehower *et al.*, 1992). There are no currently available FDA-approved p53 clinical uses of PFT-α (or analogues) as potential agent for the treatment of TCS. Zebrafish is especially suited for performing research to determine the doses, therapeutic windows, long-term safety, and effectiveness of such approaches.

5.3.2. *Antioxidant therapy.*

Recent advances in TCS-like mice indicate that dietary antioxidant supplementation protect NCCs against damage during embryogenesis and facilitate normal craniofacial development (Noack Watt *et al.*, 2016). Similar results were obtained in a TCS1-like zebrafish model when embryos developed in the presence of N-acetylcysteine (Porcel De Peralta *et al.*, 2016). Again, zebrafish is excellent for checking antioxidant supplements (Nayak *et al.*, 2018).

5.3.3. *Proteasome inhibition.*

As mentioned above, CNBP plays a role in forebrain and craniofacial development likely by controlling gene expression through G4-unfolding (Calcaterra *et al.*, 2010; David et al.,2019; Weiner *et al.*, 2011). CNBP is degraded through the proteasomal pathway and its over-expression prevents TCS-like phenotypes in zebrafish (Porcel De Peralta *et al.*, 2016). Treatment of TCS-like zebrafish embryos with proteasome inhibitors MG132 and Bortezomib (Velcade®, Millennium laboratories) abrogates CNBP degradation, attenuates neuroepithelial cell death and cell redox imbalance, and produces a robust craniofacial cartilage phenotype recovery (Rosas *et al.*, 2019). Therefore, proteasome inhibitors, which are approved for multiple myeloma and mantle cell lymphoma treatments (de Bettignies and Coux, 2010), may offer an opportunity for TCS molecular and

phenotypic manifestation's prevention. Although further development of new safe inhibitors compatible with administration during pregnancy is required, results suggest additional mechanisms operating in TCS pathogenesis and also encourage the testing of proteasome inhibitors in other TCS animal models.

6. Concluding remarks

Numerous studies conducted in different experimental models have contributed to the identification of specific genes and GRNs together with epigenetic mechanisms underlying cNCC determination, migration, and differentiation that are central to head development. In this context, the contributions from our laboratory reveal that proper zebrafish head development depends on the fine-tuning of developmental-gene expression mediated by nucleic acid binding proteins able to regulate the DNA conformation (David et al., 2016; David et al., 2019) and / or neuroepithelium redox state (Porcel de Peralta et al., 2016; Gil Rosas et al., 2019). Both the knock-down and overexpression of such proteins generate craniofacial abnormalities mainly due to aberrant craniofacial cartilages development (Sdrigotti el al., 2017; Weiner et al., 2007; Weiner et al., 2011), mimicking typical craniofacial phenotypes observed in TCS-patients (Weiner et al., 2012; Porcel de Peralta et al., 2016; Rosas et al., 2019).

Recently, both the identification of novel cNC-specific transcription factors using ChIP-Seq and RNA-Seq approaches and open enhancers through ATAC-Seq screenings has contributed to partially deciphering the cNC GRN complexity. Future researches should point to new technologies, mainly those ones based on the “omics” and the bioinformatic expertise, aimed to study entire organismal changes at every level from pre-transcriptional to post-translational regulation.

Although divergent in some specific aspects, data generated from different vertebrate models have contributed to broadly comprehend the normal processes of head development and to elucidate the aberrant processes responsible for numerous NCP. Understanding the etiology and pathogenesis of individual conditions and knowing whether they arise due to defects in cNCC determination, migration, and/or differentiation will be instrumental in designing realistic avenues for therapeutic NCP prevention.

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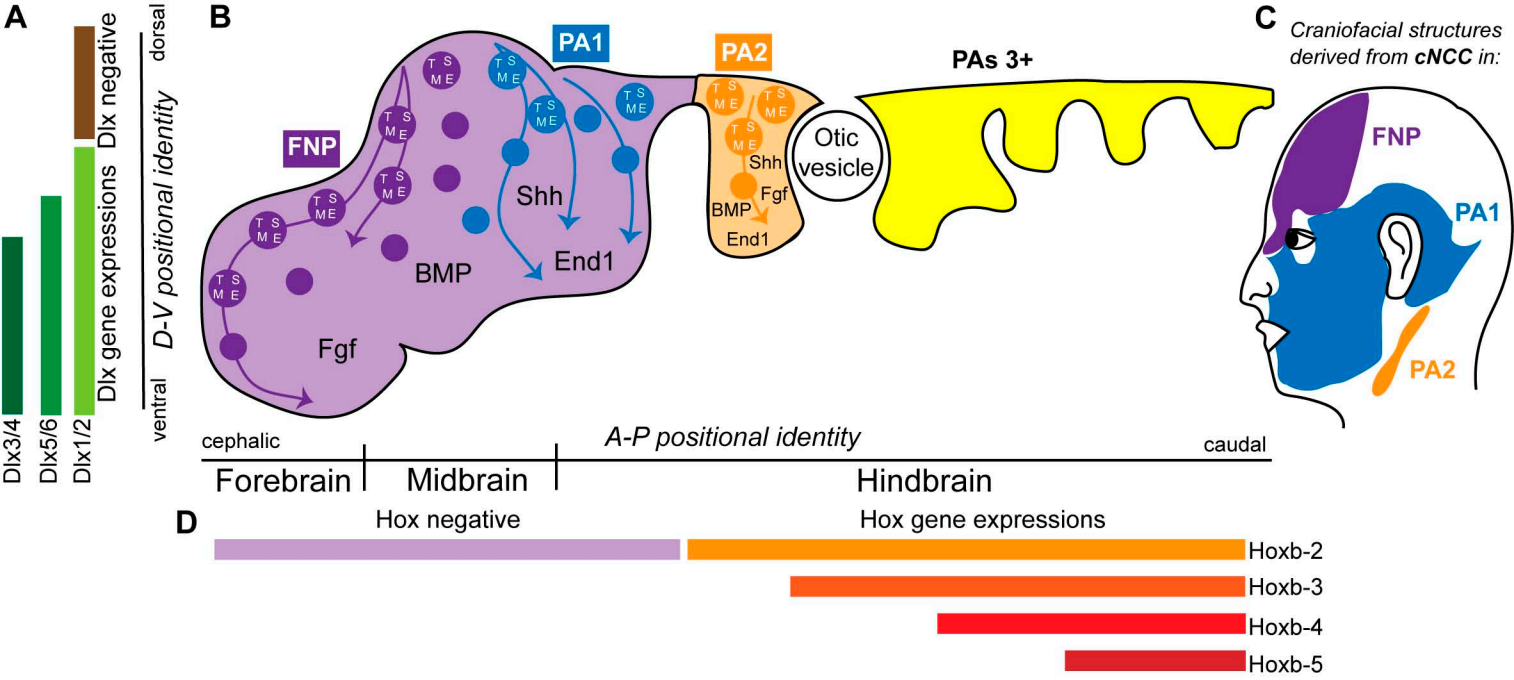
Legends

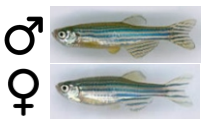
Figure 1. Segmental and directional migration of cNCCs in a representative vertebrate embryo. (A) *Dlx* dorso-ventral gene expression pattern displayed by green and brown color codes. (B) Colored arrows represent the patterns of migration of cNCCs into the frontonasal process (FNP; violet code) and pharyngeal arches 1 (PA1; light-blue code) and 2 (PA2; orange code). Migrating cNCCs express *SoxE* (S), *Twist* (T), *Ets* (E), and *Msx2* (M). The action of *End1*, *Shh*, *BMP*, and *Fgf* pathways in the FNP, PA1, and PA2 surrounding tissues regulate proliferation, differentiation, and morphogenesis in the pharynx and oral regions. (C) Craniofacial derivatives from the different cNCCs sub-populations in the human skeleton. (D) *Hox* antero-posterior gene expression pattern displayed by violet and orange-red color codes.

Figure 2. Sequential strategy for craniofacial cartilage phenotype assessing in developing zebrafish. Early fertilized embryos injected with different kind of molecules (Morpholino, CRISPR/Cas, ASO or others) or incubated in the presence of drugs are allowed to develop until 5-6 dpf. Images of Alcian Blue stained larval head cartilages are digitalized by using ImageJ software (NIH) equipped with a Plug-in allowing simultaneously assessing the lengths and angles of selected cartilages; e.g., Meckel length (ML), width of the arch formed by the Meckel cartilages; Meckel area (MA), area of the inner triangle defined by the Meckel cartilage; CeA, angle defined by ceratohyal cartilages; Ce, length of the ceratohyal cartilages; PQ, length of palatoquadrate+hyosymplectic cartilages; ceratohyal distance (CeD), distance between ceratohyal cartilages joint and lateral fins;

and cranial distance (CrD), distance between the most anterior Meckel and lateral fins. Quantitative data is provided in a data sheet ready for graphical presentation and statistical analysis (schematically shown for two parameters PQ and MA where C, control; T, treated).

Figure 3. Classification and experimental models to evaluate potential therapeutic strategies for the treatment of neurocristopathies. A) Neurocristopathies classifications according to different criteria. B) Rational pathway for the analysis of potential therapeutics for human uncharacterized craniofacial pathologies using lab experimental model animals. In the presence of uncharacterized craniofacial pathology, clinical evidences and genetic tests often suggest a link between specific genes and the disease. Experimental approaches carried out in developing animals allow not only to establish the role of these genes under normal conditions, but also to identify the mechanisms responsible for the disease. Identifying these therapeutic targets facilitates the development of pre-clinical therapeutic strategies (which can be tested in experimental models) or even clinical strategies to be tested in patients.


A
Dlx negative
Dlx gene expressions
Dlx3/4
Dlx5/6
Dlx1/2
dorsal
D-V positional identity
ventral
B
PA1
PA2
FNP
Shh
BMP
End1
Fgf
Otic vesicle
PAs 3+
C
Craniofacial structures derived from cNCC in:
FNP
PA1
PA2
cephalic
A-P positional identity
caudal
Forebrain
Midbrain
Hindbrain
D
Hox negative
Hox gene expressions
Hoxb-2
Hoxb-3
Hoxb-4
Hoxb-5

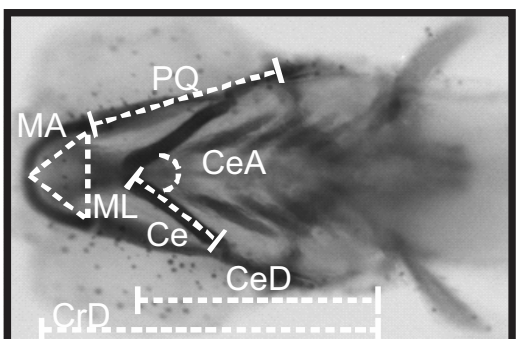


FERTILIZED EMBRYOS

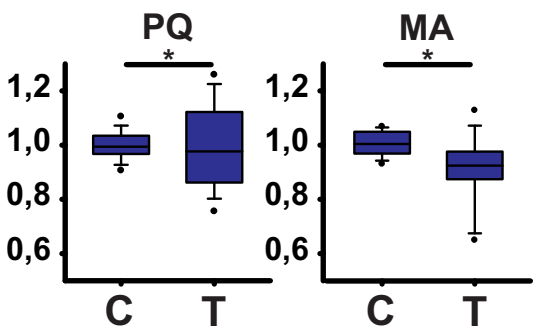
EMBRYO TREATMENT (INJECTION/INCUBATION/OTHER)

LARVAE GROWING & ALCIAN BLUE STAINING

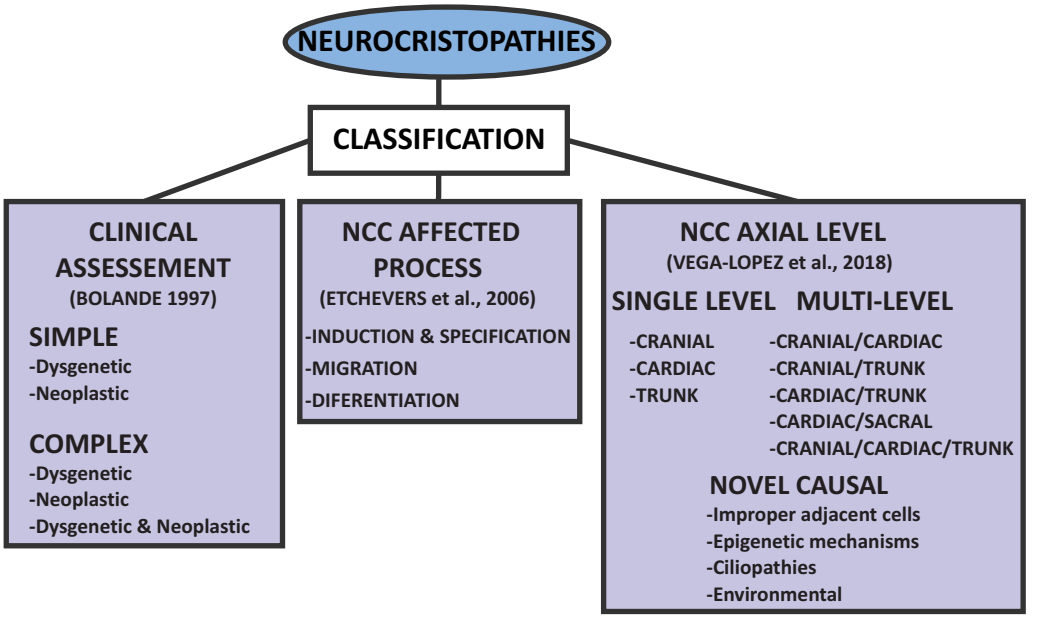
IMAGE ADQUISITION & PROCESSING (ImageJ+PLUG-IN)



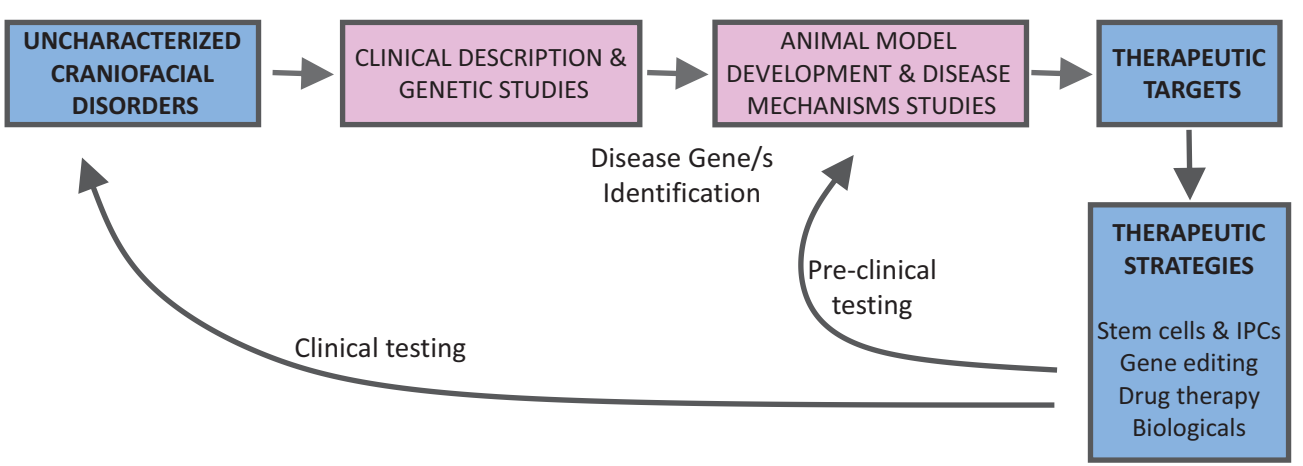
DATA ANALYSIS



A


NEUROCRISTOPATHIES
CLASSIFICATION
CLINICAL ASSESSEMENT
(BOLANDE 1997)
SIMPLE
-Dysgenetic
-Neoplastic
COMPLEX
-Dysgenetic
-Neoplastic
-Dysgenetic & Neoplastic
NCC AFFECTED PROCESS
(ETCHEVERS et al., 2006)
-INDUCTION & SPECIFICATION
-MIGRATION
-DIFERENTIATION
NCC AXIAL LEVEL
(VEGA-LOPEZ et al., 2018)
SINGLE LEVEL
-CRANIAL
-CARDIAC
-TRUNK
MULTI-LEVEL
-CRANIAL/CARDIAC
-CRANIAL/TRUNK
-CARDIAC/TRUNK
-CARDIAC/SACRAL
-CRANIAL/CARDIAC/TRUNK
NOVEL CAUSAL
-Improper adjacent cells
-Epigenetic mechanisms
-Ciliopathies
-Environmental

B


UNCHARACTERIZED CRANIOFACIAL DISORDERS
CLINICAL DESCRIPTION & GENETIC STUDIES
ANIMAL MODEL DEVELOPMENT & DISEASE MECHANISMS STUDIES
THERAPEUTIC TARGETS
Disease Gene/s Identification
THERAPEUTIC STRATEGIES
Stem cells & IPCs
Gene editing
Drug therapy
Biologicals
Pre-clinical testing
Clinical testing