Review Article

The master of craniofacial orchestra: Homeobox genes and neural crest cells

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Abstract

The role of neural crest cells and the homeobox genes in the development of craniofacial development has been a topic of research which is now directing us towards a genetic control in the patterning of the craniofacial region. These genes with their genetic domination over the development and the patterning of the head and facial region, in fact, are found to be the regulators and thus the master genes of the head and face. This review article gives an overview of the role of neural crest cells and homeobox genes in the development of the craniofacial complex.

Key words

Neural crest cells, Homeobox genes, Craniofacial development.

Introduction

With the drastic development in the field of craniofacial biology, we have been able to understand in-depth of the craniofacial development in much more detail than ever before. With the understand of the significance of the neural crest cells and genes activity for the proper development of the head and facial region of the vertebrate it is now possible to study how the particular genes act on their natural environment, there phenotypic expression and the consequences if their expression is blocked.

The role of the neural crest

First described by His on a chick embryo, neural crest cells are defined as a transient population of embryonic cells that originate from the dorsal aspect of the neural tube and migrate through the trunk and head in vertebrates to form a diverse set of cell types. Neural crest cells, the highly pluripotent cell population arising from the

surface ectoderm between the border of neural plate and epidermis, migrate extensively in a rostrocaudal sequence to produce different structures throughout the body. Before the migration can commence, the cells must undergo an event known as epithelial to mesenchymal transformation [1]. Epithelial cells are characterized by their arrangement in a single layer of cells with clearly defined apical and basal sides whereas mesenchymal cells are characterized by their arrangement and less uniform shape. The neural crest can be divided into four functional domains [2]. They are:

a. Cranial neural crest (CNC) – Gives rise to various structures of chondrocranium. These cells migrate dorsolaterally to produce the craniofacial mesenchyme that differentiates into the cartilage, bone, cranial neurons, glia, and connective tissues of the face. These cells enter the pharyngeal arches and pouches to give rise to thymic cells, odontoblasts of the tooth primordia, and the bones of middle ear and jaws.
b. Trunk neural crest – gives rise to pigment synthesizing melanocytes and dorsal root ganglion containing sensory neurons.
c. Vagal and sacral neural crest – giving rise to parasympathetic ganglia of gut.
d. Cardiac neural crest- giving rise to melaonocytes, neurons and connective tissue.

Cells from the lateral border of the crest of the neuroectoderm dissociate to form a cell population called the neural crest cells. In mammals, neural crest cells are formed during neuralation when cells at the margins of the neural folds undergo an epithelial to mesenchymal transition following an inductive interaction between neural plate and presumptive ectoderm [3]. During craniofacial development, neural crest cells especially CNC cells migrate ventrolaterally as they populate the branchial arches. Cell labeling studies have demonstrated the formation of CNC cells from rhombomeres 1 to 4, which are also involved in the formation of posterior midbrain and anterior hind brain. These cells migrate into first branchial arch and thereafter reside within maxillary and mandibular prominences. The migration of these rhombencephalic crest cells may be regulated by growth factor signaling pathways and their downstream transcription factors before they become committed to different cell types. Facial components arise from the coordination of a variety of morphogenetic events which includes cell migration and extracellular matrix remodelling, proliferation and differentiation of neural crest derived mesenchyme into skeletal and connective tissues, the assembly of musculature and the beginning stages of organogenesis [4]. It should be noted that the facial mesenchyme is derived principally from the neural crest cells and not from the embryonic third germ cells which is responsible for the development of most of the other parts of the body [3].

Migration of the neural crest cells

The neural crest cells migrate away from the neural tube and begin to migrate throughout the embryo and require two proteins - 'RhoB' and 'Slug'. 'RhoB' is important as it establishes cytoskeletal conditions that promote migration while 'Slug' activates the factors that dissociate the tight junctions between cells'. Unlike other cells in which the cell migration occurs in sheets, the migration of the neural crest cells occurs individually and generally occurs in three stages [5]:

- **Initiation:** The neural crest cells undergo an epithelial to mesenchymal transition causing the cells to break free from the neural tube and in this process adhesion connection between the neural crest cells mediated by molecules such as N-CAM, N-Cadherin and E Cadherin are down regulated. Simultaneously there is an increase in the junction between the neural crest and the extracellular matrix. This happens due to an increase in a protein integrin in the cell surface. The amount of the

intercellular space increases and finally the neural crest cells become more motile.

- **Dispersion:** The neural crest cells migrate through the extracellular matrix to reach its final destination where they proliferate and develop.
- **Cessation of migration:** Occurs as a reverse of initiation of migration. The adhesive molecules i.e. N-CAM, N-Cadherin and E-Cadherin are re-expressed. The amount of extracellular matrix is decreased and thus the migration is reduced.

The neural crest cells take either the ventral pathway or the dorsolateral pathway. The cells arising from the cranial or cephalic neural crest cell migrate dorsolaterlly to produce the craniofacial mesenchyme which gets differentiates into the cartilage, bone, cranial neurons, glia and connective tissues [3, 6]. The pathways taken by the neural crest cells leaving the hindbrain are:

- First stream consisting of mandibular arch crest migrate into mandibular arch.
- Second stream consist of hyoid arch crest and migrate to second pharyngeal arch that contain supporting elements for orofacial structures.
- Third stream consisting of branchial arch crest will get subdivided and populates the five most posterior pharyngeal arches.

The role of Homeobox genes

Homeobox genes were discovered independently by Walter J Gehring in 1983 working at the University of Basel, Switzerland and Matthew Scott and Amy Weiner who were working at Indiana University Bloomington. Homeobox is a 180 base pairs long DNA sequence found within genes that are involved in the morphogenesis in animals, plants and fungi. The homeobox encodes a 60-amino acid helix loop DNA binding within an encoded transcription factor. The region of the protein is the homeodomain and act as transcription factors that activate or inhibit the transcription of other genes [2]. Edward Lewis was the first person to identify the homeotic genes in the fly Drosophila melanogaster, which help in controlling the developmental response of groups of cells along the body's antero-posterior axis. In animals, these genes are regarded as 'master genes' of head and face with a prominent control over patterning, induction, programmed cell death and epithelial-mesenchymal interaction during craniofacial development. In the fly, the homeotic genes are predominantly clustered in two regions-Antennapedia and Bithorax-on chromosome 3 which together make up a single HOM - C complex. The first vertebrate homeobox was cloned in frog Xenopus Levis and was soon followed by cloning in mouse. The vertebrate genes are called HOX genes and consist of 39 genes both in human and mouse. These are arranged in four clusters on four different chromosomes - HOX A, HOX B, HOX C, HOX D. The expression of these genes can be seen along the dorsal axis within the central nervous system from the anterior region of hindbrain through the length of spinal cord. As the neural crest cells migrate from the rhombomeres into specific branchial arches, it retains a specific Hox code, which specifies form and pattern of different derived regions of head and neck [7]. It is interesting to note that neural crest cells destined for first branchial arch does not express Hox genes related to homeotic homeobox but relies on its subfamilies [8]. The subfamilies of Hox genes, which are of particular interest in craniofacial patterning and morphogenesis include - muscle segment *(Msx)*, distal less *(Dlx)*, orthodenticle *(Otx)*, goosecoid *(Gsc)*, Bar class *{Barx)*, paired-related *{Prx*, SHOT) & LIM homeobox [2, 6]. A number of genes containing sequences coding for DNA binding domains homologous to homeobox sequences in Drosophila have been isolated in vertebrate and their mechanism of action has been studies. Hox family genes share with Drosophila homeotic genes a genomic organization in gene clusters and an expression pattern that is similar in a number of important aspects with the homeotic

genes DNA sequence showing that it shared more than 90% homology with the peptide sequences specified by the homeobox domain of Drosophilia homeotic gene [9]. The expressions of these genes are mediated through two main groups of regulatory proteins - Growth factor family and steroid/thyroid/retinoic acid super family. The vehicles through which Hox gene information is expressed for the regulation of the growth process include fibroblast growth factor (FGF), Transforming growth factor a & b (TGFa and TGFb) and bone Morphogenetic protein 4 (BMP 4). Mutations of fly homeobox genes can lead to bizarre homeotic transformations, where one segment can even assume the phenotype of other. Thus, the most complex part of CNC migration is the understanding of how the combinations of Hox genes are expressed to specify the fate of the cells.

Muscle segment (Msx)

The Msx homeobox gene (Human ANTP class NKL subclass) family plays a crucial role in the development of craniofacial development [6]. The vertebrate Msx genes were initially cloned from mice and were identified as homologous to Drosophila muscle segment homeobox gene (Msh) [9]. Three subtypes are present Msx 1, Msx 2 and Msx 3; in which Msx 1 and Msx 2 are expressed in craniofacial development including the brachial arches especially in the region of epithelial mesenchymal organogenesis including the developing teeth. In studies done in murine, it is found that Msx 1 and Msx 2 are detected in the development and formation of skull and meninges, digital aspects of the facial primordial, associated sense organs and the teeth [10, 11]. Both the Msx 1 and Msx 2 are expressed in the sutural mesenchyme and duramater but while the expression of Msx 1 continues at a higher level in the postnatal stages of skull morphogenesis as well the level of Msx 2 expression declines [11]. During the tooth development Msx 1 is expressed in the bud stage and in the morphogenetic cap stage. Msx 1 becomes localized in the mesenchymal cells of the dental follicle and the papilla and Msx 2 becomes more expressed in the enamel organ besides expressing in dental papilla and the follicles [12]. It is found that Msx 2 plays role in the expression in the formation of the extracellular matrix and ameloblast differentiation [13]. In the late stage of morphogenesis, Msx 1 expression is absent in root sheath epithelium indicating that Msx does not plays a role in root morphogenesis [14]. Msx 1 also plays an important role in the development of the palate specially the anterior portion of the palatal shelves [15]. Targeted gene disruption of *Msx1* in mice has shown to affect the shape of several membranous calvarial bones and chondrogenic craniofacial bones, both derived from first branchial arch. The effects can be seen as cleft palate associated with loss of palatine shelves in both maxillary and palatine bones, maxillary and mandibular hypoplasia and highly penetrant arrest of tooth formation at the bud stage of development. Wolf-Hirschhorn syndrome (WHS) is a congenital human syndrome resulting from a deletion of Msx1 locus on chromosome 4. It manifests as midline fusion defects, ear defects, supernumery teeth and microcephaly. It may also cause tooth agenesis, nail dysgenesis, mental retardation, cardiac defects and variety of skeletal deformities [16].

Distal-less (Dlx)

Distal-less genes (Human ANTP class NKL subclass) [6] as the name suggest requires for the development of the limbs. There are atleast six Dlx genes in humans and named as Dlx 1 to Dlx 6. Similar to *Msx, Dlx genes* are primarily expressed in regions that give rise to highly derived or vertebrate specific structures. *Dlx* genes are mainly expressed in branchial arches in complete spatio – temporal patterns. *Dlx1* and *Dlx2* are expressed throughout the first and second arches whereas expression of *Dlx3, Dlx5* and *Dlx6* are restricted to a more distal location. In contrast to the Msx genes, the expression of Dlx 1 and Dlx 2 in the maxillary and mandibular arch mesenchyme is restricted to the region where the future molar teeth will develop specially for the ectodermal and mesenchymal

compartments of the developing tooth [17, 18]. Experiments in mice have shown that inactivation of Dlx 5 and Dlx 6 genes results in craniofacial defects including the defect of calvaria (exencephaly), reduction in the size of the eyes, cleft and dysmorphogenesis of the nasal, maxillary and mandibular structures [19].

Msx-Dlx interaction

Msx and Dlx genes are those that are expressed early enough in the CNC cells to specify its differential fate as they populate the branchial arches and subsequently shape the skull and its associated sensory structures. It is interesting to note that *Msx* expression is restricted to cells that are proliferating or dying whereas *Dlx* expression is found in regions undergoing differentiation or are capable of doing so. Accordingly *Msx and Dlx* proteins appear to have opposing transcriptional properties - *Msx* proteins function as transcriptional repressers whereas *Dlx* proteins act as activators.

The various biological and cellular activities of both *Msx and Dlx* genes are mediated through the homeoproteins they encode, which can bind to specific DNA sequences. The preferred binding site for *Msx1, Msx2, Dlx3 and Dlx5* are essentially the same –the T- A- A- T sequence. However the competition for DNA binding site does not appear to represent primary mode of regulation of neural crest cells as Msx proteins repress transcription through protein - protein interaction mediated by the homeodomain. Although this process may occur, *Msx1* and *Msx2* each can form a protein complex with *Dlx2* and *Dlx5*and this heterodimer formation has a neutralizing effect on transcriptional activities of both the *Msx* and *Dlx* proteins [19].

Role of Msx - Dlx in Tooth Development

Msx and *Dlx* genes participate in tooth development by reciprocal epithelial-mesenchymal signaling. As the epithelium of the prospective oral cavity thickens to form the dental lamina, the expression of *Msx2* localizes. Activation of *Msx1, Msx2, Dlx1* and *Dlx2* in dental mesenchyme occurs in response to BMP4 and FGF signals from the overlying epithelium. The BMP4 mediated induction of *Msx1* expression and subsequent Msx dependent activation and maintenance of BMP4 expression in the dental mesenchyme are the key steps in conferring odontogenic potential to these tissues [12, 17].

The failure of tooth development to progress past the early bud stage in *Msx1 -I-* (mutant) mice and early arrest at laminar stage in the absence of both *Msx1* and *Msx2* emphasize the role of these genes in mediating signaling events. Mice lacking *Dix1* and *Dlx2* show no defects on tooth development but *Dix1 -/-', Dlx2-/-* compound mutant mice lacking molars indicate sensitivity to *Dix1* and *Dlx2* protein levels in a limited subset of teeth. In humans, a point mutation in *Msx1* homeobox results in agenesis of second premolars and third molars in affected individuals [18].

Goosecoid (Gsc)

Goosecoid (Human PRD class) encodes a protein that acts as a transcription factor and was previously isolated from Xenopus [6]. Experiments on mice with targeted deletion of Gsc genes led to many craniofacial defects. It has also been found that the Gsc genes have some sort of autonomous in its function [20]. In wild type mice, Gsc transcripts have been detected at later stages of development in the osteogenic mesenchyme of mandible and tympanic ringbone. Mutants exhibited a hypoplastic mandible with lack of coronoid and angular process along with several defects on other bones like maxilla, palatine bone and pterygoid plates.

Barx genes

Barx genes (Human ANTP class NKL subclass) consist of transcription factor that exhibits regionalized expression within the ectomesenchyme of the first branchial arch [6]. As tooth development proceeds, Barx expression becomes more localized exclusively to the mesenchymal regions around the developing

molars to produce specific folding pattern of the dental epithelium that produce molar cusps [12, 21]. They also play a role in the development of central nervous system and are expressed in the telencephalon, diencephalon, mesenchephalon, spinal cord and in the cranial and dorsal root ganglion. Barx1 and Barx2 show complementary patterns in their expression. Barx1 appears in the mesenchyme of the maxillary and the mandibular process where as Expression of Barx2 is most prominent in mantle layer, where post- mitotic neurons are located, the palatal floor and dorsal root ganglia, mutations of which can produce cleft of secondary palate [22] hence, the association of Barx1 with Barx2 in the possible etiology of cleft lip and palate [23].

Lim genes

Lim genes (Human LIM class) have been found to play an important role in the cell type specification and differentiation during embryogenesis [6]. These are found to be related with the expression of the ectomesenchyme of the maxillary and the mandibular process and also suggested to control patterning of the first brachial arch. Experiments have shown that homeodomain proteins of Lim genes are important for craniofacial development and patterning of mammalian dentition [24]. The role of *Lhx8* gene in palatal development has been showed and in homozygous mutant mice for *Lhx8* gene, the formation and elevation of palatal shelves appeared to proceed normally but failed to make contact and fuse. Thus cleft palate was a common finding in mice carrying mutations of Lhx8 genes and can be considered as a potential gene for human cleft palate also [25, 26].

Prx genes (Pair related gene)

Prx1 and *Prx2* are closely related members of *Prx* family of homeobox genes. At 9.5 days post coitum, *Prx1* is expressed in central nervous system derived mesenchyme of Fronto nasal process, first and second branchial arches and group of cells that form maxillary process. Its expression decreases once differentiation is initiated. *Prx1* in combination with *Prx2* is essential to stabilize and maintain cell fates in craniofacial mesenchyme [6]. Rat experiments have shown that the mutation of Prx1 and Prx2 genes caused defects in the external, middle and inner ear, reduction or loss of skull bones, a reduced or sometimes cleft mandible and limb abnormalities [27]. It has also been found that Prx1 and Prx2 coordinately regulate gene expression in cells that contribute to the distal aspects of the mandibular arch mesenchyme and that Prx1 and Prx2 play a role in the maintenance of cell fate within the craniofacial mesenchyme [28]. Another paired related homeobox gene - *SHOT*- has been described recently. Two different isomers of this gene- SHOTa and SHOTb were isolated and are considered homologous to human *SHOX* and mouse *OG-12* genes [6]. The transcription factors encoded by this gene are expressed in the developing aorta, female genitalia, diencephalon, nasal capsule, palate, eyelid and limb. *SHOT was* mapped to human chromosome 3q25-q26 and OG -12 with a syntenic region on chromosome 3. This chromosomal region is involved in development of Cornelia-de- hange syndrome characterized by mental retardation and microcephaly, cleft palate, abnormally situated eyelids, nose and ear deformities as well as heart and limb defects [29].

Other transcription factors

Sonic Hedgehog (Shh)

Shh, the vertebrate homologue *oiDrosophilia* hedgehog encodes a signaling peptide, which is involved in mediating, patterning for a number of well- characterized developmental signaling centers. In craniofacial development, *Shh* is first expressed in axial mesendoderm, mutations of which lead to abnormal patterning of neural plate resulting in holoprosencephaly and cyclopia [30]. Later in facial development, *Shh* is expressed in the ectoderm of frontonasal process (FNP) and maxillary process (MXP). Transient loss of these signals can produce collapse of the facial midline and hypotelorism. Disrupting *Shh* signaling in FNP and MXP leads to interruption in their outgrowth, resulting in clefting between the

primordia; cleft lip/palate [31, 32]. It has been shown that pharmacological doses of retinoids and cholesterol analogues induce facial dysmorphogenesis in part through their misregulation of *Shh* signaling. Humans with cholesterol metabolism disorders - Smith - Lemli-Optiz syndrome exhibit holoprosencephaly and micro-cephalic characteristics, which may result from an inability of target cells to respond appropriately to *Shh*. It has also been shown that *Shh* and proteins in *Shh* signaling pathways such as Gli1, BMP2 and Ptc play key roles in regulating patterned outgrowth of the FNP and MXP and specfying the mediolateral axis of the face.

Endothelin, dHAND and eHAND

The endothelin family of signaling peptides has been implicated in development and migration of neural crest cells. Appearance of marked craniofacial and cardiac abnormalities similar to those of CATCH -22 syndrome (Cardiac defects, Abnormal facial features, Thymic hypoplasia, Cleft palate, Hypocalcemia) which is associated with chromosome-22 deletion was clearly demonstrated in mice with null mutation of endothelin -1, the gene encoding protein endothelin 1(ET-1). The other two novel bHLH (basic helix-loop- helix) proteins - *dHAND* and *eHAND* are co-expressed with endothelin-1 in developing branchial arches, aortic arch arteries and cardiac mesoderm. In endothelin null embryos, both these proteins are down regulated resulting in hypoplasia of first and second branchial arches. *Msx1,* which is implicated in growth of branchial arches, was also found to be undetectable in the mesenchyme *of dHAND* null branchial arches, thus suggesting the regulatory role played by endothelin 1 in stimulating mesenchymal expression of *dHAND* thus regulating *Msx1* expression in growing distal branchial arch [33].

Fate of Neural Crest Cells

The individual neuroectodermal cells are multipotent which are imparted positional identity by the action of homeobox gene and other transcription factors. However, at later date this regulative capacity is lost leading to the cessation of migration of neural crest cells mainly through:

- Adhesion changes - Down regulation of certain integrins and re-expression of N-CAM, N - cadherin and E-cadherin.
- Decrease in intercellular space through decline in levels of hyaluronic acid.
- Decrease in extracellular material molecules such as fibronectin, reducing the availability of migratory substrate.

With time, the precursors become progressively restricted to form NCC derivatives and eventually to individual phenotypes.

Conclusion

Many human syndromes and genetic abnormalities have now been attributed to defects in individual genes, which lose its transcriptional ability, thus its control over neural crest cell migration. With the advancements in understanding the role of genes, it is now possible to explain the cause of craniofacial defects and there magnitude if a particular gene is missing. It is therefore of utmost importance for a clinician to have an understanding of the underlying genetic mechanism to facilitate proper diagnosis and therapeutic intervention.

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