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A ROLE OF GENES IN CRANIOFACIAL GROWTH

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ABSTRACT

With the advent in the field of craniofacial biology and with the discovery of the homeobox genes, many research and investigations have shown detailed genetic control for the patterning of the craniofacial region. The Hox gene subfamily important in respect to craniofacial development are – Msx, Dlx, Otx, Gsc, Barx, Prx, and Lim. Msx-1 and Msx-2 are expressed in the brachial arches especially in the region of epithelial mesenchymal organogenesis including the developing teeth and are detected in the development and formation of skull and meninges, the digital aspects of the facial primordial, the associated sense organs and the teeth. Both the Msx-1 and Msx-2 are expressed in the sutural mesenchyme and duramater and Msx-1 is expressed in the bud stage as well as in the morphogenetic cap stage during the tooth development. Dlx-l and Dlx-2 are expressed in dental mesenchyme, epithelium of the maxillary and mandibular arch mesenchyme. Dlx-5/6 are responsible for cartilage giving rise to the cranial floor (basioccipital, basisphenoid, and sphenoid) and frontonasal prominence. In vertebrates, Goosecoid (Gsc) is expressed transiently at the rostral end of the developing brain and then re-appears in many sites, including the mesenchyme of the branchial arches, nasal capsules and mandible. Barx expression is localized exclusively to the mesenchymal regions around the developing molars. Lim genes (Human LIM class) are 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. Prx-1 and Prx-2 coordinately regulate gene expression in cells that contribute to the distal aspects of the mandibular arch mesenchyme. Shh plays an important role in regulating the craniofacial morphogenesis. Pax-9 expression is restricted to prospective tooth mesenchyme at the bud stage, marking the sites of future tooth formation. It is now clear that the role of genes in the craniofacial development is immense. With future advancement in genetic engineering, genetic treatment of craniofacial defects cannot be ruled out. In ultimate analysis, it may prove that reach of the genes is much longer than human beings have ever envisaged.

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[I] INTRODUCTION

Rapid advances in molecular genetics and bioinformatics are providing valuable information related to craniofacial growth and its control. The discovery of homeobox genes has helped us to understand its role in establishment of body plan, pattern formation and morphogenesis. The key to the determination of etiology of craniofacial anamolies and its treatability lie in the ability to differentiate the effect of genes and environment on the craniofacial skeleton in a particular individual [1].

Genetic expressions are clearly predominant during embryonic craniofacial morphogenesis, but environment is also thought to influence craniofacial morphology postnatally, particularly during facial growth [2]. It is estimated that about two third of the human genes play a role in craniofacial development. Thus, the systematic review of various genes in the process of embryogenesis and development of maxilla, mandible and craniofacial region.

[II] ROLE OF GENES IN EMBRYOGENESIS

Several major processes are involved in development of embryo which includes axis specification, pattern formation and organogenesis. The genes responsible for normal development encodes many different products, including i) signaling molecules and there receptors, ii) DNA transcription factors, iii) component of extracellular matrix, enzymes, transport system and other proteins. Each of these genetic mediators is expressed in combination of spatially and temporally overlapping patterns [3].

During embryonic development, the face and neck are derived from swellings or buds of embryonic tissue, the branchial arches that originate bilaterally on the head. The neural crest cells differentiate into most of the skeletal and connective tissue structures of the craniofacial region, while the mesoderm forms the musculature and endothelial lining of arteries of the future face and neck. The establishment of pattern in the craniofacial region is partly determined by the axial origin of the neural crest cells within each arch and partly by regional epithelial mesenchymal interactions [4]. In the mouse embryo, cranial neural crest cells originate from the posterior midbrain-hindbrain regions and migrate ventrolaterally into the branchial

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arches [5, 6]. Within the branchial arches, the different populations of crest cells do not intermingle, but instead maintain the positional cues acquired by their rostral-caudal origins in the brain. This segregation of crest cell populations is established early in organogenesis by the apoptotic elimination of crest cells from specific levels of the hindbrain, giving rise to three distinct streams of migratory crest cells. Although this patterning of crest cells depends upon their rostral caudal origin, this pattern does show some level of plasticity [7, 8]. Viz, the knockout of Hoxa-2 in mice caused the second arch to produce skeletal elements normally found in the first arch. Thus, the Hox genes can specify pattern in arches caudal to the first arch, which does not express this class of genes [9]. Further patterning of the crest cells within the arches involves a reciprocal series of epithelial mesenchymal interactions mediated by several growth factor signaling pathways [10, 11] The mammalian face develops by the coordinated growth and differentiation of five facial primordia, the single medial frontonasal prominence, the paired maxillary prominences, and paired mandibular prominences, which are located around the primitive mouth or stomodeum. As development proceeds in the frontonasal prominence, localized thickenings of the surface ectoderm called nasal placodes develop. These placodes invaginate, while their margins thicken, to form the nasal pits and the lateral and medial nasal prominences. The maxillary prominences of the first branchial arch grow toward the future midline of the face. They fuse with the lateral nasal prominence on each side, then fuse with the medial nasal prominences, and finally with the intermaxillary segment of the frontonasal process to form the upper jaw and lip. In a similar way, the paired mandibular primordia fuse along their medial edge to form the lower jaw and lip. The frontonasal prominence forms the forehead and nose. Fusion of these approaching primordia results in the formation of a bilateral epithelial seam, which is later replaced by connective tissue giving rise to a confluent lip [12, 13]. Clefts of the upper lip occur as a result of the failure of the maxillary prominence to merge with the medial nasal prominences on one side (producing a unilateral cleft) or on both sides (producing bilateral clefts). Failure of fusion of the paired mandibular prominences occurs far less frequently and results in clefts of the lower lip and jaw [14].

The NCCs are a population of highly migratory multipotent precursors that arise at the junction between the prospective neural tube and epidermis during early stages of vertebrate development [15]. Whatever the initiating mechanism is, execution of a specific differentiation program within CNCCs is likely to require modulating the activity of transcriptional regulators. Several families of transcription factors are expressed in the ectomesenchyme, including Hox, Dlx, Msx, Hand, Pax, Prx, and Fox genes [16]. For inter-BA patterning, Hoxa-2 has been shown to be necessary (in mouse) and sufficient (in chick and frog) to confer the second BA as opposed to the first BA fate.

2.1. Role of Shh in craniofacial development

Facial abnormalities in human Shh mutants have implicated the Hedgehog (Hh) pathway in craniofacial development. Although the initial formation of branchial arches (BAs) is normal, expression of several Fox genes, specific targets of Hh signaling in cranial NCCs, is lost in the mutant. The spatially restricted expression of Fox genes suggests that they play an important role in BA patterning. Removing Hh signaling in NCCs also leads to increased apoptosis and decreased cell proliferation in the BAs, which results in facial truncation that is evident by embryonic day.

Shh is expressed in the epithelium of facial primordial [17]. Due to its well established roles in patterning of other organs [18]. Shh is a good candidate for one of the molecules that pattern the facial mesenchyme. Indeed, mutations in human Shh are responsible for a subset of cases of holoprosencephaly, the congenital defect characterized by forebrain and facial abnormalities. Loss-of-function approaches using function-blocking anti-Shh antibody and gain-of-function approaches by ectopic application of Shh protein have established the importance of Shh for survival and proliferation of ectomesenchyme cells. Adding or blocking the diffusible ligand may affect multiple tissues in the facial primordia, that is, the epithelium, mesodermal mesenchyme and the ectomesenchyme. Therefore, it remains unclear whether there is a direct requirement for Hh signaling within ectomesenchyme to make craniofsacial elements, and if this is the case, whether Hh signaling may contribute to molecular patterning of the facial mesenchyme in addition to promoting cell survival and proliferation.

2.2. Expression of Shh and Ptch-1 during craniofacial development

To obtain a detailed understanding of Shh signaling during normal craniofacial development, the expression patterns of Shh and its transcriptional target Patched-1 (Ptch-1) [19] in relation to the distribution of CNCCs in the FNP and BAs was studied. CNCCs were labeled using Wnt1-Cre, an NCC-specific Cre-transgene and the R26RLacZ. At embryonic day 9.5 (E9.5) and E10.5, Shh is expressed in three epithelial populations in the developing face, that is, the ventral forebrain neuroepithelium the oral ectoderm and the pharyngeal endoderm but is absent from the mesenchyme. Expression of Ptch-1 indicates that Shh signaling occurs in both the epithelium and underlying mesenchyme.

In the MXA and FNP, only the medial half of the mesenchyme is subject to Hh signaling. On the other hand, Ptch-1 expression extends along the entire mediolateral (proximodistal) axis of the caudal half of the MNA, although its expression is dorsally restricted except at the midline. Additional sites of Shh production appear at E12.5, including the ventral nasal pit and tongue epithelium a significant part of

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the nasal, oral, and tongue mesenchyme receives Hh signaling at this stage. The mesenchyme expressing Ptch-1 contains a high density of CNCCs at all three stages examined suggesting that direct Hh signaling to this population of cells may have functional importance. In addition to Shh, another member of Hh family genes, Indian hedgehog (Ihh), has been implicated in craniofacial development; Ihh mutants have mild craniofacial abnormalities. Thus, Shh is exclusively responsible for the Hh signaling in the face prior to E12 19.

2.3. Fox genes as mediators of Hh pathway function

During craniofacial development Hh signaling regulates ectomesenchymal expression of five Fox genes, Foxc-2, Foxd-1, Foxd-2, Foxf-1, and Foxf-2, several of these induced by Shh in somites, foregut, or tissue culture. It has been proposed that the Fox genes are the major mediators of the function of Hh signaling in craniofacial morphogenesis. This hypothesis supported the mutant phenotype of Foxc-2. In particular, the absence of the palate components (palatal process of the maxilla and palatine) and the middle ear ossicles (incus and stapes) correlates with the expression of Foxc-2 in the MXA and second BAs. Foxf-2 mutants also have a cleft palate, although this is likely to be secondary to the influence of Foxf-2 on tongue morphogenesis [20]. In contrast, no craniofacial abnormalities were reported in the mutants of either Foxd-1 or Foxd-2 [21].

2.4. Foxi Class Genes – Early Craniofacial Development

Since the discovery of the highly conserved motif between the Drosophila forkhead gene and the mammalian HNF-3 transcription factor, more than 100 members of the Fox gene family have been identified from yeast to human. Foxi class genes have been identified and analyzed in several species. Mouse Foxi-1, previously called Fkh-10, is expressed in the otic vesicle at the E9.5 and the expression becomes restricted to the epithelium of endolymphatic duct and sac at the later stages. Targeted mutation of Foxi-1 causes an abnormal expansion of the membranous labyrinth, and the resulting mutant mice suffer from hearing impairment and vestibular dysfunction. Mouse Foxi-1 is also expressed in the embryonic and adult kidney from E16.5 [22]. Zebrafish foxi-1 is expressed in the early otic placode and branchial arches, and the zebrafish foxi-1 mutant and morpholino knock-down experiments show that zebrafish foxi-1 regulates dlx and Pax gene expression in the early otic placode and branchial arches. Xenopus foxi-1-a and b are mainly expressed in the neuroectodermal and mesodermal lineage during early embryogenesis and foxi-1c is expressed in the epidermal ring around the neural field and subsequently localized in placodal precursor cells. Solomon and colleagues have identified three other fox-i class genes in zebrafish. Zebrafish foxi-2 is expressed in the chordamesoderm during early somitogenesis and the retina and branchial arches during later stages. Zebrafish foxi-3-a and foxi-3-b are expressed in the epidermal mucous cells throughout embryogenesis and early larval stages. Zebrafish foxi-2, foxi-3-a, and b are not expressed in the otic lineage. Mouse Foxi genes are expressed in an early pan-placodal ectodermal domain, and also later in particular cranial placodes. Their expression shows some similarities, but also significant differences with the expression of Foxi genes in other vertebrate species.

Foxi-2 is expressed faintly in the ectoderm of the midbrain–hindbrain boundary region at the late pre-somite stage (ss). This expression is no longer visible at later stages. At E 6.5 to E10.5, more distinct but patchy expression is visible in the cranial ectoderm. At this stage, the Foxi-2 expression domain overlaps the Pax-2 expression domain, which marks the presumptive otic region in the cranial ectoderm adjacent to rhombomere 3 to 5. The Foxi-2 expression domain excludes the otic placode at later stages. Thus following hypotheses are proposed. (i) Foxi-2-positive cells in the Pax-2 domain may be future epidermal and epibranchial cells, not otic placodal cells, and migrate away from the otic region as the otic placode forms, (ii) Some of the Foxi-2-positive cells in the Pax-2 domain are future otic placodal cells and (iii) Foxi-2 expression is downregulated in these cells as otic placode forms.

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Table: 1. Gene abbreviations and references

No	Gene	Abbreviation	Reference
1.	HBG	Homeobox gene family	Burlglin, 1994; Hill, 1989; Sharpe, 1995; Favire, 1997
2.	Hox	Homeobox genes	Burlglin, 1994; Hill, 1989; Sharpe, 1995; Favire, 1997
3.	Msx	Mammalian homology to Dorsophilia muscle segment Homeobox genes	Alappat, 2003; Bendare, 2000; Akimenko, 1995
4.	Dll	Distal-less Homeobox gene in Dorsophilia	Cohen, 1998
5.	Dlx	Mammalian homology to Dorsophilia Distal-less gene	Raymond, 2002; Yang, 1998; Cohen, 1998
6.	OTX	Vertebrate homology to Orthodenticle in dorsophilia	Simeone, 1998; Acompora, 2002, Bailley-Cuif and Boncinelli 1997
7.	PAX	Paired box domain	Dahl, 1997; Peters, 1998; Mansouri, 1999
8.	Pitx	Pitutiary specific transcription factor	Szeto, 1996; Lanctot, 1997; Gage, 1999
9.	PRX	Paired related family of Homeobox genes	Meijlink, 1999; Berge, 1998; Lu, 1999
10.	Barx	Bar class of Homeobox containing genes	Tucker, 1989; Kojima, 1991; Barlow, 1999
11.	Gsc	Goosecoid gene	Yamanda,1995; Bailley-cuif and Boncinelli 1997
12.	bHLH	Basic helix-loop-helix family of transcription factor	Bailley-cuif and Boncinelli 1997
13.	Osr	Odd skipped related gene	Burlglin, 1994; Hill, 1989;
14.	Shh	Sonic Hedgehog family of genes	Echarld,1993; Hammerschidt, 1997; Nasse, 1996
15.	Ihh	Indian Hedgehog gene	Belloni, 1996
16.	Fox	Forkhead box gene family	Tukahiro Ohyama and Andrew Grover, 2004; Streit 2002
17.	Wnt	Wingless family of genes	Tomlinson, 1997; Zang and Carthero 1998
18.	Fz	Frizzeled family gene	Kuhl 2002
19.	BMPs	Bone Morphogenetic Proteins	Luo, 1995; Kim, 1998
20.	TGFs	Transforming Growth Factors	Dudas, 2004; Xu, 2006; Proetzel, 1995
21.	FGFs	Fibroblast Growth Factors	Trainor, 2002; Kim, 1998
22.	MMPs	Matrix Metalloproteinase	Rasha-Al-Mubarak, 2005
23.	Lhx	LIM-Homeobox containing genes	Maria 1998

2.5. Dlx-5 and Dlx-6 Homeobox genes

2.5.1 Role of Dlx-5 and Dlx-6 HBG in craniofacial development

Dlx homeobox genes (HBG) are mammalian homologs of the Drosophila Distal-less (Dll) gene. The Dlx/Dll gene family is of an ancient origin and appears to play a role in appendage development in essentially all species in which it has been identified. In Drosophila, Dll is expressed in the distal portion of the developing appendages and is critical for the development of distal structures. In addition, human Dlx-5 and Dlx-6 homeobox genes have been identified as possible candidate genes for the autosomal dominant form of the split-hand/split-foot.

2.5.2. Cartilage and bone defects in Dlx5/6−/− mice

Severe craniofacial cartilage defects in Dlx-5/6−/− embryos are apparent at E14.5. such as- (i) Exencephaly, (ii) Inner ear capsule and middle ear cartilages are fused and severely dysmorphic, (iii) External ear cartilage is absent, (iv) Cartilage giving rise to the cranial floor (basioccipital, basisphenoid, and sphenoid) and frontonasal prominence with severe patterning defects, (v) Cartilage from the exoccipital and ventral temporal bone primordia extending to the distal nasal capsule are distinctly condensed and fused, (vi) Maxillary and Mandibular bones are absent. However, soft tissues (skin, tongue, and muscle) are present in a manner that defined where maxillary and mandibular structures should have developed. Endochondral ossification of the exoccipital bone primordia of Dlx-5/6−/− embryos is present at E18.5. In addition, areas of membranous ossification develop that suggest an attempt to form maxilla, premaxilla and nasal bones [23].

2.5.3 Role of Dlx5/6 in control of craniofacial development

Dlx-5/6−/− mice have a multitude of craniofacial and ear

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defects including the failure of Meckel's cartilage, mandible, and calvaria formation. The craniofacial and ear defects are dramatically more severe than those observed in Dlx-5-deficient mice. Numerous genes are required for proper ear and craniofacial development, including Prx-1, Prx-2, Msx-1, Msx-2, Endothelin-1(Edn1), and Endothelin-A receptor (ETA). Mice null for Prx-1/2, Msx-2, Edn-1, or ETA have craniofacial and ear defects that have some similarity to a portion of the defects in Dlx-5/6−/− mice [23].

2.6. Msx homeobox genes

2.6.1. Role of Msx homeobox gene family in craniofacial development

Vertebrate Msx genes are unlinked, homeobox-containing genes that bear homology to the Drosophila muscle segment homeobox gene. These genes are expressed at multiple sites of tissue-tissue interactions during vertebrate embryonic development. Inductive interactions mediated by the Msx genes are essential for normal craniofacial, limb and ectodermal organ morphogenesis.

Vertebrate craniofacial tissues form multiple embryonic tissues including the cranial neural crest derived cells, prechordal mesoderm, and the embryonic craniofacial ectoderm. Normal craniofacial morphology develops as a consequence of complex interactions between these embryonic tissues, and requires precise regulation of cell movement, growth, patterning, and differentiation of craniofacial tissues. Genetic studies have revealed the involvement of numerous genes in these processes, including genes encoding a variety of transcription factors, growth factors and receptors [24]. Mutations in genes that influence any of these processes would cause craniofacial abnormalities, such as facial clefting and craniosynostosis, which are among the most frequent congenital birth defects in humans. Among the critical factors involved in craniofacial development are members of the Msx homeobox gene family. The vertebrate Msx genes were initially cloned from mice and identified as homologous to the Drosophila muscle segment homeobox gene (msh) [25, 26]. Subsequently, Msx genes have been isolated from a variety of organisms, including ascidians, sea urchin, zebrafish [27], frogs [28], birds [29], and humans. The mammalian Msx gene family consists of 3 physically unlinked members, named Msx-1, Msx-2, and Msx-3. Msx-3 is only expressed in the dorsal neural tube, in a pattern resembling that of the prototypical Drosophila Msh gene [30]. However in, developing vertebrate embryos, Msx-1 and Msx-2 are widely expressed in many organs, particularly at the sites where epithelial-mesenchymal interactions take place. Most notably, Msx-1 and Msx-2 are strongly expressed in the developing Msx genes encode transcription repressors. The Msx proteins are regulatory proteins that function as transcriptional repressors in vitro and in vivo [31, 32], important modulators of craniofacial, limb, and nervous system development.

Protein-protein interactions, which engage residues within their homeodomain guide target gene selection and transcription regulation [33]. The Msx homeodomain interacts directly with the TATA binding protein (TBP), the core component of the general transcription complex to execute transcription repression importantly; this ability to interact with members of the basal transcription machinery and affect transcription is not contingent upon their DNA-binding function. Msx proteins also interact with other homeodomain proteins to regulate transcription. Heterodimers formed between Msx-1 and other homeodomain proteins such as Dlx-2, Dlx-5, Lhx-2 and Pax-3 result in mutual functional antagonism in vitro [34]. The tissues in which expression of Msx-1 overlaps these other proteins there may be such a regulatory mechanism in place. Although, Msx-1 and Msx-2 show similar DNA binding site preference as well as the ability to repress transcription they display different biochemical properties by virtue of unique N-terminal domains, which confer Msx-2 with a greater affinity for DNA while rendering Msx-1 a more potent repressor. Three dimensional structure of Msx-1 homeodomain/DNA complex reveals two major arm of the homeodomain, which tracks the minor groove of the DNA. Secondly, the DNA bound by the Msx-1 homeodomain with other shows a 28° bend compared to the normal 21° observed homeodomain proteins.

2.6.2. Expression of Msx genes during craniofacial development

Expression of Msx-1 and Msx-2 are seen at multiple sites of tissue-tissue interaction including the craniofacial regions. Through the course of murine craniofacial development, both Msx-1 and Msx-2 are detected in the forming skull and meninges, the distal aspects of the facial primordia, the associated sense organs, and teeth. In the developing skull, Msx-1 and Msx-2 are expressed in the suture mesenchyme and dura mater. While Msx-1 expression extends into the postnatal stages of skull morphogenesis, Msx-2 registers a sharp decline in expression after birth. Reports of a weak, diffuse expression of Msx-1 in the palatal mesenchyme provided the first evidence that Msx-1 may have a direct role in palate development. A more detailed analysis by Zhang et al., (2002) [35] has reported that Msx-1 expression in the palatal mesenchyme is confined to the anterior portion of the developing palatal shelves [36].

2.7. Role of Lhx-6 and Lhx-7, in craniofacial development

Many homeobox containing genes are, however, found dispersed outside the HOM-C/Hox clusters. Such genes often encode polypeptides which, in addition to the homeodomain, contain additional domains (such as paired, POU or LIM domains which can regulate the transcriptional activity of the protein [37]. LIM homeodomain proteins [38], are characterised by the association of two LIM domains

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(cysteine-rich motifs involved in intra- and intermolecular interactions) with a homeodomain and constitute a family of transcriptional regulators that were originally identified by sequence homology between the C. elegans genes lin-11 and mec-3 and the mammalian transcription factor islet-1. Genetic studies in both invertebrates and vertebrates have shown that LIM homeodomain encoding genes are required for the control of cell fate specification and differentiation In vertebrates, targeted mutagenesis of Lhx gene has established that they are necessary for the differentiation of specific cell types, such as subclasses of CNS neurons (isl-1) or subpopulations of endocrine cells of the anterior pituitary. Finally, misexpression studies have indicated that the Lmx-1 gene is a critical component of the molecular mechanisms which lead to establishment of the dorsal phenotype of the vertebrate limb bud [39]. Lhx-6 and Lhx-7 are expressed in overlapping subdomains of the first branchial arch and the basal forebrain and the expression in the first arch primordia is under the control of signals from the overlying oral epithelium. One of these signals is likely to be FGF-8, which is expressed in the oral epithelium and is capable of inducing expression of both Lhx-6 and Lhx-7 in mandibular mesenchyme in vitro.

Low levels of Lhx-6 and Lhx-7 transcripts were first detected by whole-mount in situ hybridisation in a subdomain of the first branchial arch of E9.5 embryos At E10.5; both genes are expressed at high levels, and with an overlapping pattern, in the maxillary and mandibular processes of the first branchial arch. However, expression is restricted to the subdomains bordering the cleft that separates the two facial primordial. Sections of hybridised embryos showed that Lhx-6 and Lhx-7 transcripts were restricted to the neural crest-derived ectomesenchyme and were excluded from the overlying oral epithelium. No expression is detectable in other branchial arches. It appears, therefore, that the expression domains of Lhx-6 and Lhx-7 coincide with the regions of the first arch mesenchyme which acquire odontogenic potential and contribute to the formation of individual teeth. Consistent with this, expression of Lhx-6 and Lhx-7 in the branchial region at subsequent developmental stages is primarily associated with developing teeth. Expression of Lhx-6 and Lhx-7 during mouse odontogenesis analysed by in situ hybridisation on sections of embryos ranging from E12.5-P2 showed, expression of both genes was identical and distributed uniformly in the ectomesenchyme adjoining the oral epithelium. By the tooth bud stage (E13.5), expression is downregulated from most regions of the mandibular and maxillary mesenchyme, but is maintained (and indeed upregulated) in the mesenchyme adjacent to the epithelial thickenings which constitute the tooth primordial. Similarly, at subsequent stages of tooth cap and bell high levels of Lhx-6 and Lhx-7 expression are restricted to the mesenchymal component of the developing tooth. Finally, by postnatal day 2, expression of both Lhx-6 and Lhx-7 genes is downregulated in the developing teeth. Overall, data indicates that prior to initiation of tooth development; Lhx-6 and Lhx-7 are expressed uniformly in the odontogenic mesenchyme of the first branchial arch, whereas during odontogenesis, expression is restricted to the mesenchyme participating in the formation of individual teeth.

[III] GENES INVOLVED IN CRANIOFACIAL SUTURE GROWTH

3.1. Expression of BMP-2 and BMP-4

The BMP signaling pathway interacts with FGF, Shh, and Wnt signaling pathways and regulates the expression of several critical transcription factors, such as Runx-2/Cbfa-1, Msx-1, and Msx-2. Although no mutations in the genes encoding the different BMP isoforms have yet been found in human cranial sutures (CS), it has been suggested that BMP signaling is crucial in suture formation of the human bones. Both BMP-2 and BMP-4 are present in the osteogenic fronts of cranial sutures, and high expression of BMP-2 was observed in the mesenchyme during palatal fusion [40]. Deficiency of BMP signaling in mouse neural crest cells shows multiple defects in craniofacial skeleton, such as cleft palate and a hypotrophic mandible. BMP signaling is also found to induce and upregulate the expression of homeobox gene Dlx-5, a critical factor for the development of both the craniofacial skeleton and teeth. From early tooth initiation to crown morphogenesis, the BMP/Msx signaling loop mediates reciprocal interactions between the epithelium and the mesenchyme.

During embryonic stages, BMP-2 mRNA is detected in the OFs and weakly in parietal bones. After birth, the expression of BMP-2 is greatly reduced. BMP-4 mRNA, on the contrary, is localised in the OFs at high levels and at a lower level in mesenchyme of sagittal suture until E17. At E15 BMP-4 is also expressed in the developing falx cerebri and in the dura. From the end of embryonic stage (E18), the expression of BMP-4 was decreased and the analysis of serial sections showed that expression is not continuous in the OFs, indicating a patched pattern. Both BMP-2 and 4 transcripts are expressed in epidermis of skin and from E16 in hair follicles, however, as with Msx probes the hair pigment also contributed somewhat to the authentic expression.

3.2. Expression of Shh and Ptc

The first weak signs of Shh gene expression are found at E17 in posteromedial OFs of the parietal bones. From E18 onwards, Shh transcripts are expressed in a patched pattern in the OFs in sagittal suture as well as in metopic suture. Expression of Ptc, is identified as Shh receptor, is not detected by whole-mount in situ hybridisation until E18. From E18 onwards, Ptc transcripts are localised in the OFs in sagittal and metopic sutures. The patterns of Ptc and Shh expression were remarkably similar. Control tissues hybridised with sense probes gave negative result. Interestingly, neither Shh nor Ptc is expressed in the area of the coronal suture during these stages [41].

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Table: 2. Genes causing various human birth defects

No	Gene	Syndrome	Birth defect
1.	*BORA1*	Branchio-oto-renal	External ear anomalies, hearing loss, kidney defects
2.	*COL2A1*	Stickler	Skeletal dysplasia, cleft palate, nearsightedness
3.	*EMX2*	Schizencephaly	Clefting of the cerebral cortex
4.	*GLI3*	Greg	Premature closure of cranial sutures, extra digits
5.	*GLI3*	Polydactyly type A	Extra posterior digits
6.	*HOXA13*	Hand-foot-genital	Hypoplasia of first digit, kidney and genital defects
7.	*HOXD13*	Synpolydactyly A1	Extra digits that are fused with each other
8.	*IHH*	Brachydactyly	Short finger and toes
9.	*IRF6*	Van der Wounde	Cleft lip/plate, with lip pits
10.	*IRF6*	Popliteal pterygium	Cleft lip/palate, webbing across joints
11.	*LMX1*	Nail-patella	Anamolies of bone, kidneys, fingernails
12.	*MSX1*	-	Cleft lip/palate, missing teeth
13.	*NOG*	Multiple synostosis	Abnormal fusion of bone, hearing loss
14.	*TP63*	Ectodermal dysplasia	Limb, teeth, hair defects
15.	*PAX2*	-	Kidney and optic nerve defects
16.	*PAX3*	Waardenburg	Hypopigmentation, hearing impairment
17.	*PAX6*	Aniridia	Hypoplasia or aplasia of the irides
18.	*PAX9*	Oligodontia	Missing teeth
19.	*SHH*	Holoprosencephaly	Lack of midline cleavage of brain
20.	*SOX9*	Campomelic dysplasia	Skeletal defects, sex reversal
21.	*SOX10*	Hirschsprung	Bowel Hypomotility
22.	*TBX3*	Ulnar-mammary	Upper limb anamolies, breast and genital anamolies
23.	*TBX5*	Holt-Oram	Anterior upper limb anamolies, heart defects
24.	*TBX22*	-	Ankyloglossia, cleft palate
25.	*TCOF1*	Treacher Collins	Mid –face hypoplasia, small jaw, external ear defects
26.	*WT1*	Denys-Drash	Kidney defects, sex reversal
27.	*DHCR7*	Smith-Lemli-Optiz	Mental retardation, Syndactyly, multiple organ defect

3.3. Different signalling pathways may regulate suture maintenance prenatally and postnatally

In situ hybridisation analysis shows that the expression of Msx-2 and BMP-4 is correlated with the prenatal activity of dura mater. Postnatally, neither of these genes are detected in this location. Although the expressions of Msx-1 and Msx-2 are overlapping, they showed clear differences and, in vitro, BMP-4 induced the expression of both Msx-1 and Msx-2, whereas FGF-4 induced the expression of Msx-1 only. In dental mesenchyme also, FGFs preferentially regulate Msx-1, but not Msx-2. These findings are in line with a showing that the functions of Msx-1 and Msx-2 genes are modulated differentially by their non-conserved N-terminal regions. However, it is shown that Msx-1 and Msx-2 have similar DNA binding and transcriptional properties suggesting redundant functions of the two genes.

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Whole-mount in situ hybridization analysis show that Shh as well as its receptor, Ptc, started to be expressed at the end of embryonic development. Their expression appeared as patches on the OFs of the midline sutures. This indicates that, firstly, the target tissue for Shh signalling is in the OF and, secondly, there are site-specific differences in Shh signalling in the calvaria, which may reflect the difference in sutural architecture. The coronal suture whose OFs are overlapping apparently lacked Shh expression, whereas as Shh is seen in end-to-end type midline sutures. Thus it is suggest that the Shh signalling may be involved in regulating cranial suture development and intramembranous bone formation. It is possible that Shh has an analogous effect on intramembranous bone development as has been shown for Ihh, another hedgehog family member, in endochondral bone formation, where Ihh controls the differentiation of hypertrophic chondrocytes. Postnatally the expression of BMP-2, BMP-4 and Msx-2 is discontinuous along the OFs apparently reflecting a patched pattern thus resembling the expression of Shh and Ptc. It is speculated that Shh may interact with BMPs in the OF through a Ptc-dependent pathway which may be involved in the prevention of precocious sutural closure [41].

3.4. Expression of Bone sialoprotein (Bsp) and Twist

Bsp was first expressed at E12 just lateral to the temporal cartilages, in a strip medial and superior to the eye extending occipitally. From these ossification centres in the frontal and parietal bones, the expression spread toward the apex of the cranium where the osteogenic fronts approximate to form a suture (E15), two osteogenic fronts and intervening mesenchyme. Until E17 Bsp is expressed throughout the calvarial bones, most notably on their outer surfaces. In contrast, osteoclast are found mainly on the endocranial surfaces and so, as the calvaria expands, there is an intimate balance between bone apposition and resorption, thus maintaining bone thickness and shape. After E17, transcripts became more restricted to areas of high activity, notably the sutures. Bsp expression clearly demarcating the developing calvarial bones and illustrated the approximation of their osteogenic fronts. At E10, Twist is intensely expressed in mesenchyme throughout the first and second branchial arches, as well as in the mesenchyme surrounding the developing eye and cranial mesenchyme just beneath the epithelium. Expression then comes more restricted so that, by E14, transcripts are seen bordering areas of condensing calvarial mesenchyme. These condensations consist of osteoprogenitors that differentiate into functioning osteoblasts, the temporal, frontal and parietal bones being thereby initiated. Twist mRNA was also detected close to developing cartilages. From E15 to P1, Twist continued to be expressed in the calvarial mesenchyme and, as in earlier stages, not in mature osteoblasts. Postnatally its intensity is decreased gradually.

3.5. Expression of FGFR-1-B, FGFR-1-C, FGFR-2-B, FGFR-2-C, FGFR-3B, FGFR-3-C and FGFR-4

FGFRs are expressed at numerous locations during early mouse development including the craniofacial area and, although FGFRs have been detected in developing bones and sutures, little is known about their detailed expression during calvarial bone development. Twist proteins are conserved basic helix-loop-helix transcription factors (bHLH) and Inhibitors of differentiation (Ids) are conserved dominant negative helix-loop-helix proteins (dnHLH). Both have been implicated as regulators of mesoderm differentiation and myogenesis in both Drosophila and vertebrate development though, in contrast to Drosophila Twist, murine Twist is thought to act as a suppresser rather than an activator of myogenesis. Although, Id-1 lacks a DNA binding domain, it inhibits bHLH's function by suppressing their heterodimerization through direct protein-protein interactions. Early osteoblastic cell cultures have been shown to express both Twist and Id, with expression decreasing as maturity increases.

3.6. Expression of FGF-2

FGF-2 demonstrates a more restricted expression patterns, being associated with osteoblast differentiation in the suture. FGF-2 is expressed in the mesenchyme of the calvarial sutures and more weakly in the developing calvarial bones and the underlying dura mater. Expression decreased in intensity after E16. Expression of this potential ligand, although overlapping that of the FGFRs, is not primarily expressed in the osteogenic fronts and is also more extensively expressed in the mid-sutural mesenchyme, suggesting paracrine functions.

BEK, a splicing alternative of FGFR-2, is intensely expressed in the OFs of parietal bones of E15 and E17 mouse embryos, and transcripts are also detected in the superficial dermis of skin. Postnatally BEK is expressed at the same location but its intensity is diminished. Interestingly at P6 these areas of expression appeared to join above and below the mid-sutural mesenchyme, possibly indicating the forming periosteum sheathing this area. At E15, FGF-9 is expressed with high intensity in the dural layers, the calvarial mesenchyme and the overlying epidermis. By E17 transcripts are most notably located in the dura mater and endocranial portion of the mesenchyme and dermis. Postnatally, expression is still noted in the calvarial mesenchyme at a diminished level. FGF-4 is not present in these tissues between E15 and P6.

The FGFRs are high-affinity tyrosine kinase receptors, which together with co-factors mediate the effects of FGFs. They are transmembrane glycoproteins with two or three extracellular immunoglobulin domains. These binding domains differ between alternative splice variants, which are of particular interest as they possess different ligand-binding specificities as well as exhibiting unique temporospatial expression patterns

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suggesting unique functions. In addition, many of the human mutations in FGFR-1/FGFR-3 causing disorders in bone development are found in or close to the third immunoglobulin (III) domain. The expression pattern of FGFR-1B is generally weak compared to the other FGFRs and transcripts are not seen in either the developing calvarial bone or sutures.

Very low levels of FGFR-1C expression is detected in the calvarial bones, most notably in the osteogenic fronts between E15 and 17, major sites of osteoblastic condensation and differentiation. FGFR-1C is also expressed in many cartilages in the developing head. FGFR-2B (kgfr) is expressed in the osteogenic fronts of the parietal bones (E15-17). In addition, transcripts are located in developing epithelia, notably the skin including hair follicles. FGFR- 2C (bek) is found in similar locations to FGFR-2B but at generally much stronger intensity. Postnatally, expression is at the same locations but diminished in intensity. In comparison to FGFR-2B, FGFR-3B is weakly and FGFR-3C strongly expressed in many cartilages of the head. The majority of this cartilage does not contribute to the calvarial bones, which form directly from mesenchyme. FGFR-3C mRNA is also detected with low intensity in the head periostea and sutural osteogenic fronts Transcripts of FGFR-4 are not detected in the developing calvarial bone or mesenchyme. However, FGFR-4 mRNA is detected strongly in developing muscle, notably in the developing temporalis between the epithelium and the underlying calvaria.

3.7. Muscle segment homeobox-containing (Msx) transcription factors

Msx-1 and Msx-2 are transcription factors expressed in overlapping patterns at multiple sites of tissue interactions during vertebrate development. In particular, they have been associated with epithelial– mesenchymal interactions during craniofacial⁄dental development, as targets of BMP and FGF signaling. For instance, BMP-2 and BMP-4 induce the upregulation of Msx gene expression in tooth explants as well as in rhombomeres and several FGFs induce the expression of Msx-1 in dental mesenchyme. Msx-1 and Msx-2 have also been associated with the differentiation of neural crest-derived intramembraneous bones in the skull. Msx-2 deficient mice exhibit defective proliferation of osteoprogenitors in the developing calvaria and have defects of skull ossification and persistent interparietal foramina. Transgenic mice overexpressing the Msx-2 mutation appear to have different phenotypes depending on which promotor is used, varying from precocious bone formation with accelerated suture closure to craniofacial defects with aplasia of the interparietal bone. Msx-1 and Msx-2 also determine the position and shape of teeth (so-called field model, linking patterning of tooth type to spatial expression of homeobox genes in the dental mesenchyme).
Msx-1 is expressed in the mesenchyme of sagittal suture and the dura mater during embryonic and postnatal stages. Msx-2 is intensely expressed in the sutural mesenchyme and the dura mater during embryonic stages. Interestingly, after birth, the expression of Msx-2 is dramatically diminished in the mesenchyme and it completely disappeared from the dura mater. Furthermore, analysis of serial sections reveals that the expression of Msx-2 is no longer continuous after birth around the OFs, indicating a patched pattern of expression. Msx-1 and Msx-2 transcripts are intensely expressed in hair follicles. However, the hair pigment also contributed an additional component to the authentic expression.

3.8. Twist transcription factor

Twist is a helix–loop–helix transcription factor that plays a role in cranial neural tube morphogenesis, Twist is expressed very early as a negative regulator of osteoblast differentiation and its expression decreases with maturity, i.e. Twist is expressed by osteoprogenitors but not by mature osteoblasts. FGF-2⁄FGF-4 and Twist exhibit overlapping expression patterns, both being intensively expressed in the midsutural mesenchyme between the calvarial bones and in the mesenchyme during early tooth initiation. It was shown recently that Twist is one of the integrating parts of the Shh, FGF, BMP, and Msx-2 signaling pathways mediating a number of common effects at the cellular level during development of, e.g. the cranial structures, limbs, the palate, and teeth. Mutations in the Twist gene cause Saethre–Chotzen syndrome, resulting from a loss-of-function mechanism. In contrast to FGFR and Msx-2 mutations, these are mostly deletions or nonsense mutations. Twist knockout mice die before osteogenesis has started, with a failure of the cranial neural folds to fuse and defects in the head mesenchyme. Experimental animal studies further support the idea that FGF signaling may lie both up- and downstream of Twist.

3.9. Otx- Genes

Another family of evolutionary conserved homeodomain factors with critical regulatory roles in the determination of head structures during development is the Otx genes, vertebrate homologs to Orthodenticle in Drosophila. The overlapping patterns of expression of Otx and Emx genes in the rostral region of the developing brain, together with functional studies, have suggested that these genes, analogous to the Hox code for hindbrain development, provide a combinatorial code for rostral brain development [42]. Otx-2 homozygous null mutants die early in embryogenesis and fail to develop structures anterior to R3. The phenotypic abnormalities in Otx-l homozygous mutants indicated its essential role in the formation of cortex in the adult brain.

In addition to its expression in the rostral region of the developing brain, Otx-2 is also expressed in neuroectoderm from the forebrain up to the mid-hind-brain isthmus. Interestingly, Otx-2 heterozygote animals exhibit otocephaly and abnormalities in midbrain crest derivatives of the first arch, suggesting a role for Otx-2 in pre-migratory CNCC originating from the midbrain region. In Otx-2+/- mice, the elements that were shown to be derived solely from midbrain

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crest (distal Meckel's cartilage, dentary, maxilla, and palatine) are lacking or severely reduced, suggest ing that defects in these animals may be due to deficiencies in CNNC derived from the midbrain region. However, in Otx-2+/- mice, the hindbrain-derived, first-arch elements (malleus, incus, and pterygoid) are almost unaffected. Analysis of the knock-in mice in which Otx-2 was replaced with Otx-l showed that most Otx-2 functions, including the Otx-2 haploinsufficiency causing the otocephalic phenotype, are replaceable with those of Otx-l.

[VI] PALATAL DEVELOPMENT AND FUSION

4.1. Tissue patterning

The wingless (wnt) and frizzled (fz) family genes were first characterized in Drosophila, where they specify tissue patterning and cell-fate determination during embryonic development [40, 41]. The homologous wnt and fz family members in mammals have also been reported to function in tissue specification [43, 44]. Wnt-5a and its receptor, fz, are seen to be both down-regulated (–2.7 and –1.9, respectively). This was confirmed by immune-histo-chemistry. Their role in palatogenesis is as yet unidentified; although Wnt-5a shows strong expression in the dental papilla mesenchyme [45] and mutant murine embryos have defects in the face and organs that extend from the body [46]. Barx-1 is a homeobox gene that is expressed in developing facial primordia, including the maxillary mesenchyme [47], targets of fibroblast growth factor FGF-8, which in early chick embryo ectoderm defines the maxillo-mandibular region through epithelial–mesenchymal interactions and subsequent upregulation of homeobox genes in the local mesenchyme [48]. Upregulation of Barx-1 expression before contact of palatal shelves and subsequent downregulation after fusion is seen.

4.2. Neural development

Zic-3 belongs to a class of genes responsible for neural development [49], and the expression of murine Zic genes suggests an essential role in body pattern formation. At ED-13.5, Zic-3 is expressed in peripheral zones of limb mesenchyme and presumptive muscle, but subsequently its expression decreases [50]. Zic-3 mRNA was downregulated within palatal shelves between ED-13.5 and 14.5, but immunohistochemistry revealed negligible levels of Zic-3 protein expression at all stages. This suggests that Zic-3 mRNA levels may be insignificant, and the changes seen may be minimal. The role of Zic-3 in palate development is unknown, but Zic-3 null mice have complex congenital heart disease, neural tube defects, disturbances of laterality, and vertebral and rib defects, but no cleft palate [51]. Sox-1 transcripts are first detected in the neural fold ectoderm at the headfold stage, and during early somitogenesis are expressed in the neuroectoderm [52]. Mesenchyme from the facial primordia is derived from neural crest cells that have migrated from the neuroectoderm. Proliferation of neural crest cell-derived mesenchyme is an important part of palatal shelf formation Proteins containing LIM domains play important roles in a variety of fundamental biological processes, including cytoskeleton organization and organ development [53]. It is noted that muscle LIM protein increased fourfold between ED14.5 and 15.5 in the murine palate. Lhx-8, a LIM homeobox gene, is expressed in the mesenchyme of the mouse palatal structures throughout their development, and nullizygous mutants for Lhx-8 are known to have cleft palate [54].

Maternally expressed gene (Meg-1) is probably responsible for the imprinted effects of prenatal growth retardation or growth promotion caused by maternal or paternal duplication of proximal chromosome 11, with reciprocal deficiencies.

4.3. Molecular control of secondary palate development

Targeted gene mutations in mice have revealed a number of molecular determinants of PS growth. In these, the PS is hypoplastic and either remain in a vertical position, leading to a wide cleft, or manage to elevate but remain apart. Organogenesis is governed by interactions between adjacent tissues layers. Organs as diverse as the lung, neural tube, tooth; hair and palate share several signaling pathways, although the developmental outcome is different. This emphasizes the notion of 'common notes—different melodies', where similar molecular networks are used during ontogeny of several organs but regulate different processes. Thus, insights gained from the biological events operating during embryogenesis of one organ can be used to shed light into those acting in other organs. Early experimental studies indicated a role for epithelial– mesenchymal interactions in the regional specification of PS epithelia and growth of the PS [55, 56]. Studies identified several molecular networks operating between the PS epithelium and mesenchyme during the different steps of palatogenesis. These include signaling molecules and growth factors such as Shh, members of the TGF-β superfamily, including BMPs and TGF-βs, FGFs, their receptors, effectors and targets. Transcription factors play fundamental roles in tissue patterning, growth and differentiation. Msx-1, the LIM-homeobox containing Lhx-8, the short stature homeobox Shox-2 and the odd-skipped related-2 (Osr-2) genes have been shown to be expressed in the growing PS. Targeted mutations of these genes generate cleft palate (CP) with minor or no craniofacial anomalies, indicating an intrinsic requirement of these factors during palatogenes [57, 58]. The CP in mice lacking Msx-1 (Msx-1−/−) has been shown to be caused by altered mesenchymal proliferation [59]. Msx-1 and Msx-2 genes are bona fide targets of BMP signaling in different developing embryonic sites including the tooth, cranial sutures, hair follicle and neural tube, where they act to regulate morphogenesis and differentiation [60, 61, 62,

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63]. Further, in both the embryonic tooth and palate, Msx-1 has been shown to be necessary for expression of BMP-4 and/or BMP-2. Interestingly, exogenous BMP-4 or a mesenchymally expressed BMP-4 transgene are capable to rescue the tooth developmental arrest and CP, respectively, in Msx-1−/− mice. Further elegant experiments 59 indicated that Msx-1 and BMP-4 function in an autoregulatory loop in regulating mesenchymal proliferation in the anterior palate. Nestin-Cre-mediated removal of type I BMP receptor (BMP-R1A; Alk-3) as well as BMP-4 activities demonstrated distinct functions for BMP signaling in lip fusion and secondary palate development in mice [64]. Ablation of BMP-R1A function in both the epithelium and mesenchyme of lip and palate primordia was found to generate bilateral cleft lip and palate. Altered cell proliferation and misexpression of Barx-1 and Pax-9 in the palate as well as precocious cell death in the fusing lip seem to be the cause of the clefting in the BMP-R1a mutants. In these, expression of other important factors such as Msx-1, Tbx-22 and Osr-2 is unchanged. However, conditional removal of BMP-4 activity resulted in isolated cleft lip. The latter phenotype seems at odds with the previously demonstrated important role for mesenchymal BMP-4 in the developing palate. Keratin 14-Cre-mediated targeted mutation of BMP-R1a, which inactivates this receptor in ectodermally derived tissues, including tooth, skin and palatal epithelia, has been shown to affect tooth and hair follicle development. However, the palate seems to develop normally in mutant mice. Altogether, these observations indicate that BMP-R1A functions primarily within the PS mesenchyme. Targeted inactivation of Osr-2 indicates a role for this transcription factor in medio-lateral patterning of the PS. In Osr-2−/− mice, the proliferation defects in the PS mesenchyme and the delayed elevation of the PS seem to be independent of Msx-1, BMP, Shh and Tbx-22 inputs but may be linked to Pax-9 and Osr-1 function .

Other studies addressed the role of FGF signaling during early palate development by analyzing mouse embryos lacking the functions of FGF-10 and FGFR-2b [65]. In the FGF-10−/− and FGFR-2b−/− mutants, altered cell proliferation within both the PS mesenchyme and epithelium as well as increased apoptosis within the epithelium seem to be the primary causes of CP. Those studies also revealed an interesting epithelial–mesenchymal signaling loop. By signaling via its receptor FGFR-2b in the PS epithelium, the mesenchymally derived FGF-10 brings not only about epithelial proliferation and survival but also induces expression of Shh within the epithelium. Shh, in turn, signals to the mesenchyme and stimulates cell proliferation. In general, signaling activities are subject to tight spatio-temporal control and in many instances too much or too little of a good thing can be detrimental to a developing organ. This is well illustrated in anomalies caused by deregulated Hh and FGF signaling. While FGF-10/FGFR-2b activity plays a crucial role during palatogenesis, it appears to be subject to a tight spatio-temporal regulation as shown in mice lacking Shox-2. Shox-2−/− mice develop a very rare type of palatal clefting that may also be found in humans and other mammalians the soft palate is intact, whereas the hard palate is cleft. Abnormal proliferation and apoptosis are likely at the core of the clefting. Surprisingly, a number of protagonists implicated in palatogenesis, including Msx-1, BMP-4, Pax-9, Lhx-8, Osr-2, TGF-β-3 and Jag-2, are found to be expressed normally. In contrast, FGF-10 and FGFR-2c were expressed at ectopic sites within the PS mesenchyme of the Shox-2−/− mice. These studies re-emphasize the importance of a fine tuning of the timing and sites of signaling activities for normal development to take place. TGF-β peptides activate the membrane receptor serine/ threonine kinase quaternary complex made of two type II and two type I receptors. The type I TGF-β receptor Alk-5 has been shown to play a key role in craniofacial and palate development [66]. The craniofacial anomalies of Alk-5 mutants were more severe than those in corresponding mutants lacking the function of the TGF-β type II receptor (TGFβ-RII) in cranial neural crest derivatives [67]. Those striking differences have been suggested to be due to Alk-5 function in mediating signalings by ligands other than TGFβ1–3 and to the ability of Alk-5 to function with type II receptors other than TGF-βRII. In contrast to embryos lacking TGF-βr2 in the PS mesenchyme, which displays reduced cell proliferation, the Alk-5-deficient PS mesenchyme seems to be hyperproliferative and to undergo massive apoptosis, again pointing to differences in the signaling functions of these two receptors. In humans, abnormally high TGF-β activity impinges upon palate formation as demonstrated in individuals bearing mutations in TGF-βR1 or TGF-βR2 [68]. These findings indicate that while signaling activities of type I and type II TGF-β receptors are crucial, the amplitude of such signals must be tightly controlled for normal palatogenesis. With the exception of the developing limb, organs consisting of an epithelium and a mesenchyme express the Hedgehog family members, Shh or Ihh, in the epithelial compartment, whereas targets and effectors of the Hedgehog pathway are found in both tissue layers, indicating Shh and Ihh activities at a distance from their sources. In the developing palate, Shh is produced in the PS epithelium, whereas its membrane receptor Patched-1 (Ptc-1) is present in both the epithelium and mesenchyme. The Hedgehog transcriptional effectors Gli1–3 are expressed in the PS mesenchyme but are present at low levels in the PS epithelium as well (AGL). Abrogation of Shh function in the palate epithelium generates CP. In contrast, epithelial loss of function of Smoothened (an obligatory and nonredundant component for all Hh signaling) does not generate CP, implying that the PS mesenchyme is the major target for Shh action. However, this does not exclude the possibility of an indirect action of Shh on the PS epithelium via Shh-induced mesenchymal inputs. Shh has been shown to act as a powerful mitogen in numerous developmental and neoplastic contexts. In vitro cultures show that Shh stimulates PS mesenchymal proliferation. Other in vitro studies have shown that Shh induces/ maintains BMP-2 expression, and that BMP-2 mediates Shh mitogenic effects on PS mesenchyme.

After vertical growth, the PS elevates into a horizontal position, and further extension allows contact between the

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opposing PS. Some genetic disruptions affect this second phase of PS growth. For instance, mice lacking TGF-βr2 in the PS mesenchyme develop a CP due to reduced extension of the horizontal PS, and paracrine TGF-β3 signaling in the PS mesenchyme seems to be required for this growth phase [69]. Similarly, embryos lacking platelet-derived growth factor-c (PDGF-C) activity show normal PS growth up to E13.5; however, after a delayed lifting, the hypoplastic PS are unable to about [70]. Loss of function of single minded-2 (Sim-2) in mice generates either a complete cleft of the secondary palate or a cleft of its posterior-most portion [71]. The complete cleft seems to be caused by lack of outgrowth of the PS which is, however, able to elevate. The PS of Sim-2−/− mice are hypocellular between E14.5 and E16.5, and histochemical staining suggested the presence of abnormally high amounts of hyaluronan. This aspect is interesting in light of the known role of hyaluronan (hyaluronic acid), a major component of the extracellular matrix, in regulating cell proliferation, differentiation and migration.

Targeted gene ablation in mice identified several factors playing a determinant role in palate fusion. These include TGF-β3 [72, 73], the forkhead domain-containing transcription factor Foxe-1, epidermal growth factor receptor EGFr and PDGF-C. Loss of function of these factors generates CP with no or minor other craniofacial anomalies. In vitro explant cultures showed that PS from TGF-β3, EGFr and PDGF-C mutants fail to fuse owing to failure of the MES to degenerate. Importantly, studies in humans identified a mutation within the forkhead domain of FOXE-1 in siblings with thyroid agenesis, CP and choanal atresia and associated TGF-β3 with nonsyndromic CP. Cell–cell junctional complexes are essential for cell survival, morphogenesis, proliferation and differentiation.

4.4. Molecular control of palatal shelf fusion

During the last few years, extensive efforts have been made to shed light upon the role of TGF-β3 during palatal fusion. Adhesion of the MEE upon PS contact is a necessary step for fusion. TGF-β3, which is expressed in the MEE before and during PS fusion, mediates MEE adhesion of the opposing PS through filopodia and chondroitin sulfate proteoglycans at the apical surface of MEE cells and to be necessary for apoptosis of the regressing MES. Importantly, in the absence of TGF-β3 [74], MEE cells display altered distribution of E-cadherin, α- and β-catenins and impaired cell–cell adhesion. Early studies on fusion processes in different systems consistently show the presence of filopodia at the tip of fusing epithelial sheets. Thus, TGF-β3 plays a crucial role during the different steps of MEE adhesion and fusion [75]. Other studies implicated TGF-β3 in controlling the remodeling of the extracellular matrix through regulation of the expression of MMP-13, MP-2 and Tissue inhibitor of metalloproteinase-2. These studies indicate that TGF-β3 signaling operates not only in the MEE, but is also involved in mediating epithelial mesenchymal interactions leading to tissue changes that regulate palatal fusion. The effects of TGF-β3 on MES regression seem to be mediated by the TGF-β type II and the TGF-β type I receptor (Alk-5)/Smad pathway as shown by loss and gain of function studies in vitro and in vivo.

[V] TRANSCRIPTION FACTOR IN MANDIBULAR MORPHOGENESIS

5.1. Msx genes

At early stages of development of mandible, the expression of Msx-l and Msx-2 in the mandibular arch is limited to the mesenchyme in the medial region and is excluded from the mesenchyme in the lateral region [76, 77]. In addition to its expression in the medial region, Msx-l is also expressed in the mesenchyme surrounding the hyomandibular cleft. Tissue recombination and bead implantation studies indicate that the expression of Msx genes in the developing mandible and other facial processes is dependent on signals derived from the overlying epithelium [78, 79, 80]. In the developing mandible at early stages, the expression of Msx-l in the medial region is correlated with areas undergoing expansion which contain highly proliferative and undifferentiated mesenchyme cells. In Msx-l-deficient mice, the medial part of the mandible is truncated. Studies in the developing chick mandible also suggest that Msx genes may be involved in delineating the non-chondrogenic region at the midline region (symphysis).

This possibility is supported by observations that, in contrast to control explants, explants from chick mandibular arches treated with Msx-2 antisense oligonucleotides, formed cartilage in the medial region, resulting in the fusion of the 2 bilateral rods of cartilage at the midline. It is also shown that overexpression of Msx-2 also inhibits chondrogenesis in organcultured mouse mandibles [81]. Although lack of Msx-l does not appear to disturb the mandibular symphysis, Msx-l/Msx-2 knock-outs display severe abnormalities in the developing mandible. In humans, mutation of one copy of Msx-1 results in single- tooth agenesis, and specific point mutations in the homeodomain in one copy of Msx-2 result in Boston-type craniosynostosis [82].

5.2. Dlx genes

Members of the Dlx family are expressed in the craniofacial region in both ectoderm and mesenchyme [83]. Among these, Dlx-2, Dlx-3, and Dlx-5 are expressed at the junction of the neural plate and surface ectoderm, suggesting that they may be expressed by pre-migratory and migratory CNCC cells [84, 85]. In situ hybridization studies on E9.5-E1O mouse embryos showed that Dlx-l and Dlx-2 are expressed in the mesenchyme of both proximal and distal regions of all BAs, while Dlx-3, Dlx-5, and Dlx-6 are expressed predominantly in mesenchyme of the distal regions. For example, in the first branchial arch, Dlx-l and Dlx-2 are expressed in the maxillary processes (the proximally located component) as well as in the mandibular

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processes (the distally located component) and Dlx-3, Dlx-5, and Dlx-6 are expressed only in the mandibular processes. In the developing mandible, Dlx genes are expressed in a lateral-to-medial gradient. Similarly, in the second (hyoid) arch, Dlx-l and Dlx-2 are expressed throughout the proximo-distal axis, while Dlx-3, Dlx-5, and Dlx-6 are expressed only in the more distal (close to the midline) region of the second arch. These patterns of expression suggested that Dlx genes play essential roles in proximo-distal patterning of the BAs. This possibility is supported by phenotypic abnormalities in mice lacking Dlx-l, Dlx-2, Dlx-l/Dlx-2, and Dlx-5. Mice lacking Dlx-l, Dlx-2, and Dlx-l/Dlx-2 exhibited abnormalities similar, but not identical, to those of maxillary and proximal (caudal) hyoid arch derived structures. However, in these mutant mice, the skeletal components of the mandibular arch and distal hyoid arch appeared to be normal [86]. On the other hand, one of the most noticeable abnormalities in the mice homozygous for a targeted deletion of Dlx-5 is in the developing mandible [87]. At early stages (E13-E14), the mandibular arch and the Meckel's cartilage of Dlx-5 mutants are shorter than those in wild-type embryos and exhibit abnormalities in the caudal region of Meckel's cartilage.

At its caudal end, Meckel's cartilage in Dlx-5 mutants is bifurcated and gives rise to an ectopic novel cartilage, which becomes surrounded by bone later in development. At birth, the mandibles of Dlx-5 mutant mice are shortened, lack the coronoid process, and contain mis-shapen condylar and angular processes.

The phenotypic abnormalities in the mandibular arches of Dlx-5 mutants suggest essential (non-redundant) roles of Dlx-5 for proper development of mandibular processes and the skeleton of the caudal region of the mandibular arch. Analyses of the branchial arches in Dlx-5 mutants indicate that the absence of Dlx-5 did not affect cell proliferation or apoptosis but expanded the territory of the proliferating cells within the first branchial arch. In situ hybridization analysis indicated decreases of Gsc expression in the mesenchyme of the frontonasal processes and in the mandibular and hyoid mesenchyme of the Dlx-5 mutants. Unlike other members of the Dlx family, Dlx-5 and Dlx-6 are also expressed in developing bones, cartilage, and teeth, suggesting that the Dlx-5 and Dlx-6 genes may play roles in the multi-step process of skeletal differentiation and/or morphogenesis [88,89] Dlx-5 is also expressed at specific stages of osteoblast differentiation in vitro and could repress osteocalcin gene expression [90]. Interestingly, lack of Dlx-5 results in hypo-mineralization of calvaria and expression of osteocalcin in the periosteum at birth.

In humans, there is evidence implicating Dlx-5 and Dlx-6 genes as candidate genes for ectrodactyly (split hand/foot malformations, SHFM1). Often, these patients also have cleft palate and deafness. A frame-shift mutation in Dlx-3 is also associated with taurodontism and enamel hypoplasia in humans with Tricho-dento-osseous syndrome [91].

5.3. Goosecoid (Gsc genes)

In vertebrates, Gsc is expressed transiently at the rostral end of the developing brain and then re-appears at E9.5-E10.5 in many sites, including the mesenchyme of the branchial arches [92]. In the mandibular arch, Gsc is strongly expressed in the mesenchyme in the region of the hyomandibular cleft. Gsc null mutants die soon after birth, with rib cage malformations and multiple craniofacial defects, including abnormalities in the mandibular arch and middle ear structures. In the mandibles of these mutants, although the condylar process appears normal, the coronoid and angular processes are severely reduced in size, and the mandible is shortened in length. Furthermore, Meckel's cartilage is not enclosed by the mandibular bones but is embedded in a novel groove that extends along the entire length of the mandible. Studies showed that abnormalities in the mandible of Gsc null mutants are due to the absence of cells destined to express Gsc in these mutants, suggesting the essential roles of Gsc in the initial proliferation and/or survival of Gsc expressing cells [93].

5.4. Pitx Genes

Pitx-l (Ptx-l /POTX) and Pitx-2 (RIEG, Otx-2, Otlx-2, Brx-l, Arp-l) are 2 members of a vertebrate multigene family with overlapping and distinctive patterns of expression during embryogenesis. Pitx-l was originally identified as a factor interacting with the pituitary-specific transcription factor Pit-i and POMC promoter. Pitx-l is expressed in the pituitary gland throughout its development, in the lateral plate mesoderm of the caudal half of the embryo which leads to its expression exclusively in the hindlimb and not the forelimb, in the first branchial arch as well as its derivatives, and in oral ectoderm. The patterns of expression suggest that Pitx-l is a critical transcription factor involved in specification of the hindlimb and development of the pituitary gland and structures derived from the first branchial arch. This possibility was supported by phenotypic abnormalities in null mutants for Pitx-l and mis-expression of Pitx-l in the chick wing bud. Pitx-l null mutants die immediately or shortly after birth and are readily recognizable by their shortened mandibular arch [95]. These mutants exhibit abnormalities in the limb and in the derivatives of the first BA, including cleft palate, significantly shortened tongue and mandible, a novel bone surrounding Meckel's cartilage, and lack of gonial bones. In situ hybridization analysis indicated that the expression of several markers expressed early in the first branchial arch-including Msx-l, Msx-2, Gsc, Shh, BMP-2/4, Wnt-5a, and Pitx-2-is unaltered in Pitx-l null mutant mice, suggesting that defects in the craniofacial structures similar to those observed in the hindlimb may be due to defects in proliferation and/or abnormal chondrogenesis of mesenchymal cells. Interestingly, it has been suggested that, in humans, mutant Pitx-1 alleles might be responsible for a subset of patients with Treacher-Collins syndrome (other syndromes shown in **table 2**). Pitx-2/RIEG was initially identified by positional cloning of the gene responsible for Rieger Syndrome in humans. This

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autosomal dominant haplo insufficiency syndrome is characterized by anterior chamber ocular abnormalities and other features of various degrees of severity, including dental hypodontia, umbilical abnormalities, cardiac defects, and mild craniofacial dysmorphism. Pitx-2 is expressed in many tissues during development, including the craniofacial mesenchyme and epithelium of the first and second branchial arches. Experimental evidence indicated a role for Pitx-2 downstream of sonic hedgehog and nodal in a genetic pathway regulating laterality of heart, gut, and other asymmetric organs. The direct role of Pitx-2 in left-right determination and development of other structures, including the development of the mandibular processes [76], is provided by abnormalities in mice deficient for Pitx-2. Pitx-2 mutant mice die at around E14-15 and exhibit lung isomerization and defects in cardiac positioning and pituitary development. In addition, Pitx-2 null mutants exhibited cleft palate and abnormalities in the maxillary and mandibular arches, including severely hypoplastic mandible and arrested tooth buds. In situ hybridization analysis indicated the absence of FGF-8 and altered domains of expression of BMP-4, Msx-l, and Msx-2 in the developing facial processes in Pitx-2 null mutants, suggesting that the facial abnormalities may be mediated by changes in the patterns of expression of these genes.

5.5. Pax genes

Pax genes encode a family of transcription factors characterized by an evolutionary conserved paired box domain, a 384-bp DNA binding motif that regulates embryonic development by the control of target genes. In mammals, the Pax gene family consists of 9 members that, based on the presence or absence of structural motifs, are divided into 4 subgroups. The function and role of Pax genes in embryonic development have been elucidated by analysis of naturally occurring mouse mutants, targeted inactivation of several Pax genes in mice, and human syndromes. A common feature of all Pax mutants is reduction in size and malformation or loss of specific organs [96]. Among all Pax genes, members of group I (Pax-l, Pax- 9), group III (Pax-3, Pax-7), and group IV (Pax-6) are expressed in the developing facial processes. Analyses of various mutants have indicated essential roles for Pax-3, Pax-6, and Pax-7 in the development of CNCC-derived structures in the upper face. However, in these mutants, no abnormalities were observed in CNCC-derived structures of the lower face [76]. On the other hand, phenotypic abnormalities in the Pax-9 null mutant indicate essential roles for Pax-9 for the lower face. Pax-9-deficient mice lacked structures derived from pharyngeal pouches, such as thymus and parathyroid glands. In the developing mandible, in addition to abnormalities in the developing teeth, the alveolar ridge and the coronoid process are absent in Pax-9 null mutants.

5.6. Prx genes

Prx-l (previously called Mhox) and Prx-2 (previously called S8) are closely related members of the paired-related family of homeobox genes that are co-expressed in a variety of sites, including the craniofacial mesenchyme. Studies in developing mice and chickens indicate that Prx-l and Prx-2 are co-expressed in the CNCC-derived mesenchyme of the frontonasal process, in the mesenchyme of the first and second branchial arches, and in the pre-osteogenic areas [97]. In the developing mandible, at early stages of development, high levels of expression of both genes are detected in the mesenchyme of the medial region. In addition to the medial region, Prx-l is also expressed in the cells around the first branchial groove. As development proceeds, the expression of both genes is downregulated in the mandibular process but maintained in the maxillary and nasal processes.

Although Prx-2 null mutant mice show no obvious craniofacial and skeletal abnormalities, Prx-l null mutant mice show defects in skeletal elements derived from the maxillary processes and the caudal part of the mandibular processes, including hypoplastic coronoid, condylar, and angular processes and malformed malleus. In addition, Meckel's cartilage in Prx-l mutants displayed abnormal sigmoidal morphology. Studies indicate that, in the Prx-l null mutants, cells fated to express Prx-l are initially present in the regions that give rise to the defective structures, but disappear later, suggesting that Prx-l product may be required for the maintenance (proliferation and/or survival) of specific subpopulations of CNCC-derived mesenchymal cells in the branchial arches. In contrast to single mutants, Prx-l/Prx-2 double-knock-out mice exhibited severe craniofacial abnormalities, including pointed snout, the absence of external ears, and severely shortened lower jaws. Double-mutant mice also had novel phenotypes, including abnormalities in the medial region of the developing mandibles, lower incisors, and Meckel's cartilage. Approximately 8% of the newborn double-mutants generated exhibited clefts in the mandible and tongue, whereas the mandibular processes of the double-mutant mice generated lacked the midline symphysis and were fused. In these double-mutants, either a single incisor arrested in the bud stage or no incisors were present. The arrested incisor tooth buds showed decreases in the expression of Pax-9 and patched. Furthermore, in these doublemutants, most of Meckel's cartilage was absent. The phenotypic abnormalities in Prx-l and Prx-l/Prx-2 mutants indicate redundant but essential roles for Prx-l and Prx-2 in the signaling network regulating epithelial-mesenchymal interactions that promote outgrowth and skeletogenesis in the mandible. Other members of the paired-related family of homeobox genes in vertebrates include Alx-3, Alx-4, and Cart-l. These genes are also expressed in the distal part of the mandibular arch. However, no phenotypic abnormalities in the developing mandible have been reported in mice lacking these genes. It is possible that, similar to Prx-l and Prx-2, the absence of abnormalities in the mandibular arch in these knock-outs may be due to functional redundancies.

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5.7. Barx genes

Barx-l and Barx-2 are members of the vertebrate Bar class of homeobox-containing genes homologous to Drosophila BarH-l and BarH-2. Three mouse and two chick homologues of the Barx genes have been isolated. Tissue distribution studies showed that these genes are expressed in many sites, including the facial processes. Studies in developing mice showed that, in the developing maxilla and mandible, Barx-l is expressed in the mesenchyme, and Barx-2 is expressed in the overlying epithelium [98].

However, studies in chick embryos indicate that, unlike in the mouse, Barx-l is expressed in both epithelium and mesenchyme of the maxillary and mandibular processes. Furthermore, in chick embryos, Barx-2b-which is 80% and 61% identical to mouse Barx-2 and Barx-l, respectively-is expressed prominently in myogenic populations in the craniofacial region, in the mesoderm of the BA, and in areas of the forming bones. During embryogenesis, Barx-2b is expressed at the tips of the outgrowing maxilla and mandible but disappears by stage 30. In both the chick and mouse, the mesenchymal domain of Barx-l expression is restricted to the lateral region, where FGF-8 is expressed by the overlying epithelium. The expression of Barx-l is excluded from the mesenchyme in the medial region in which BMP-4 is expressed in the overlying epithelium. The expression of Barx-l is also excluded from the central core corresponding to the regions forming Meckel's cartilage and the muscle of the mandibular process.

These patterns of expression suggest the involvement of signals derived from the overlying epithelium (FGF-8/BMP-4) in regulating the spatial patterns of Barx-l expression in the mandibular mesenchyme. In fact, studies in mouse mandibles indicate that beads soaked in FGF-8 can induce/maintain expression of Barx-l in the lateral mandibular mesenchyme. On the other hand, beads soaked in BMP-4 inhibited expression of Barx-l in the lateral mandibular mesenchyme expression. Inhibition of BMP-4 signaling by application of Noggin protein during the early stages of mandibular development resulted in ectopic expression of Barx-l in the mesenchyme in the medial region. Similar antagonistic interactions between BMP and FGF signaling also restrict expression of Barx-l to the maxillary mesenchyme in the posterior region, suggestive of the involvement of Barx-l in patterning of the facial processes.

5.8. HAND genes

dHAND and eHAND, 2 members of the bHLH (basic helixloop- helix) family of transcription factors, are co-expressed in many regions, including the medial region of the developing mandible. Deletion of the dHAND gene in mice resulted in embryonic death at Ell secondary to cardiac failure and many abnormalities, including severely hypoplastic first and second BAs. Molecular analyses indicated that although Prx-l, Dlx-2, eHand, and Msx-2 are expressed at normal levels, Msx-l expression is not detectable in the medial region of the developing mandible of E9.5 dHAND null mutants.

Studies indicate that the hypoplastic mandible in the dHand-/- mutant is not due to defects in the migration of neural crest cells into the branchial arch and occurs secondary to programmed cell death. The unchanged patterns of expression of eHAND in the dHAND mutant suggest that eHAND is unable to compensate fully for dHAND in the branchial arch and that these genes may have some unique roles in the development of the developing mandible [99].

[VI] CONCLUSION

Considering the embryogenesis of craniofacial form, development is genetically determined through neural crest cell migration and through the expression of homeobox gene information. Epithelial-mesenchymal interaction during the process of craniofacial patterning, induction and programmed cell death is mediated by regulatory molecules and growth factor super families controlled by gene expression. So role of genes in craniofacial development is immense and further studies in future may suggest more specific genes. The practical application of this knowledge in the diagnosis and treatment planning will be beneficial. With further advancement in genetic engineering genetic treatment planning of craniofacial defects cannot be ruled out.

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Authors declare no conflict of interests.

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