We are IntechOpen, the world's leading publisher of Open Access books
Built by scientists, for scientists

4,800
Open access books available

122,000
International authors and editors

135M
Downloads

154
Countries delivered to

Our authors are among the
TOP 1%
most cited scientists

12.2%
Contributors from top 500 universities

WEB OF SCIENCE™

Selection of our books indexed in the Book Citation Index in Web of Science™ Core Collection (BKCI)

Interested in publishing with us?
Contact book.department@intechopen.com

Numbers displayed above are based on latest data collected.
For more information visit www.intechopen.com

Chapter 9

Regulatory Functions of *Pax1* and *Pax9* in Mammalian Cells

V. Sivakamasundari, Petra Kraus and Thomas Lufkin

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.71920

Abstract

Pax1 and *Pax9* are paired-box transcription factors, which play vital roles in axial skeletogenesis, thymus organogenesis, palatogenesis and odontogenesis among others. The importance of these closely related transcription factors can be perceived from the various human anomalies associated with their disruption. Vertebral column abnormalities such as kyphoscoliosis, seen in Jarcho-Levine and Klippel-Feil syndromes, secondary cleft palate, oligodontia/ hypodontia (missing teeth) and thymus developmental defects have all been associated with mutations in *PAX1* and/or *PAX9*. In this chapter, we describe the molecular functions of *Pax1* and *Pax9* in various tissues during mouse development.

Keywords: *Pax1*/*Pax9*, intervertebral disc, palatogenesis, odontogenesis, thymus

1. Introduction

A cell is the functional unit of any living organism and the genome is its underlying blueprint. Transcription factors (TFs) are proteins that bind to the DNA in a sequence-specific manner, where they modulate (activate, repress or insulate) the expression of a particular set of genes. Spatio-temporal regulation of a combination of genes, the "gene battery", is the basis of individual cell type determination in a multicellular organism [1].

Gene regulation is a tremendous feat. A single gene can be regulated by multiple TFs, acting on multiple cis-regulatory elements (CREs), in different cells and at different times (i.e. spatio-temporal regulation). Non-coding RNAs (e.g. microRNAs, small nucleolar RNAs etc.) also play a role at a post-transcriptional level [2]. This complex interplay of the various trans-factors acting on the CREs to determine a gene battery can be mapped into a transcriptional

INTECH
open science | open minds

© 2018 The Author(s). Licensee InTech. This chapter is distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/3.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

network. Such networks execute downstream processes like specification, commitment and differentiation of stem cells or progenitors into a particular lineage during development. Dysregulation of transcriptional networks manifests as aberrations in the cells which in turn results in developmental defects or diseases [1, 3].

In this chapter, we will describe the roles of two developmental TFs – *Pax1* and *Pax9*, in mammalian development. The importance of studying the closely related *Pax1* and *Pax9* can be appreciated from the various human anomalies associated with them. Vertebral column abnormalities such as kyphoscoliosis, seen in Jarcho-Levine and Klippel-Feil syndromes, secondary cleft palate, oligodontia/hypodontia (missing teeth) and thymus developmental defects have all been associated with mutations or SNPs in *PAX1* and/or *PAX9* [4–7].

The role of *Pax1* was discovered serendipitously, involving a spontaneous mouse mutant with a kinked tail – named "*undulated*". This mouse mutant carried a point mutation in *Pax1*, which resulted in vertebral anomalies, whereby certain segments of the lumbar vertebrae were missing. This led to a misalignment of the vertebral column hence the kinked tail phenotype. More spontaneous variants of the *undulated* (*un*) mutant were discovered, all of which mapped to some defect in the *Pax1* gene or deletion of its entire locus [8]. *Pax1* paralog, *Pax9*, was also mapped and shown to have a role in the development of various organs. What is more intriguing is how well-conserved the functions of these genes are, such that the defects observed in the loss-of- function *Pax1* or *Pax9* mouse models are phenocopied in humans as well. Thus, analyses of such mouse models help us to glean into the functions of these genes and decipher what organs they are important in.

Pax1 and *Pax9* have a variety of roles in multiple tissues (e.g. scapula, pelvic girdle, limb and salivary gland epithelium) yet their functions have been most extensively studied in axial skeletogenesis, palatogenesis, odontogenesis, and thymus development [9–12]. Hence, in this chapter we will focus on their regulatory functions in the context of these tissues.

2. The evolutionary history of *Pax1* and *Pax9*

Pax genes are a family of developmental TFs with crucial functions in early patterning and organogenesis. The paired box, encoding a highly conserved segment of 128 amino acids with DNA-binding activity, was initially identified in the *Drosophila melanogaster* genes: *paired* (*prd*), and *gooseberry* (*gsb*) by Markus Noll and team in 1986 [13].

Similarity to the paired box led to the identification of the *Pax* gene family in other vertebrates and invertebrates. The ancestral *proto-pax* existed prior to the Cambrian explosion, and the two-rounds of whole genome duplication during or prior to this period, and subsequent divergence with uneven deletion events are believed to have given rise to the various paralogs and orthologues in the vertebrates and invertebrates [14]. The paired box is believed to have originated through domestication of the *Tc1/mariner* transposon, which is prevalent in all orders of living organisms. Currently, *Pax* genes have been identified in all orders of the metazoan species, with nine in mammals (human and mouse), and up to fifteen in *Danio rerio* [15].

The *Pax* genes are divided into two supergroups (PAXB-like and PAXD-like) and four subgroups/subfamilies (I to IV) based on their sequence similarity, the combination of functional domains they possess and overlapping regions of tissue expression. PAXB group contain the paired-domain (with two Helix-Turn-Helix, HTH motifs) (PD), octapeptide motif (HSVSNILG) (OP), and paired type homeodomain (PTHD) (full or truncated). The PAXD-like group contains an additional paired type homeodomain tail (PTH). It is as yet unclear whether *proto-pax* originated from PAXB or PAXD supergroups. These supergroups are further categorized as four subfamilies in vertebrates: Group I (*Pax1* and *Pax9*), Group II (*Pax2, Pax5, Pax8*), Group III (*Pax3, Pax7*) and Group IV (*Pax4, Pax6*) (**Figure 1**) [15].

Pax1 and *Pax9* belong to the same subfamily (Group 1/PAXD-like), containing only the PD and OP. Mouse *Pax1* and *Pax9* share a high amino acid sequence similarity of 79%, diverging mainly at their C-terminal ends. Their paired-domains share 98% identity and differ only at five sites - at the first two amino acids of the PD and at positions 82, 89 and 93 of the proteins, which belong to the C-terminal half of the PD [16]. The amino acid substitution from Tyr to Phe at position 2 of the PD is described to be class-specific [17]. Between species, *Pax* orthologs are highly conserved whereby the coding sequences of human PAX1 and mouse Pax1 share 88.1% identity while the PD share 100% identity. Similarly the PD of human PAX9 and mouse Pax9 share 100% identity, while overall identity is 98% [16, 18]. This high conservation in mouse has allowed it to serve as a suitable model to study the functions of *Pax* genes.

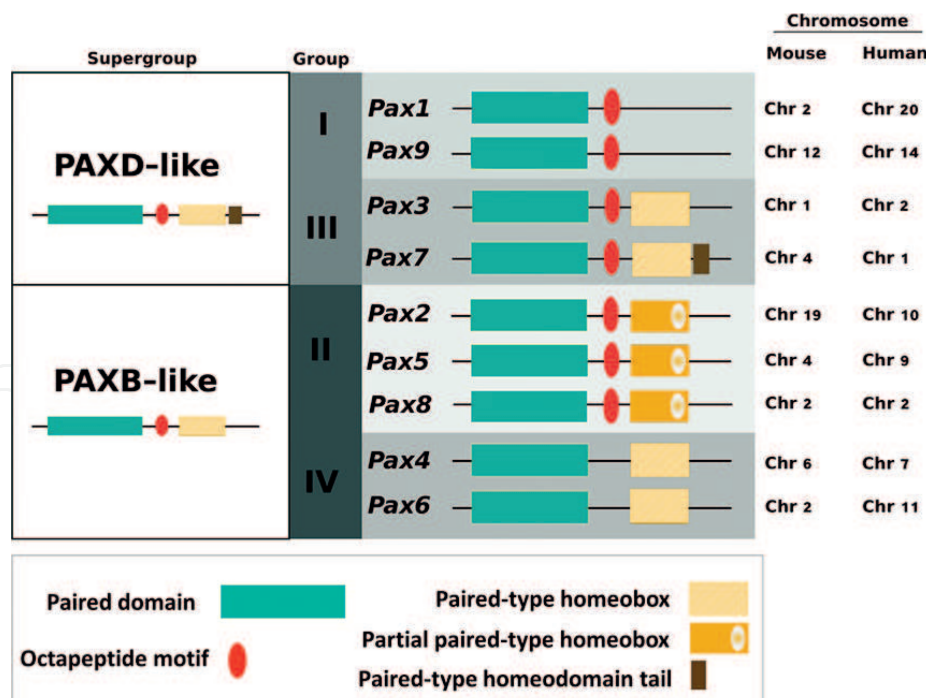

Figure 1. *Pax* genes, structure and grouping in mouse and human. *Pax* genes are divided into supergroups and subgroups. The PAXD-like supergroup is defined by the additional presence of a paired type homeodomain tail. The PD and PTHD have DNA-binding ability and so are drivers of the transcriptional program. The OP is believed to assist in protein-protein interactions, mostly mediating repressive effects of the TFs.

3. Paired domain and DNA recognition

Pax TFs execute their function through their DNA-binding ability aided by the PD and/or homeodomain. DNA-binding ability of the PD was initially demonstrated through *in vitro* biochemical assays on *Drosophila* prd protein binding to the e5 sequence from the *even-skipped* promoter [19]. Since Pax1 and Pax9 do not possess a homeodomain, they are fully reliant upon the PD for binding specificity and affinity. The PD of Pax1 recognizes a 24 bp sequence [20].

Biochemical and crystallographic studies revealed that the PD is a bipartite structure with the N-terminal (PAI) and C-terminal (RED) sub domains, each with a helix-turn-helix (HTH) motif [15, 19, 21, 22]. These subdomains recognize a non-palindromic consensus sequence with two half sites (5′ and 3′) positioned on adjacent major grooves on the same side of the DNA. The PAI subdomain recognizes the 3′ half site of the consensus sequence while RED recognizes the 5′ half site [22]. Our own analysis of *in vivo* Pax9 binding sites in the intervertebral disc (IVD) anlagen revealed a motif "5′-C/A G/A CGTGAACCG-3′" that highly resembles the 3′ half site of the consensus PD motif "5′-GCG G/T A/G AC G/C G/A-3′" (**Figure 2**) [19, 23].

While the PAI domain is most critical for DNA binding, in some scenarios, the Pax protein can bind solely through the RED domain. For instance, in the *undulated* mutants, point mutation

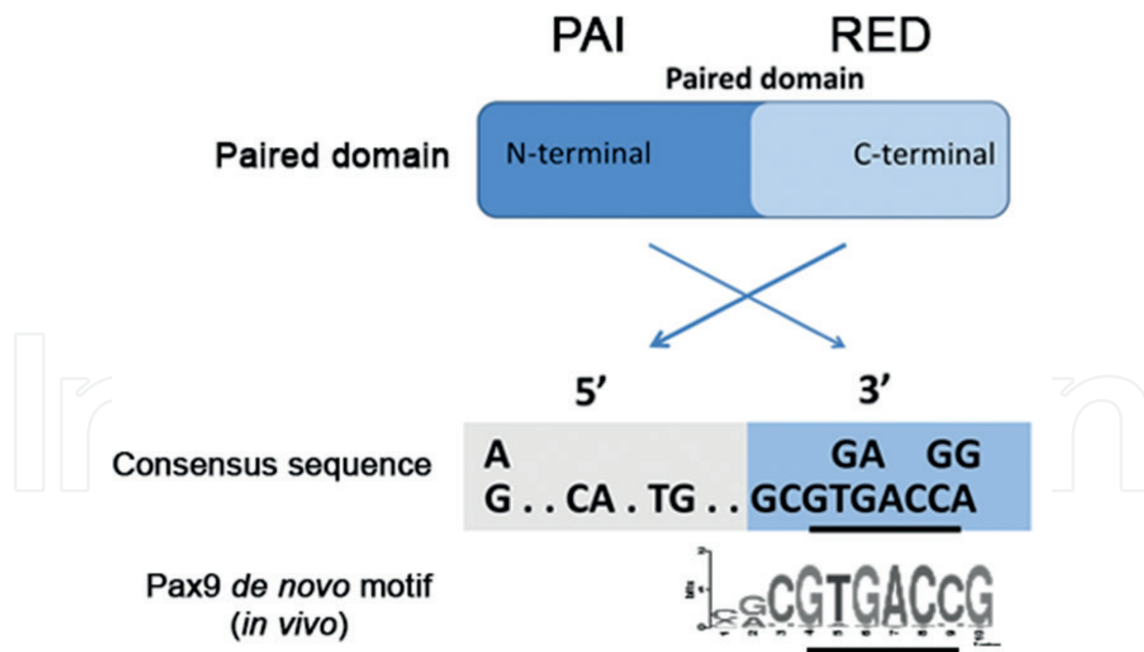

Figure 2. Paired domain and consensus recognition sequence. The paired domain consists of the N-terminal (PAI) and C-terminal (RED) domains. RED recognizes the 5′ half site sequence while PAI recognizes the 3′ half site sequence. The pentanucleotide motif "GGAAC" described by Chalepakis et al. [20] as the core DNA-binding motif of paired domain is underlined. In E12.5 mouse IVD anlagen, Pax9 recognizes an *in vivo* motif resembling the 3′ half site. Abbreviation(s): IVD, intervertebral disc.

http://dx.doi.org/10.5772/intechopen.71920

in the N-terminal half of the PD in Pax1 drastically reduced its binding affinity and altered the specificity, and so resulted in its loss of function [20]. In contrast, particular isoforms of Pax6 and Pax8 bind DNA exclusively through their REI subdomains [24, 25]. Moreover, binding to both half sites by both subdomains confers greater affinity and specificity *in vitro*. The truncated form of Pax5 PD (missing the last 36 amino acid residues of the PD) retained the capacity to bind to a subset of the sequences bound by the complete form, albeit with lower affinity [19]. Thus, these subdomains are modular. Their ability to bind independently or in combination is postulated to confer greater diversity in the repertoire of sequences that can be bound by the PD.

4. Expression patterns of *Pax1* and *Pax9* in mouse development

Like numerous other developmental TFs, Pax TFs are characterized by spatio-temporally restricted expression during embryogenesis, playing essential roles in early patterning and organogenesis. They can be generalized to have a role in proliferation, migration, condensation and differentiation functions in different cell types. Their expression is often down-regulated or turned off in terminally differentiated tissues. Dysregulation of *Pax* gene expression often results in various developmental abnormalities and has also been observed in various cancers such as esophageal squamous cell carcinoma, non-small cell lung cancer and cervical intraepithelial neoplasia [26–28].

During development, *Pax1*/*Pax9* are the only *Pax* genes not expressed in neural tissues but instead are expressed in the endoderm- and mesoderm-derived tissues [29]. They share similar tissue sites of expression namely the foregut epithelium, sclerotome, pharyngeal pouch endoderm and limb bud mesenchyme [30]. However, unlike *Pax1*, *Pax9* is expressed in neural crest-derived tissues. Both *Pax* genes begin to be expressed in the somites and foregut as early as E8.5, the pharyngeal pouches at E9.0, limb buds at E10.0 to E11.5 and thymus anlagen at E12.5 [9, 16, 31]. In tissues where they are co-expressed, especially the sclerotome-derived axial skeleton, they are known to have redundant, compensatory roles. On the other hand, they are unable to rescue each other's functions in tissues where they are not co-expressed.

5. Pleiotropic roles of *Pax1* and *Pax9* in mouse development

Developmental TFs are pleiotropic. While the very definition of pleiotropy has several meanings in development, evolution and genetics, here we employ the definition of one gene affecting multiple phenotypes [32]. *Pax1* and *Pax9* are no exception. They have multiple roles and act on different tissues which are derived from different germ layers. Therefore, when disrupted, they exhibit complex phenotypes depending on which tissues are disrupted during development.

5.1. *Pax1* and *Pax9* in sclerotome-derived IVD of the axial skeleton

The axial skeleton is a critical load-bearing structure of the vertebral body plan and also functions to protect essential spinal nerves. It is composed of the metameric arrangement of vertebral bodies (VBs) connected by fibrocartilaginous intervertebral discs (IVDs) [33].

Axial skeletogenesis in mouse is a precisely coordinated series of processes; an interplay between the notochord and paraxial mesoderm-derived somites. It begins with the specification of the ventral somites into sclerotome by Sonic hedgehog (Shh) signals emanating from the notochord and floor plate of the neural tube [34–37]. Shh acts partly by antagonizing Wnt signals from the dorsal neural tube and surface ectoderm and BMP signals from the dorsal neural tube or lateral plate mesoderm.

Throughout IVD development, *Pax1* and *Pax9* share largely overlapping expression domains. *Pax1* expression can be detected in the de-epithelializing ventral somites as early as E8.5, while *Pax9* expression is detected slightly later at E9.0. These sclerotomal cells proliferate and then migrate to surround the notochord and form the mesenchymal prevertebrae. By E11.5, these give rise to metameric condensations along the anteroposterior (A/P) axis. Within these condensed segments, *Pax1* is uniformly expressed in rostral and caudal regions, while *Pax9* remains restricted to the caudal portion, but by E12.5, *Pax1* also becomes restricted to the caudal half which will give rise to the IVD anlagen [16, 38, 39]. Sclerotomal cells in close proximity to the notochord give rise to VBs and IVDs while the lateral regions develop into the proximal parts of the ribs, vertebral pedicles and laminae of the neural arch. Subsequently, the condensed portions of the prevertebrae give rise to the IVD and the less condensed regions give rise to the VB. Formation of these condensations is mandatory for the subsequent chondrogenesis into IVD segments of the axial skeleton [16, 40–42].

By E12.5, *Pax1* and *Pax9* expression are restricted to the IVD and are not expressed in the VB. Within the IVD anlagen *Pax1* and *Pax9* expression domains differ slightly. While *Pax1* is strongly expressed in the medial segment, *Pax9* is stronger in the lateral regions. Then the distinction between IVD and VB becomes more apparent at E13.5. The IVD mesenchyme further differentiates into the inner cartilaginous annulus fibrous (IAF) and outer annulus fibrous (OAF) at around E14.5. *Pax1* remains expressed in the IVD and perichondrium of the VB, while *Pax9* is weakly expressed in the IVD. At E15.5, their expression declines within the IAF and become restricted to the OAF. *Pax9* is no longer detected in the vertebral column at E16.5 but mild *Pax1* expression has been detected in the OAF [16, 23, 39, 43].

5.1.1. Regulation of Pax1 and Pax9 and their role in sclerotome maintenance

Pax1 and *Pax9* can be regulated by multiple mechanisms in the somites and sclerotome. Shh induces the expression of *Pax1*, *Pax9* and *Mesenchyme forkhead-1* (*Mfh1*) in the ventral somites which communicate its proliferative function [35, 37]. *Pax1*, *Pax9* and *Mfh1* are vital for maintaining the sclerotome cell numbers. In fact, *Pax1* and *Mfh1* genetically interact as *Pax1*$^{-/-}$*Mfh1*$^{-/-}$ mutants show reduced cell proliferation [35]. Noggin (Nog) also induces *Pax1* expression in

the absence of Hh signaling (in *Shh*$^{-/-}$ mutants) [44, 45]. Other factors which do not independently induce *Pax1*/*Pax9* expression but can regulate their expression in the somites are *Pbx1*/*Pbx2* and *Meox1*/*Meox2*. In both *Pbx1*$^{-/-}$*Pbx2*$^{-/-}$ mutants and *Meox1*$^{-/-}$*Meox2*$^{-/-}$ mutants, *Pax1* and *Pax9* expression is diminished in the somites/sclerotome, although *Pax9* to a lesser extent [46, 47]. Furthermore, Pax1 potentially auto-regulates itself as *Pax1*$^{-/-}$ mutants show reduced *Pax1* mRNA expression. *Pax9* however is independent of *Pax1* in the sclerotome, as *Pax1*$^{-/-}$ mutants do not show any reduction in *Pax9* mRNA [23]. Thus, *Pax1* and *Pax9* can be regulated by different upstream regulators most of which remain to be identified.

5.1.2. Molecular functions of Pax1 and Pax9 in axial skeletogenesis

The roles of *Pax1* and *Pax9* in vertebral column development were first identified through spontaneous mouse mutants – *undulated* (*un*) [48], *Undulated short-tail* (*Un*^{s}) [49], *undulated-extensive* (*un*^{ex}) [50] and *undulated intermediate* (*un-i*) [51] – which encompass a mutation in *Pax1* or deletion of the loci containing *Pax1* [8]. Subsequent gene-targeted knock-out models of *Pax1* [9] and *Pax9* [30] and generation of compound mutants revealed their synergistic, gene-dosage dependent, redundant roles in axial skeletogenesis [23, 52].

Pax1$^{-/-}$ mice exhibit a characteristic short, kinked tail phenotype with defects in the vertebral column (cervical and lumbar), scapula (loss of acromion process) and sternum (inappropriate ossification of some of the inter sternebrae). Within the vertebrae, the lumbar regions show a more pronounced phenotype of split vertebrae with loss of IVDs and formation of a ventral rod-like cartilaginous structure. They also lack the pharyngeal pouch derivatives thymus and parathyroid glands. However, these mice were viable and fertile. Even though *Pax1*$^{+/-}$ show an overall normal phenotype externally, they possess slight abnormalities in the vertebral column and sternum with varying penetrance, indicating haploinsufficiency of *Pax1* in these structures [9].

Contrary to *Pax1*$^{-/-}$ mice, *Pax9*$^{-/-}$ mutants surprisingly do not possess any vertebral column defects. Instead they show defects in all the pharyngeal pouch-derived structures. They exhibit cleft secondary palate, and lack all teeth, both of which are derived from 1st pharyngeal pouch. Further, they lack thymus, parathyroid glands and ultimobranchial bodies, which are derived from the 3rd and 4th pharyngeal pouches. They also display preaxial polydactyly of fore- and hind-limbs. These mice display post-natal lethality, and inability to feed owing to a cleft palate. While *Pax9*$^{+/-}$ mutants did not exhibit any overt defects, a hypomorphic allele, *Pax9*^{neo} showed that *Pax9* is haploinsufficient for tooth development, but not for other structures [30, 53].

Considering the overlapping expression domains in the vertebral structures, compound mutants of *Pax1* and *Pax9* were generated [52]. Increasing severity in vertebral column defects was observed with successive loss of *Pax1* and *Pax9* alleles. The most severe phenotype was displayed by *Pax1*$^{-/-}$*Pax9*$^{-/-}$ mutants that exhibited a complete loss of VB and IVDs, no caudal vertebrae and malformed proximal parts of the ribs. These vertebral column abnormalities, however, were more severe than those seen in individual null mutants of *Pax1* and *Pax9*, indicating their synergistic roles in the vertebral column. The lack of vertebral elements did

not result from lack of sclerotome specification, since sclerotomal cells were present in compound mutants, albeit in reduced numbers. Therefore it was hypothesized that *Pax1/Pax9* are required to maintain the proliferative capacity of the sclerotomal cells. Intriguingly, it was discovered that *Pax9* was unable to fully compensate for the loss of *Pax1* but *Pax1* could fully rescue *Pax9* deficiency in the axial skeleton. Notably, *Pax1* was unable to rescue orofacial defects seen in *Pax9*-null mutants since *Pax1* is not expressed in the dental primordia [52].

From these studies and others from our lab, it became evident that *Pax1/Pax9* have dual roles in axial skeletogenesis: (1) they maintain sclerotome cells in sufficient numbers and in appropriate locations for IVD anlagen formation through the regulation of proliferation and cell migration; (2) they contribute to the IVD mesenchymal condensation process through the activation of early chondrogenic genes (*Sox5, Bmp4, Co2a1, Acan, Wwp2*), likely in conjunction with *Sox* trio, TGF-b and BMP pathways. In fact, we will observe in the later parts of this chapter that proliferation, migration and mesenchymal condensation are fundamental functions of *Pax1* and *Pax9*, themes which will be replayed in the development of dental mesenchyme and thymus.

A certain number of sclerotomal cells are necessary for a critical size of condensation to form, upon which endochondral ossification can occur. As mentioned earlier, *Pax1* is known to genetically interact with *Mfh1*, another TF expressed in the sclerotome, to synergistically control sclerotome proliferation [35]. Indeed, regulation of proliferation could be a general conserved function among *Pax* genes; *Pax5* is known to regulate B cell proliferation and *Pax6* diencephalic precursor cells proliferation [54, 55]. We further confirmed a role for *Pax1/Pax9* in cell proliferation through a combinatorial approach of performing transcriptomic profiling on *Pax1*- and *Pax9*-specific cells and identifying the direct binding targets using Chromatin immunoprecipitation sequencing (ChIP-seq) [23]. Befitting their dosage effect on axial skeletogenesis, increasing numbers of targets were dysregulated with increasing loss of *Pax1* and *Pax9* alleles. Especially, a substantial number of genes associated with proliferation were affected only upon the loss of three ($Pax1^{+/-}Pax9^{-/-}$ and $Pax1^{-/-}Pax9^{+/-}$) or four ($Pax1^{-/-}Pax9^{-/-}$) alleles of *Pax1/Pax9* compared to the loss of two alleles ($Pax1^{-/-}$). Corroborating this, phenotypical decrease in the number of sclerotomal cells was more apparent in mutants with the loss of three or four alleles [23].

Besides proliferation, *Pax1* and *Pax9* also have roles in cell motion, adhesion and mesenchymal condensation through extracellular matrix (ECM) organization. Sclerotomal cells become mislocalized to the lateral sides in E14.5 $Pax1^{-/-}Pax9^{-/-}$ embryos; a defect not observed in $Pax1^{-/-}$ mutants. Cellular motion associated genes were also dramatically affected in the double null mutants, thus affirming the role of *Pax1* and *Pax9* in regulating cell motion [23].

The cell-type-specific molecular approach also revealed novel functions of *Pax1/Pax9* in regulating genes associated with collagen fibrillogenesis and cartilage development independent of *Sox9*, like *Col2a1, Bmp4, Acan, Sox5* and *Wwp2*. *Col2a1, Wwp2* and *Sox5* are also directly regulated by Pax9 in the vertebral column, and a single copy of *Pax1* or *Pax9* can independently maintain transcription of these critical IVD genes [23]. Additionally, Pax1 has been shown to induce *Acan* in chick presomitic mesoderm explants, independent of *Shh* [56]. A further confirmation of genetic linkage of these genes with *Pax1/Pax9* is that knock-out mouse mutants of

	Gene	Expression sites in developing embryo	Function	References
1	*Col2a1*, Collagen Type II, apha 1	(1) Sclerotome (2) Vertebral, intervertebral disc, tail, limb and craniofacial cartilage condensations (3) Limb, head and shoulder mesenchyme	(1) Major ECM component of cartilage (2) Collagen fibrillogenesis (3) Cartilage development (4) TGF-beta tethering in extracellular matrix (ECM) to modulate its signaling.	[23, 52, 57, 61–63]
2	*Acan*, Aggrecan	(1) Vertebral, intervertebral disc, tail, limb and craniofacial cartilage condensations (2) Limb, head, nasal mesenchyme	(1) Major ECM component of cartilage (2) Cartilage development (3) Water retention and maintain osmotic pressure in cartilage	[61–63]
3	*Sox5*, SRY-box-containing gene 5	(1) Vertebral, intervertebral disc, tail, limb and craniofacial cartilage condensations (2) Forebrain	(1) ECM synthesis (2) Cartilage development (3) Chondrocyte differentiation	[23, 60–65]
4	*Wwp2*, WW domain containing E3 ubiquitin	(1) Maxilla and mandible (2) Vertebral and intervertebral disc condensations	(1) Ubiquitylation of proteins (2) Mono-ubiquitylates Sox9 and enhances its transcriptional activity (3) Forms a complex with Sox9: Sox9-Wwp2-Med25 complex which drives *Col2a1* expression. (4) Palatogenesis	[23, 62, 67]
5	*Bmp4*, Bone morphogenetic protein 4	(1) Limb and head mesenchyme (2) Nasal pit epithelium (3) Vertebrae and intervertebral disc cartilage condensations (4) Dental and palatal mesenchyme	(1) Growth factor to activate BMP signaling (2) BMP signaling promotes ECM production and chondrocyte proliferation (3) Cartilage development and chondrocyte differentiation (4) Bmp4 up-regulates cartilage marker genes likes *Acan, Sox5, Sox6* and *Sox9*.	[23, 62, 68, 71]

Abbreviation(s): ECM, extracellular matrix.

Table 1. Expression sites and functions of selected *Pax1*/*Pax9* downstream targets essential in axial skeletogenesis.

Col2a1 [57], *Acan* [58], *Wwp2* [59] and *Sox5* [60] exhibit axial skeletal and craniofacial defects that phenocopy $Pax1^{-/-}Pax9^{-/-}$ mutants (**Table 1**) [52, 57, 61–68].

Importantly, *Pax1*/*Pax9* and *Sox5*/*Sox6* were linked by a negative feedback loop in the vertebral column. This *Pax-Sox* network might be essential in the segregation of IAF and OAF. *Sox5*

and *Sox6* play redundant but vital roles in IVD morphogenesis by regulating the timely maturation of chondroblasts and promoting inner annulus differentiation [60]. They are known to regulate ECM genes *Col2a1* and *Acan* in conjunction with Sox9 as *Sox* trio (*Sox5*/*Sox6*/*Sox9*) [60, 63, 66, 69]. On the other hand, *Pax1* and *Pax9* are down-regulated during the maturation of pre-chondrogenic cells into chondrocytes in the IAF and become restricted to the fibrotic OAF. Cell-type-specific analysis of EGFP-targeted *Sox5*$^{-/-}$*Sox6*$^{-/-}$ mutants (generated in our lab by a similar strategy as the *Pax1*/*Pax9* alleles) revealed that *Sox5*/*Sox6* repressed *Pax1*, while *Pax1*/*Pax9* positively regulated *Sox5* in the IVD anlagen cells [70]. This negative feedback circuit between *Pax* and *Sox* could therefore explain the initial co-expression of *Sox* and *Pax* in the IVD mesenchyme at E12.5-E13.5, and the subsequent restriction of *Pax1*/*Pax9* to the OAF by E15.5 [23].

Pax1 and *Pax9* also have a subsequent role in IVD differentiation through their connection with *Sox5*/*Sox6*, BMP and TGF-b pathways. First, TGF-b and BMP components - *Smad3*, *Tgfbr2*, *Tgfb3* and *Bmp4* are all expressed in the IVD anlagen at E12.5 and become restricted to the OAF by E14.5 [23, 71]. Second, TGF-b signaling is essential to maintain the boundary between VB and IVD, by preventing the inappropriate chondrogenic differentiation in the future IVD segment of the sclerotome and promoting annulus fibrosus development of the IVD [71–73]. Conversely, BMP signaling promotes chondrogenic differentiation of sclerotome cells by regulating the *Sox* trio and cartilage genes (*Acan* and *Wwp2*) [71]. Third, *Pax1*/*Pax9* regulate *Bmp4* and BMP- and TGF-b- regulated targets in the IVD anlagen (**Figure 3**). The continued expression of *Pax1*/*Pax9*, *Bmp4* and TGF-b pathway components in the OAF at E14.5 suggests their involvement in further differentiation of the OAF [23].

In terms of compensatory roles, compared to *Pax9*, *Pax1* is the more dominant player in axial skeleton development. The primary reason is that *Pax1* has the ability to fully compensate for *Pax9* deficiency in the vertebral column, by up-regulating its own expression through auto-regulation. *Pax9*$^{-/-}$ mutants show upregulated *Pax1* expression. The inverse, however, is not true as *Pax9* is incapable of upregulating itself in *Pax1*$^{-/-}$, thus being unable to match the dosage required to rescue *Pax1* function [23, 30, 52]. While dosage may partly explain the defect, the high homology shared between the PD of Pax1 and Pax9 makes one wonder if Pax9 can truly regulate all of the Pax1 targets if knocked into the *Pax1* locus. In fact, *Pax1* and *Pax9* can independently regulate some of the same set of critical IVD genes (e.g. *Sox5*, *Col2a1* and *Wwp2*). Thus, a *Pax9*-knock-in to *Pax1* locus would abrogate any temporal and spatial differences between *Pax1* and *Pax9*, and allow us to investigate if *Pax9* is truly capable of performing the functions of *Pax1* or if both inherently regulate different set of targets.

In humans, *PAX1* and *PAX9* have been linked to Jarcho-Levine and Klippel-Feil syndromes, characterized by vertebral anomalies such as kyphoscoliosis or vertebral segmentation defects that phenocopy *Pax1*$^{-/-}$*Pax9*$^{-/-}$ mouse mutants [4, 74, 75]. Indeed, several of the *Pax1*/*Pax9* regulated genes have been associated with similar axial skeleton defects [23]. Of these, mutations in *ACAN* have been linked to spondyloepiphyseal dysplasia (SEMD) and mutations in *COL2A1* is responsible for certain forms of SEMD [76, 77]. Identification of *Pax1*/*Pax9* as upstream regulators of these genes suggests that dysregulation of *PAX1*/*PAX9* function can reduce the levels of downstream targets like *Acan* and *Col2a1* which in turn lead to vertebral anomalies.

http://dx.doi.org/10.5772/intechopen.71920

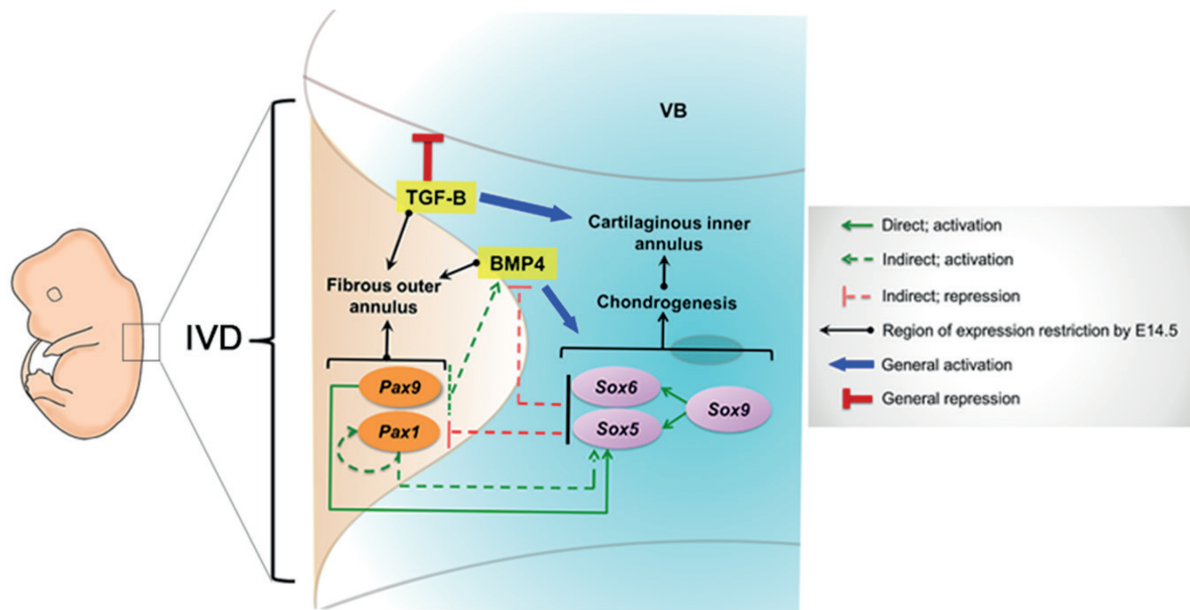

Figure 3. Schematic of *Pax*-*Sox*-TGFb-BMP4 network in the development of embryonic IVD. TGF-b signaling maintains the boundary between vertebral body (VB) and intervertebral disc (IVD), by preventing the inappropriate chondrogenic differentiation in the future IVD segment. *Bmp4* is regulated by *Pax1*/*Pax9* and the *Sox* trio. *Bmp4* itself regulates the *Sox* trio. The negative feedback loop mechanism between *Pax1*/*Pax9* and *Sox5*/*Sox6*, and their connection to *Bmp4* is postulated to be essential in the segregation of IAF and OAF during IVD development. At E14.5, expression of *Bmp4*, *Pax1*, *Pax9* and *Tgfb3* are restricted to the OAF while the *Sox* trio is retained in the IAF. Abbreviations: VB, vertebral body; IVD, intervertebral disc; TGF-b, transforming growth factor, beta; BMP4, bone morphogenetic protein 4.

5.2. *Pax1* and *Pax9* in pharyngeal-derived tissues

The pharyngeal endodermal pouches (Pp) are pockets that develop successively from the foregut endoderm in a rostro-caudal fashion. They are depressions found in between the branchial/pharyngeal arches which form in the cranial lateral parts of the embryo. These Pp and arches encompass cells derived from the three different germ layers – ectoderm, endoderm and mesoderm- as well as neural crest-derived mesenchyme [78, 79].

Each Pp gives rise to different craniofacial and glandular structures. The 1st Pp (Pp1) gives rise to the maxillary and mandibular structures, 3rd Pp (Pp3) gives rise to thymus and parathyroid glands, and the 4th Pp (Pp4) gives rise to the ultimobranchial bodies which subsequently give rise to thyroid C cells. The 2nd Pp (Pp2) is known to give rise to the palatine tonsil epithelium in all mammals except rodents, and in non-mammals (e.g. avian) it is suspected to give rise to salivary glands, although the latter remains to be investigated in more species [78, 79].

5.2.1. Pax9 in palatogenesis

Pp1-derived maxillary and mandibular prominence are the foundation structures for proper palatogenesis and odontogenesis. The shared developmental ontology of palate and teeth thus result in the co-occurrence of orofacial clefts and tooth agenesis when genes underlying Pp development are disrupted [80]. In fact, the molecular networks that regulate palatogenesis

and odontogenesis share mostly the same set of genes, although the hierarchy and connections between them is tissue-dependent.

Palate, the roof of the mouth, is the structure that helps separate the nasal from the oral cavity. It consists of the anterior hard palate and posterior soft palate. The primary palate forms the anterior portion, and is derived from the medial nasal process. The pair of medial outgrowth of the maxillary processes form the palatal shelves which elevate horizontally above the dorsum of the tongue and fuse to form the secondary palate [81]. *Pax9* is expressed in the neural crest-derived medial nasal process at E10.5, which subsequently develops into the maxillary prominence (upper jaw). *Pax9* then begins to be expressed in the palatal shelf mesenchyme at E12.5 onwards in a posterior-to-anterior gradient.

Pax9$^{-/-}$ mutants exhibit deficiency in primary palate outgrowth. Also, their palatal shelves are abnormally shaped and fail to elevate, resulting in failure of palatal fusion [30, 82]. Conditional knock-out of *Pax9* specifically in neural crest cells (*Pax9flox/Wnt1-Cre*) showed definitive proof that defects in the neural crest-derived mesenchymal components are the underlying basis for the palatal defects seen in *Pax9*-null mutants [83]. Disrupted anterior-posterior (A/P) patterning of the palatal shelves and decreased posterior palate mesenchymal proliferation are believed to be underlying cause of the palate defects in *Pax9*-null mutants [82].

Current studies begin to reveal a molecular network involving *Pax9, Msx1, Bmp4, Osr2, Fgf10* and *Shh* in palatogenesis. In *Pax9*-deficient mutants, *Shh* in the palatal epithelium and rugae, and *Msx1*, *Bmp4*, *Osr2* and *Fgf10* in the palate mesenchyme were all reduced, indicating *Pax9* is located upstream of these factors in the network hierarchy. Studies suggest that *Pax9* modulates A/P patterning through the *Bmp4/Shh* axis, and palate growth and elevation through *Osr2/Fgf10/Shh* cascade, whereby both *Shh* and *Pax9* independently regulate *Osr2* (**Figure 4**) [81, 82, 84, 85]. A more recent study has shown the involvement of Wnt signaling downstream of *Pax9* to play a role in palate elevation as well. How these multiple factors are integrated in this complex morphogenetic process remains to be fully understood. Especially, we still lack information on which targets are directly regulating each other and how these networks are integrated at a single cell level.

Contrary to *Pax9*, *Pax1* is not expressed in the dental and palatal mesenchyme. This explains the differential phenotypic abnormalities seen in *Pax1*$^{-/-}$ vs. *Pax9*$^{-/-}$ mutants. *Pax1*$^{-/-}$ mutants never exhibit the striking craniofacial defects - cleft secondary palate, defective primary palate and tooth agenesis seen in *Pax9*$^{-/-}$ mutants [9, 30]. *Pax1*, however, is expressed in a different domain of the facial mesenchyme, but its function in this tissue remains to be investigated [16, 43].

5.2.2. Pax9 in odontogenesis

Even though anatomical differences exist between mouse and human odontogenesis, the genetic basis of tooth development is conserved between vertebrates [53]. In humans, among the orofacial developmental defects, two most common anomalies are tooth agenesis and orofacial clefts. Worldwide, about 1 in 1000 individuals suffers from oligodontia [86]. Dominant heterozygous mutations in PAX9 have been identified to be the underlying genetic cause

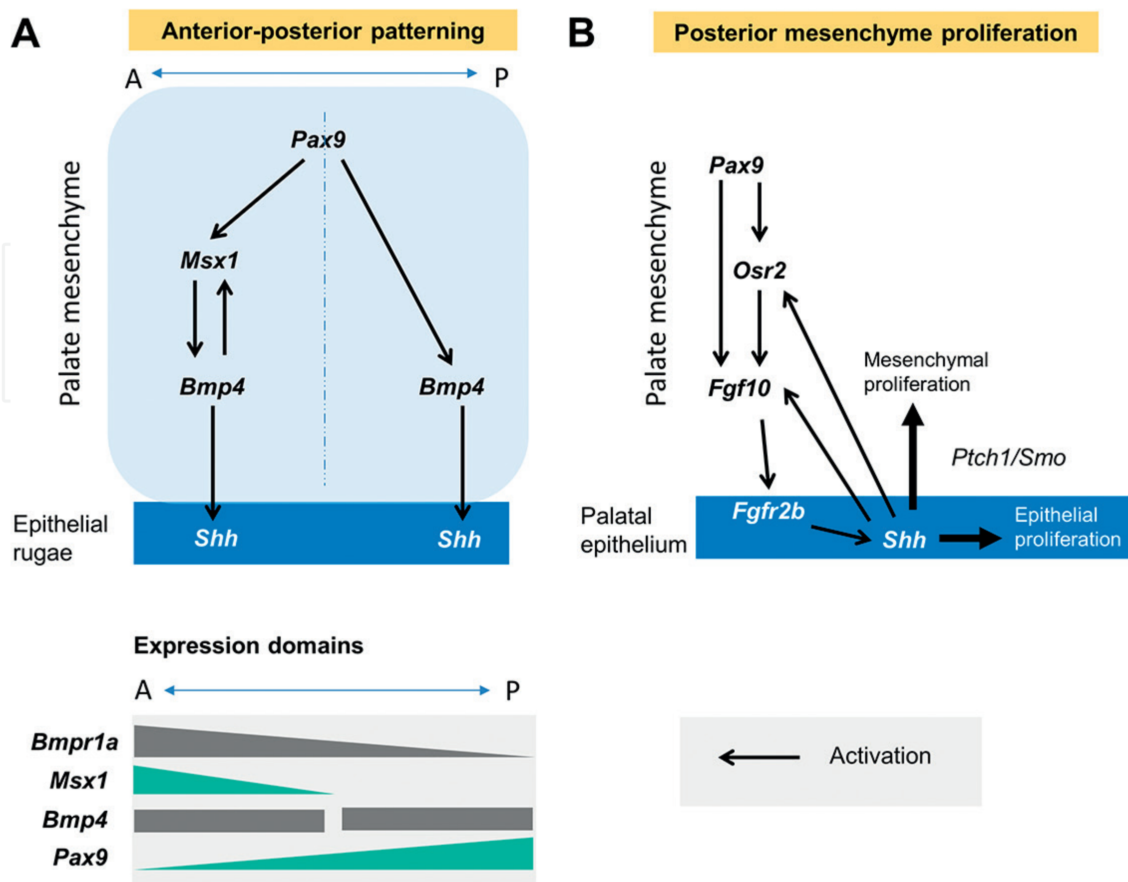

Figure 4. Pax9 molecular network in palatogenesis. (A) *Shh* expressing epithelial rugae serve as signaling centers for coordinating the A/P patterning and anterior outgrowth of the palate. *Shh* expression is maintained in the anterior epithelium by *Msx1*/*Bmp4* and in posterior epithelium by *Pax9*-mediated *Bmp4*, independent of *Msx1*. The expression level of the various factors in the anteroposterior axis is illustrated below. *Bmp4* expression was not detected in the mid-region of the palate at E12.5-E13.5 [82]. (B) *Pax9* regulates mesenchymal proliferation in the posterior palate through the *Osr2*/*Fgf10*/*Shh* axis. Shh also regulates *Osr2* independently. This mesenchymal-epithelial signaling in the palate is essential to maintain proliferation of both the mesenchyme and epithelium [85]. Abbreviations: A/P, anterior-posterior; A, anterior; P, posterior.

of non-syndromic forms of tooth agenesis in some cases [80]. Identification of the genetic cascade involved in odontogenesis in mouse will therefore greatly assist in rectifying tooth agenesis in humans. Elucidation of these pathways is also important for stem cell directed therapies for tooth agenesis.

Similar to the palate, dental mesenchyme is also derived from cranial neural crest cells, and so show defects in *Pax9*-null and *Pax9*-cKO mutants. After patterning during early embryonic stages, which determine the sites, size of tooth field and type of teeth that should develop, tooth development at the specified regions begins. Tooth development happens through a succession of morphogenetic changes and differentiation involving the proverbial epithelial-mesenchymal interactions for signal exchange - between the dental mesenchyme (of the Pp1-derived mandible and maxilla), and the overlying dental epithelium [87]. It involves a back-and-forth, dynamic “developmental power” shift between the epithelium and mesenchyme throughout development.

The epithelium and mesenchyme together go through a series of stages from the epithelial thickening (at E11.0) to bud (E13.5), cap (E14.5), bell (E16.5-E18.5) and tooth eruption stages. Tissue recombination experiments early on showed that oral epithelium from E9.0 to E11.5 possessed odontogenic potential to induce tooth development in the underlying non-dental, neural crest-derived mesenchyme, but not in the non-neural crest-derived limb mesenchyme [88, 89]. This tooth inductive potential then shifts to the dental mesenchyme. Indeed the dental mesenchyme was able to induce tooth development when combined with a non-oral epithelium, but the dental epithelium had lost this ability at E13.0. In a similar manner, at E14.5, the odontogenic potential shifts to the epithelial enamel knot, a transient signaling core that drives the progression from cap to bell stages [90].

In early tooth morphogenesis, *Pax9* is known to play dual roles in patterning the dental mesenchyme: (1) maintenance of *Bmp4* mesenchymal expression to drive tooth progression from bud to cap stage; (2) restricting *Msx1*/*Bmp4* signal mediated dental mesenchyme proliferation to the buccal side by maintaining *Osr2* expression on the lingual side.

Pax9 is not needed for tooth bud initiation, but is required for its subsequent progression to the cap stage. *Pax9* is initially induced in the dental mesenchyme of prospective molar and then incisor regions at E10.0 by diffusible FGF8 signals derived from the oral epithelium. In turn, *Pax9* expression is restricted to specific domains by the counter inhibition of Bmp4 from the epithelium and Bmp2 in the lateral mandibular mesenchyme [91]. Once initiated *Pax9* expression is maintained and is no longer dependent on inductive signals from the oral epithelium. *Pax9* remains expressed in tooth mesenchyme up to E16.5 performing its role in patterning, proliferation and condensation. Hypomorphic *Pax9* mutants revealed a gene-dosage dependency on *Pax9* for tooth formation. In these mice, decreased *Pax9* levels led to reduction in number of dental mesenchymal cells, hence defective mesenchymal condensation and subsequent developmental delay in molar development. However, *Pax9*-null mutants exhibit a dramatic phenotype where they lack all teeth [30, 53].

In vivo and *in vitro* studies revealed more complexity in the tooth morphogenetic process, involving a *Pax9*/*Msx1*/*Bmp4*/*Osr2* signaling axis [92, 93]. *Pax9*-null mutants showed reduced *Msx1*, *Bmp4* and *Osr2* expression in the dental mesenchyme suggesting that it is on top of the network hierarchy. In addition, *Pax9* and *Msx1* are co-expressed in the dental mesenchyme and synergistically regulate tooth development through *Bmp4*. Single homozygous mutants of *Pax9*$^{-/-}$ and *Msx1*$^{-/-}$ show cleft palate with arrested tooth development [30, 94]. *Msx1*-null mutants however showed reduction only in *Bmp4* but not in *Pax9* or *Osr2* [95]. Although *Pax9* is upstream of *Msx1*, it is not necessary for *Msx1* expression during tooth initiation at E12.5, but is required for its activation at later stages (E13.5-E14.5). In turn, both Pax9 and Msx1 interact at the protein level to synergistically drive *Bmp4* expression [92, 96], which appears to be primarily driven by the paired domain of Pax9. The epistatic relationship between *Pax9*, *Msx1* and *Bmp4* was further evident through the partial rescue of dentition defects in *Pax9*$^{+/-}$*Msx1*$^{+/-}$ mutants by re-expression of *Bmp4* [92].

BMP4 signaling is required downstream of *Pax9* and *Msx1* for tooth morphogenesis to progress from the bud to the cap stage, failure of which will result in tooth agenesis. Mice with

http://dx.doi.org/10.5772/intechopen.71920

neural crest-specific inactivation of *Bmp4* (*Bmp4f/f;Wnt1Cre*) exhibit arrested development at the bud-stage in mandibular molar teeth [95]. While in early tooth initiation Bmp4 from the oral epithelium has a repressive role on *Pax9*, once *Pax9* expression becomes independent of epithelial signals, the *Bmp4*/*Pax9* hierarchy becomes inverted and *Bmp4* is no longer able to inhibit *Pax9*. Rather *Bmp4* expression becomes dependent on Pax9 and Msx1 [92].

Besides *Pax9*, another layer of patterning of the dental field is driven by *Osr2*, a negative regulator of odontogenic potential, mediated by its inhibition of *Bmp4* in the lingual region. Both *Osr2* and *Bmp4* are expressed in opposing gradients in the dental mesenchyme: *Osr2* is expressed in a lingual-buccal gradient while *Bmp4* is expressed in a buccal-lingual gradient. Moreover, *Osr2*$^{-/-}$ mutants exhibit supernumerary teeth lingual to molars. Genetic inactivation of *Osr2* in *Msx1*$^{-/-}$;*Bmp4*^{cKO} mice rescued the dental defects. Additionally, *Bmp4* expression in the dental mesenchyme was rescued in the *Msx1*$^{-/-}$*Osr2*$^{-/-}$ mutants. Osr2 could stably interact with Msx1 at the protein level and weakly with Pax9, suggesting a potential competition between Osr2 and Pax9 in partnering with Msx1 to drive *Bmp4* expression [93]. These observations thus put forth a more defined but complex regulatory mechanism at play in the dental domain (**Figure 5**).

In humans, mutations in paired domain of *PAX9*, which in turn lead to defective PAX9 function, or mutations in the conserved regulatory elements of *PAX9*, which lead to reduced PAX9 levels, have been associated with autosomal dominant hypodontia [97–101]. In certain severe cases of non-syndromic oligodontia, the heterozygous deletion of *PAX9* locus, or mutations in

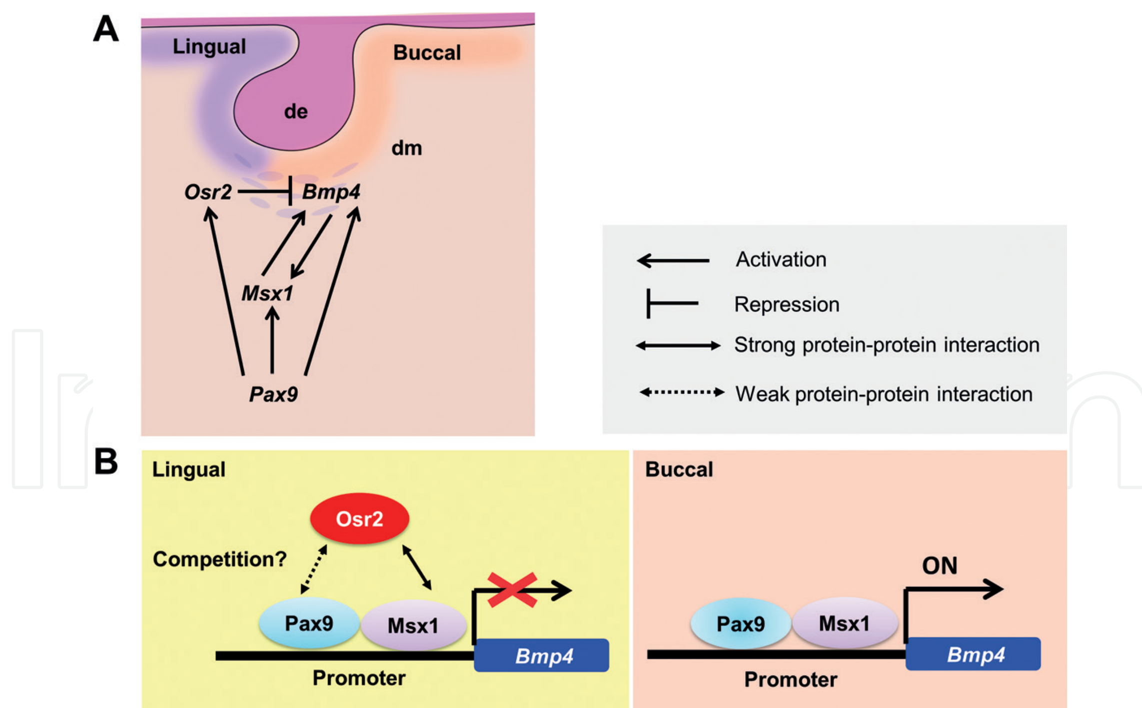

Figure 5. Pax9 molecular network in odontogenesis. (A) *Pax9* regulates a *Msx1*/*Bmp4* axis in the dental mesenchyme. *Osr2* expression in the lingual side restricts *Bmp4* to the buccal mesenchyme. *Pax9* is postulated to indirectly regulate *Osr2* expression. (B) Pax9 and Msx1 interaction at the protein level to regulate *Bmp4* expression. *In vitro*, Osr2 also has the ability to strongly bind to Msx1, and weakly to Pax9. Osr2 may compete with Pax9 for Msx1 to inhibit Bmp4 expression. Abbreviation(s): de, dental epithelium; dm, dental mesenchyme.

the initiation codon of *PAX9* have been noted [102, 103]. Considering the crucial role of *Pax9* in regulating BMP signaling for tooth morphogenesis to progress from bud to cap stage, it can be discerned that in humans with defective PAX9 function, tooth morphogenesis would be incomplete, resulting in missing teeth.

5.2.3. Pax1 and Pax9 in thymus development

The thymus is a bi-lobular epithelial organ surrounded by a mesenchymal capsule, located in the thoracic cavity. It is the niche site for T cell selection and maturation. The parathyroid glands, on the other hand, are endocrine glands located adjacent to the thyroid gland and produce parathyroid hormone for calcium homeostasis [104].

During development, the entire thymic epithelial component (cortical and medullary) is derived from the Pp3 endoderm. But proper formation of a functional thymus requires interaction with the surrounding neural crest-derived mesenchymal capsule [105, 106]. The mesenchymal capsule is essential for the proper thymic epithelial cell (TEC) proliferation and differentiation (by secreting FGF signals) and the collective migration of the thymic rudiment into their appropriate final location - the thoracic cavity, above the heart [107].

Although *Pax9* is known to be expressed in neural crest-derived mesenchyme, it has clear endodermal contributions for thymus development as it is expressed only in the endoderm-derived epithelium of the Pp [83, 108]. Unlike the sclerotome, *Pax9* is first expressed in the Pp3 endoderm at E9.5, while *Pax1* is only weakly detected at this stage [31]. *Pax1* expression becomes stronger a day later at E10.5 along with *Pax9* [16, 31]. Both *Pax1* and *Pax9* are expressed in the E12.5 thymic anlagen and become restricted to the thymic cortical epithelial cells by E14.5 [31]. Their expression remains in a subset of cortical epithelial cells in adults [109]. Since both thymus and parathyroid glands are derived from the Pp3, their formation is closely interconnected during development and show defects in the absence of *Pax1* or *Pax9* [104].

Hoxa3 is the earliest known regulator of Pp patterning toward parathyroid and thymic fates [110]. Even though the thymus and parathyroid glands develop from the same primordium, parathyroid patterning is initiated by E9.5, marked by *Gcm2* expression, whereas thymus epithelium marker *Foxn1* is detected only around E11.0. While *Shh*/*Tbx1*/*Gcm2* pathways are essential for parathyroid patterning, the *Hoxa3*/*Pax1*/*9*/*Eya1*/*Six1*/*4* axis drives thymus anlage formation and patterning [109, 111]. The hierarchy of genes within the latter cascade however remains to be clarified. Both *Pax1* and *Pax9* are down-regulated in E10.5 thymic primordia in *Hoxa3*$^{-/-}$ mutants [112]. While *Hoxa3* is not essential for initiation of *Pax1* and *Pax9* in the primordium, it is essential for their maintenance later. *Pax1* and *Pax9* expression is normal in the *Eya1*-null and *Six1*/*Six4*-null mutants indicating they are upstream of *Eya1* and *Six1*/*Six4* in this cascade [113]. But this is complicated by the observation that *Eya1*$^{-/-}$*Six1*$^{-/-}$ mutants show reduced *Pax1* but unaltered *Pax9* expression [114]. Regardless of the hierarchy, it is clear that *Pax1* and *Pax9* have important roles in thymus/parathyroid development.

Pax1 loss of function mutants exhibit a hypoplastic thymus with defects in thymocyte maturation [8, 31]. Furthermore, $Hoxa3^{+/-}Pax1^{-/-}$ compound mutants show a more drastic thymus phenotype than single null mutants. They possess hypoplastic thymi that are ectopically located due to delay in separation from the pharynx, indicating that *Hoxa3* and *Pax1* genetically interact and synergize to regulate proliferation of the thymus primordium [115].

A more drastic phenotype has been described for *Pax9* targeted-null mutants, whereby all the Pp3 and Pp4 derivatives - the entire thymus, parathyroid gland and ultimobranchial bodies – are absent [30]. However a subsequent study showed that $Pax9^{-/-}$ mutants indeed possess a hypoplastic, rudimentary thymic structure, colonized by T cell precursors, albeit ectopically localized in the larynx owing to failure of separation from the pharynx [116]. Furthermore, *Pax9* mutants exhibit defects in certain lymphocyte (T cell) subtypes. These data indicated that *Pax9* is not necessary for thymic primordium formation, but essential for its correct localization and normal thymopoiesis [116].

6. Conclusion

Accumulating evidences suggest the emergence of a central role of *Pax1* and *Pax9* in cell proliferation, cell motility and ECM regulation for condensation. Despite increasing knowledge of how these two TFs are interconnected with other factors, a myriad of questions still remain unanswered. For example, what tissue-restricted co-factors do Pax1 and Pax9 interact with to regulate the formation of axial skeleton and pharyngeal-derived tissues? If the PD of Pax1 and Pax9 are highly conserved, can Pax1 compensate for Pax9 and *vice versa* in the above-mentioned tissues if knocked-into the locus of its paralog? Furthermore, substantial progress in understanding the thymic and parathyroid development remains to be made. The exact molecular mechanisms of *Pax1* and *Pax9* initiation and their downstream targets are yet unknown in these tissues. Future studies on enriched specific cell-types and emerging state-of-the-art technologies will allow us to interrogate these questions at a single-cell resolution. High throughput technologies such as single cell transcriptomics, spatial transcriptomics (FISSEQ, MERFISH), multi-parameter profiling of proteins at single cell (CyTOF) and spatial levels (Imaging mass cytometry) will help to elucidate the pathways and the regulatory networks governing the development of these tissues [117–120]. These technologies in combination with ChIP-seq and utilization of the various gene-targeted mouse models will help to accelerate our understanding of these factors and their gene regulatory networks in the years to come.

Acknowledgements

We thank Dr. Massimo Nichane and Dr. Kevin Johnson for their valuable inputs on the manuscript. This work was supported by the Bayard and Virginia Clarkson Endowment Fund granted to Thomas Lufkin.

Author details

V. Sivakamasundari[1*], Petra Kraus[2] and Thomas Lufkin[2*]

*Address all correspondence to: siva.v@jax.org and tlufkin@clarkson.edu

1 The Jackson Laboratory for Genomic Medicine, Farmington, CT, USA

2 Department of Biology, Clarkson University, Potsdam, NY, USA

References

[1] Arnone MI, Davidson EH. The hardwiring of development: Organization and function of genomic regulatory systems. Development. 1997;**124**:1851-1864

[2] Walhout AJ. Unraveling transcription regulatory networks by protein-DNA and protein-protein interaction mapping. Genome Research. 2006;**16**:1445-1454. DOI: 10.1101/gr.5321506

[3] del Sol A, Balling R, Hood L, Galas D. Diseases as network perturbations. Current Opinion in Biotechnology. 2010;**21**:566-571. DOI: 10.1016/j.copbio.2010.07.010

[4] Bannykh SI, Emery SC, Gerber JK, Jones KL, Benirschke K, Masliah E. Aberrant Pax1 and Pax9 expression in Jarcho-Levin syndrome: Report of two Caucasian siblings and literature review. American Journal of Medical Genetics. Part A. 2003;**120A**:241-246. DOI: 10.1002/ajmg.a.20192

[5] Giampietro PF, Raggio CL, Reynolds CE, Shukla SK, McPherson E, Ghebranious N, et al. An analysis of PAX1 in the development of vertebral malformations. Clinical Genetics. 2005;**68**:448-453. DOI: 10.1111/j.1399-0004.2005.00520.x

[6] Bonczek O, Balcar VJ, Sery O. PAX9 gene mutations and tooth agenesis: A review. Clinical Genetics. 2017;467-476. DOI: 10.1111/cge.12986

[7] Lee JK, Park JW, Kim YH, Baek SH. Association between PAX9 single-nucleotide polymorphisms and nonsyndromic cleft lip with or without cleft palate. The Journal of Craniofacial Surgery. 2012;**23**:1262-1266. DOI: 10.1097/SCS.0b013e31824e27c7

[8] Dietrich S, Gruss P. undulated phenotypes suggest a role of Pax-1 for the development of vertebral and extravertebral structures. Developmental Biology. 1995;**167**:529-548. DOI: 10.1006/dbio.1995.1047

[9] Wilm B, Dahl E, Peters H, Balling R, Imai K. Targeted disruption of Pax1 defines its null phenotype and proves haploinsufficiency. Proceedings of the National Academy of Sciences of the United States of America. 1998;**95**:8692-8697

[10] Aubin J, Lemieux M, Moreau J, Lapointe J, Jeannotte L. Cooperation of Hoxa5 and Pax1 genes during formation of the pectoral girdle. Developmental Biology. 2002;**244**:96-113. DOI: 10.1006/dbio.2002.0596

[11] LeClair EE, Bonfiglio L, Tuan RS. Expression of the paired-box genes Pax-1 and Pax-9 in limb skeleton development. Developmental Dynamics. 1999;**214**:101-115. DOI: 10.1002/(SICI)1097-0177(199902)214:2<101:AID-AJA1>3.0.CO;2-4

[12] Hofmann C, Drossopoulou G, McMahon A, Balling R, Tickle C. Inhibitory action of BMPs on Pax1 expression and on shoulder girdle formation during limb development. Developmental Dynamics. 1998;**213**:199-206. DOI: 10.1002/(SICI)1097-0177(199810)213:2<199:AID-AJA5>3.0.CO;2-B

[13] Bopp D, Burri M, Baumgartner S, Frigerio G, Noll M. Conservation of a large protein domain in the segmentation gene paired and in functionally related genes of Drosophila. Cell. 1986;**47**:1033-1040

[14] Short S, Holland LZ. The evolution of alternative splicing in the Pax family: The view from the Basal chordate amphioxus. Journal of Molecular Evolution. 2008;**66**:605-620. DOI: 10.1007/s00239-008-9113-5

[15] Paixao-Cortes VR, Salzano FM, Bortolini MC. Origins and evolvability of the PAX family. Seminars in Cell & Developmental Biology. 2015;**44**:64-74. DOI: 10.1016/j.semcdb.2015.08.014

[16] Neubuser A, Koseki H, Balling R. Characterization and developmental expression of Pax9, a paired-box-containing gene related to Pax1. Developmental Biology. 1995;**170**:701-716. DOI: 10.1006/dbio.1995.1248

[17] Walther C, Guenet JL, Simon D, Deutsch U, Jostes B, Goulding MD, et al. Pax: A murine multigene family of paired box-containing genes. Genomics. 1991;**11**:424-434

[18] Stapleton P, Weith A, Urbanek P, Kozmik Z, Busslinger M. Chromosomal localization of seven PAX genes and cloning of a novel family member, PAX-9. Nature Genetics. 1993;**3**:292-298. DOI: 10.1038/ng0493-292

[19] Czerny T, Schaffner G, Busslinger M. DNA sequence recognition by Pax proteins: Bipartite structure of the paired domain and its binding site. Genes & Development. 1993;**7**:2048-2061

[20] Chalepakis G, Fritsch R, Fickenscher H, Deutsch U, Goulding M, Gruss P. The molecular basis of the undulated/Pax-1 mutation. Cell. 1991;**66**:873-884

[21] Jun S, Desplan C. Cooperative interactions between paired domain and homeodomain. Development. 1996;**122**:2639-2650

[22] Xu W, Rould MA, Jun S, Desplan C, Pabo CO. Crystal structure of a paired domain-DNA complex at 2.5 A resolution reveals structural basis for Pax developmental mutations. Cell. 1995;**80**:639-650

[23] Sivakamasundari V, Kraus P, Sun W, Hu X, Lim SL, Prabhakar S, et al. A developmental transcriptomic analysis of Pax1 and Pax9 in embryonic intervertebral disc development. Biology Open. 2017;**6**:187-199. DOI: 10.1242/bio.023218

[24] Epstein JA, Glaser T, Cai J, Jepeal L, Walton DS, Maas RL. Two independent and interactive DNA-binding subdomains of the Pax6 paired domain are regulated by alternative splicing. Genes & Development. 1994;**8**:2022-2034

[25] Kozmik Z, Czerny T, Busslinger M. Alternatively spliced insertions in the paired domain restrict the DNA sequence specificity of Pax6 and Pax8. The EMBO Journal. 1997;**16**:6793-6803. DOI: 10.1093/emboj/16.22.6793

[26] Tan B, Wang J, Song Q, Wang N, Jia Y, Wang C, et al. Prognostic value of PAX9 in patients with esophageal squamous cell carcinoma and its prediction value to radiation sensitivity. Molecular Medicine Reports. 2017;**16**:806-816. DOI: 10.3892/mmr.2017.6626

[27] Hsu DS, Acharya CR, Balakumaran BS, Riedel RF, Kim MK, Stevenson M, et al. Characterizing the developmental pathways TTF-1, NKX2-8, and PAX9 in lung cancer. Proceedings of the National Academy of Sciences of the United States of America. 2009;**106**:5312-5317. DOI: 10.1073/pnas.0900827106

[28] Luan T, Hua Q, Liu X, Xu P, Gu Y, Qian H, et al. PAX1 methylation as a potential biomarker to predict the progression of cervical intraepithelial neoplasia: A meta-analysis of related studies. International Journal of Gynecological Cancer. 2017;**27**:1480-1488. DOI: 10.1097/IGC.0000000000001011

[29] Strachan T, Read AP. PAX genes. Current Opinion in Genetics & Development. 1994;**4**:427-438

[30] Peters H, Neubuser A, Kratochwil K, Balling R. Pax9-deficient mice lack pharyngeal pouch derivatives and teeth and exhibit craniofacial and limb abnormalities. Genes & Development. 1998;**12**:2735-2747

[31] Wallin J, Eibel H, Neubuser A, Wilting J, Koseki H, Balling R. Pax1 is expressed during development of the thymus epithelium and is required for normal T-cell maturation. Development. 1996;**122**:23-30

[32] Paaby AB, Rockman MV. The many faces of pleiotropy. Trends in Genetics. 2013;**29**:66-73. DOI: 10.1016/j.tig.2012.10.010

[33] Raj PP. Intervertebral disc: Anatomy-physiology-pathophysiology-treatment. Pain Practice. 2008;**8**:18-44. DOI: 10.1111/j.1533-2500.2007.00171.x

[34] Fan CM, Tessier-Lavigne M. Patterning of mammalian somites by surface ectoderm and notochord: Evidence for sclerotome induction by a hedgehog homolog. Cell. 1994;**79**:1175-1186

[35] Furumoto TA, Miura N, Akasaka T, Mizutani-Koseki Y, Sudo H, Fukuda K, et al. Notochord-dependent expression of MFH1 and PAX1 cooperates to maintain the proliferation of sclerotome cells during the vertebral column development. Developmental Biology. 1999;**210**:15-29. DOI: 10.1006/dbio.1999.9261

[36] Johnson RL, Laufer E, Riddle RD, Tabin C. Ectopic expression of Sonic hedgehog alters dorsal-ventral patterning of somites. Cell. 1994;**79**:1165-1173

[37] Koseki H, Wallin J, Wilting J, Mizutani Y, Kispert A, Ebensperger C, et al. A role for Pax-1 as a mediator of notochordal signals during the dorsoventral specification of vertebrae. Development. 1993;**119**:649-660

[38] DiPaola CP, Farmer JC, Manova K, Niswander LA. Molecular signaling in intervertebral disk development. Journal of Orthopaedic Research. 2005;**23**:1112-1119. DOI: 10.1016/j.orthres.2005.03.008

[39] Wallin J, Wilting J, Koseki H, Fritsch R, Christ B, Balling R. The role of Pax-1 in axial skeleton development. Development. 1994;**120**:1109-1121

[40] Christ B, Wilting J. From somites to vertebral column. Annals of Anatomy. 1992;**174**:23-32

[41] Brand-Saberi B, Christ B. Evolution and development of distinct cell lineages derived from somites. Current Topics in Developmental Biology. 2000;**48**:1-42

[42] Sivakamasundari V, Lufkin T. Bridging the gap: Understanding embryonic intervertebral disc development. Cell & Developmental Biology. 2012;**1**(2). pii: 103. PMID: 23106046

[43] Sivakamasundari V, Kraus P, Jie S, Lufkin T. Pax1(EGFP): New wildtype and mutant EGFP mouse lines for molecular and fate mapping studies. Genesis. 2013;**51**:420-429. DOI: 10.1002/dvg.22379

[44] McMahon JA, Takada S, Zimmerman LB, Fan CM, Harland RM, McMahon AP. Noggin-mediated antagonism of BMP signaling is required for growth and patterning of the neural tube and somite. Genes & Development. 1998;**12**:1438-1452

[45] Chiang C, Litingtung Y, Lee E, Young KE, Corden JL, Westphal H, et al. Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function. Nature. 1996;**383**:407-413. DOI: 10.1038/383407a0

[46] Capellini TD, Zewdu R, Di Giacomo G, Asciutti S, Kugler JE, Di Gregorio A, et al. Pbx1/Pbx2 govern axial skeletal development by controlling Polycomb and Hox in mesoderm and Pax1/Pax9 in sclerotome. Developmental Biology. 2008;**321**:500-514. DOI: 10.1016/j.ydbio.2008.04.005

[47] Mankoo BS, Skuntz S, Harrigan I, Grigorieva E, Candia A, Wright CV, et al. The concerted action of Meox homeobox genes is required upstream of genetic pathways essential for the formation, patterning and differentiation of somites. Development. 2003;**130**:4655-4664. DOI: 10.1242/dev.00687

[48] Balling R, Deutsch U, Gruss P. undulated, a mutation affecting the development of the mouse skeleton, has a point mutation in the paired box of Pax 1. Cell. 1988;**55**:531-535

[49] Blandova, Y.R., and Egorov, I.U. Sut allelic with un. Mouse News Letters. 1975;**52**:43

[50] Wallace ME. An inherited agent of mutation with chromosome damage in wild mice. The Journal of Heredity. 1985;**76**:271-278

[51] Adham IM, Gille M, Gamel AJ, Reis A, Dressel R, Steding G, et al. The scoliosis (sco) mouse: A new allele of Pax1. Cytogenetic and Genome Research. 2005;**111**:16-26. DOI: 10.1159/000085665

[52] Peters H, Wilm B, Sakai N, Imai K, Maas R, Balling R. Pax1 and Pax9 synergistically regulate vertebral column development. Development. 1999;**126**:5399-5408

[53] Kist R, Watson M, Wang X, Cairns P, Miles C, Reid DJ, et al. Reduction of Pax9 gene dosage in an allelic series of mouse mutants causes hypodontia and oligodontia. Human Molecular Genetics. 2005;**14**:3605-3617. DOI: 10.1093/hmg/ddi388

[54] Wakatsuki Y, Neurath MF, Max EE, Strober W. The B cell-specific transcription factor BSAP regulates B cell proliferation. The Journal of Experimental Medicine. 1994; **179**:1099-1108

[55] Warren N, Price DJ. Roles of Pax-6 in murine diencephalic development. Development. 1997;**124**:1573-1582

[56] Rodrigo I, Hill RE, Balling R, Munsterberg A, Imai K. Pax1 and Pax9 activate Bapx1 to induce chondrogenic differentiation in the sclerotome. Development. 2003;**130**:473-482

[57] Aszodi A, Chan D, Hunziker E, Bateman JF, Fassler R. Collagen II is essential for the removal of the notochord and the formation of intervertebral discs. The Journal of Cell Biology. 1998;**143**:1399-1412

[58] Watanabe H, Nakata K, Kimata K, Nakanishi I, Yamada Y. Dwarfism and age-associated spinal degeneration of heterozygote cmd mice defective in aggrecan. Proceedings of the National Academy of Sciences of the United States of America. 1997;**94**:6943-6947

[59] Zou W, Chen X, Shim JH, Huang Z, Brady N, Hu D, et al. The E3 ubiquitin ligase Wwp2 regulates craniofacial development through mono-ubiquitylation of Goosecoid. Nature Cell Biology. 2011;**13**:59-65. DOI: 10.1038/ncb2134

[60] Smits P, Lefebvre V. Sox5 and Sox6 are required for notochord extracellular matrix sheath formation, notochord cell survival and development of the nucleus pulposus of intervertebral discs. Development. 2003;**130**:1135-1148

[61] Visel A, Thaller C, Eichele G. GenePaint.org: An atlas of gene expression patterns in the mouse embryo. Nucleic Acids Research. 2004;**32**:D552-D556. DOI: 10.1093/nar/gkh029

[62] Diez-Roux G, Banfi S, Sultan M, Geffers L, Anand S, Rozado D, et al. A high-resolution anatomical atlas of the transcriptome in the mouse embryo. PLoS Biology. 2011;**9**:e1000582. DOI: 10.1371/journal.pbio.1000582

[63] Han Y, Lefebvre V. L-Sox5 and Sox6 drive expression of the aggrecan gene in cartilage by securing binding of Sox9 to a far-upstream enhancer. Molecular and Cellular Biology. 2008;**28**:4999-5013. DOI: 10.1128/MCB.00695-08

[64] Smits P, Li P, Mandel J, Zhang Z, Deng JM, Behringer RR, et al. The transcription factors L-Sox5 and Sox6 are essential for cartilage formation. Developmental Cell. 2001;**1**: 277-290

[65] Lefebvre V, Behringer RR, de Crombrugghe B. L-Sox5, Sox6 and Sox9 control essential steps of the chondrocyte differentiation pathway. Osteoarthritis and Cartilage. 2001;**9**(Suppl A):S69-S75. PMID: 11680692. 89

[66] Lefebvre V, Li P, de Crombrugghe B. A new long form of Sox5 (L-Sox5), Sox6 and Sox9 are coexpressed in chondrogenesis and cooperatively activate the type II collagen gene. The EMBO Journal. 1998;**17**:5718-5733. DOI: 10.1093/emboj/17.19.5718

[67] Nakamura Y, Yamamoto K, He X, Otsuki B, Kim Y, Murao H, et al. Wwp2 is essential for palatogenesis mediated by the interaction between Sox9 and mediator subunit 25. Nature Communications. 2011;**2**:251. DOI: 10.1038/ncomms1242

[68] Takae R, Matsunaga S, Origuchi N, Yamamoto T, Morimoto N, Suzuki S, et al. Immunolocalization of bone morphogenetic protein and its receptors in degeneration of intervertebral disc. Spine (Phila Pa 1976). 1999;**24**:1397-1401

[69] Lefebvre V, Huang W, Harley VR, Goodfellow PN, de Crombrugghe B. SOX9 is a potent activator of the chondrocyte-specific enhancer of the pro alpha1(II) collagen gene. Molecular and Cellular Biology. 1997;**17**:2336-2346

[70] Lee WJ, Chatterjee S, Yap SP, Lim SL, Xing X, Kraus P, et al. An integrative developmental genomics and systems biology approach to identify an in vivo Sox trio-mediated gene regulatory network in murine embryos. BioMed Research International. 2017;**2017**:8932583. DOI: 10.1155/2017/8932583

[71] Sohn P, Cox M, Chen D, Serra R. Molecular profiling of the developing mouse axial skeleton: A role for Tgfbr2 in the development of the intervertebral disc. BMC Developmental Biology. 2010;**10**:29. DOI: 10.1186/1471-213X-10-29

[72] Baffi MO, Moran MA, Serra R. Tgfbr2 regulates the maintenance of boundaries in the axial skeleton. Developmental Biology. 2006;**296**:363-374. DOI: 10.1016/j.ydbio.2006.06.002

[73] Baffi MO, Slattery E, Sohn P, Moses HL, Chytil A, Serra R. Conditional deletion of the TGF-beta type II receptor in Col2a expressing cells results in defects in the axial skeleton without alterations in chondrocyte differentiation or embryonic development of long bones. Developmental Biology. 2004;**276**:124-142. DOI: 10.1016/j.ydbio.2004.08.027

[74] McGaughran JM, Oates A, Donnai D, Read AP, Tassabehji M. Mutations in PAX1 may be associated with Klippel-Feil syndrome. European Journal of Human Genetics. 2003;**11**:468-474. DOI: 10.1038/sj.ejhg.5200987

[75] Tracy MR, Dormans JP, Kusumi K. Klippel-Feil syndrome: Clinical features and current understanding of etiology. Clinical Orthopaedics and Related Research. 2004 Jul;**424**:183-90. PMID: 15241163

[76] Gleghorn L, Ramesar R, Beighton P, Wallis G. A mutation in the variable repeat region of the aggrecan gene (AGC1) causes a form of spondyloepiphyseal dysplasia associated with severe, premature osteoarthritis. American Journal of Human Genetics. 2005;**77**:484-490. DOI: 10.1086/444401

[77] Nakane T, Tando T, Aoyagi K, Hatakeyama K, Nishimura G, Coucke IP, et al. Dysspondyloenchondromatosis: Another COL2A1-related skeletal dysplasia? Molecular Syndromology. 2011;**2**:21-26. DOI: 10.1159/000333098

[78] Graham A, Smith A. Patterning the pharyngeal arches. BioEssays. 2001;**23**:54-61. DOI: 10.1002/1521-1878(200101)23:1<54:AID-BIES1007>3.0.CO;2-5

[79] Grevellec A, Tucker AS. The pharyngeal pouches and clefts: Development, evolution, structure and derivatives. Seminars in Cell & Developmental Biology. 2010;**21**:325-332. DOI: 10.1016/j.semcdb.2010.01.022

[80] Phan M, Conte F, Khandelwal KD, Ockeloen CW, Bartzela T, Kleefstra T, et al. Tooth agenesis and orofacial clefting: Genetic brothers in arms? Human Genetics. 2016;**135**:1299-1327. DOI: 10.1007/s00439-016-1733-z

[81] Lan Y, Xu J, Jiang R. Cellular and molecular mechanisms of palatogenesis. Current Topics in Developmental Biology. 2015;**115**:59-84. DOI: 10.1016/bs.ctdb.2015.07.002

[82] Zhou J, Gao Y, Lan Y, Jia S, Jiang R. Pax9 regulates a molecular network involving Bmp4, Fgf10, Shh signaling and the Osr2 transcription factor to control palate morphogenesis. Development. 2013;**140**:4709-4718. DOI: 10.1242/dev.099028

[83] Kist R, Greally E, Peters H. Derivation of a mouse model for conditional inactivation of Pax9. Genesis. 2007;**45**:460-464. DOI: 10.1002/dvg.20295

[84] Lan Y, Ovitt CE, Cho ES, Maltby KM, Wang Q, Jiang R. Odd-skipped related 2 (Osr2) encodes a key intrinsic regulator of secondary palate growth and morphogenesis. Development. 2004;**131**:3207-3216. DOI: 10.1242/dev.01175

[85] Rice R, Spencer-Dene B, Connor EC, Gritli-Linde A, McMahon AP, Dickson C, et al. Disruption of Fgf10/Fgfr2b-coordinated epithelial-mesenchymal interactions causes cleft palate. The Journal of Clinical Investigation. 2004;**113**:1692-1700. DOI: 10.1172/JCI20384

[86] Rakhshan V, Rakhshan H. Meta-analysis of congenitally missing teeth in the permanent dentition: Prevalence, variations across ethnicities, regions and time. International Orthodontics. 2015;**13**:261-273. DOI: 10.1016/j.ortho.2015.06.008

[87] Cobourne MT, Mitsiadis T. Neural crest cells and patterning of the mammalian dentition. Journal of Experimental Zoology. Part B, Molecular and Developmental Evolution. 2006;**306**:251-260. DOI: 10.1002/jez.b.21084

[88] Mina M, Kollar EJ. The induction of odontogenesis in non-dental mesenchyme combined with early murine mandibular arch epithelium. Archives of Oral Biology. 1987;**32**:123-127

[89] Lumsden AG. Spatial organization of the epithelium and the role of neural crest cells in the initiation of the mammalian tooth germ. Development. 1988;**103**(Suppl):155-169. PMID: 3250849

[90] Tucker A, Sharpe P. The cutting-edge of mammalian development; how the embryo makes teeth. Nature Reviews. Genetics. 2004;**5**:499-508. DOI: 10.1038/nrg1380

[91] Neubuser A, Peters H, Balling R, Martin GR. Antagonistic interactions between FGF and BMP signaling pathways: A mechanism for positioning the sites of tooth formation. Cell. 1997;**90**:247-255

[92] Ogawa T, Kapadia H, Feng JQ, Raghow R, Peters H, D'Souza RN. Functional consequences of interactions between Pax9 and Msx1 genes in normal and abnormal tooth development. The Journal of Biological Chemistry. 2006;**281**:18363-18369. DOI: 10.1074/jbc.M601543200

[93] Zhou J, Gao Y, Zhang Z, Zhang Y, Maltby KM, Liu Z, et al. Osr2 acts downstream of Pax9 and interacts with both Msx1 and Pax9 to pattern the tooth developmental field. Developmental Biology. 2011;**353**:344-353. DOI: 10.1016/j.ydbio.2011.03.012

[94] Satokata I, Maas R. Msx1 deficient mice exhibit cleft palate and abnormalities of craniofacial and tooth development. Nature Genetics. 1994;**6**:348-356. DOI: 10.1038/ng0494-348

[95] Jia S, Zhou J, Gao Y, Baek JA, Martin JF, Lan Y, et al. Roles of Bmp4 during tooth morphogenesis and sequential tooth formation. Development. 2013;**140**:423-432. DOI: 10.1242/dev.081927

[96] Ogawa T, Kapadia H, Wang B, D'Souza RN. Studies on Pax9-Msx1 protein interactions. Archives of Oral Biology. 2005;**50**:141-145. DOI: 10.1016/j.archoralbio.2004.09.011

[97] Nieminen P, Arte S, Tanner D, Paulin L, Alaluusua S, Thesleff I, et al. Identification of a nonsense mutation in the PAX9 gene in molar oligodontia. European Journal of Human Genetics. 2001;**9**:743-746. DOI: 10.1038/sj.ejhg.5200715

[98] Frazier-Bowers SA, Guo DC, Cavender A, Xue L, Evans B, King T, et al. A novel mutation in human PAX9 causes molar oligodontia. Journal of Dental Research. 2002;**81**:129-133

[99] Das P, Hai M, Elcock C, Leal SM, Brown DT, Brook AH, et al. Novel missense mutations and a 288-bp exonic insertion in PAX9 in families with autosomal dominant hypodontia. American Journal of Medical Genetics. Part A. 2003;**118A**:35-42. DOI: 10.1002/ajmg.a.10011

[100] Mendoza-Fandino GA, Gee JM, Ben-Dor S, Gonzalez-Quevedo C, Lee K, Kobayashi Y, et al. A novel g.-1258G>A mutation in a conserved putative regulatory element of PAX9 is associated with autosomal dominant molar hypodontia. Clinical Genetics. 2011;**80**:265-272. DOI: 10.1111/j.1399-0004.2010.01529.x

[101] Mues G, Kapadia H, Wang Y, D'Souza RN. Genetics and human malformations. The Journal of Craniofacial Surgery. 2009;**20**(Suppl 2):1652-1654. DOI: 10.1097/SCS.0b013e3181b2d3f3

[102] Das P, Stockton DW, Bauer C, Shaffer LG, D'Souza RN, Wright T, et al. Haploinsufficiency of PAX9 is associated with autosomal dominant hypodontia. Human Genetics. 2002;**110**:371-376. DOI: 10.1007/s00439-002-0699-1

[103] Klein ML, Nieminen P, Lammi L, Niebuhr E, Kreiborg S. Novel mutation of the initiation codon of PAX9 causes oligodontia. Journal of Dental Research. 2005;**84**:43-47. DOI: 10.1177/154405910508400107

[104] Gordon J, Manley NR. Mechanisms of thymus organogenesis and morphogenesis. Development. 2011;**138**:3865-3878. DOI: 10.1242/dev.059998

[105] Gordon J, Wilson VA, Blair NF, Sheridan J, Farley A, Wilson L, et al. Functional evidence for a single endodermal origin for the thymic epithelium. Nature Immunology. 2004;**5**:546-553. DOI: 10.1038/ni1064

[106] Blackburn CC, Manley NR. Developing a new paradigm for thymus organogenesis. Nature Reviews. Immunology. 2004;**4**:278-289. DOI: 10.1038/nri1331

[107] Foster KE, Gordon J, Cardenas K, Veiga-Fernandes H, Makinen T, Grigorieva E, et al. EphB-ephrin-B2 interactions are required for thymus migration during organogenesis. Proceedings of the National Academy of Sciences of the United States of America. 2010;**107**:13414-13419. DOI: 10.1073/pnas.1003747107

[108] Feng J, Jing J, Sanchez-Lara PA, Bootwalla MS, Buckley J, Wu N, et al. Generation and characterization of tamoxifen-inducible Pax9-CreER knock-in mice using CrispR/Cas9. Genesis. 2016;**54**:490-496. DOI: 10.1002/dvg.22956

[109] Manley NR, Condie BG. Transcriptional regulation of thymus organogenesis and thymic epithelial cell differentiation. Progress in Molecular Biology and Translational Science. 2010;**92**:103-120. DOI: 10.1016/S1877-1173(10)92005-X

[110] Manley NR, Capecchi MR. The role of Hoxa-3 in mouse thymus and thyroid development. Development. 1995;**121**:1989-2003

[111] Bain VE, Gordon J, O'Neil JD, Ramos I, Richie ER, Manley NR. Tissue-specific roles for sonic hedgehog signaling in establishing thymus and parathyroid organ fate. Development. 2016;**143**:4027-4037. DOI: 10.1242/dev.141903

[112] Chisaka O, Capecchi MR. Regionally restricted developmental defects resulting from targeted disruption of the mouse homeobox gene hox-1.5. Nature. 1991;**350**:473-479. DOI: 10.1038/350473a0

[113] Xu PX, Zheng W, Laclef C, Maire P, Maas RL, Peters H, et al. Eya1 is required for the morphogenesis of mammalian thymus, parathyroid and thyroid. Development. 2002;**129**:3033-3044

[114] Zou D, Silvius D, Davenport J, Grifone R, Maire P, Xu PX. Patterning of the third pharyngeal pouch into thymus/parathyroid by Six and Eya1. Developmental Biology. 2006;**293**:499-512. DOI: 10.1016/j.ydbio.2005.12.015

[115] Su D, Ellis S, Napier A, Lee K, Manley NR. Hoxa3 and pax1 regulate epithelial cell death and proliferation during thymus and parathyroid organogenesis. Developmental Biology. 2001;**236**:316-329. DOI: 10.1006/dbio.2001.0342

[116] Hetzer-Egger C, Schorpp M, Haas-Assenbaum A, Balling R, Peters H, Boehm T. Thymopoiesis requires Pax9 function in thymic epithelial cells. European Journal of Immunology. 2002;**32**:1175-1181. DOI: 10.1002/1521-4141(200204)32:4<1175:AID-IMMU1175>3.0.CO;2-U

[117] Crosetto N, Bienko M, van Oudenaarden A. Spatially resolved transcriptomics and beyond. Nature Reviews. Genetics. 2015;**16**:57-66. DOI: 10.1038/nrg3832

[118] Chen KH, Boettiger AN, Moffitt JR, Wang S, Zhuang X. RNA imaging. Spatially resolved, highly multiplexed RNA profiling in single cells. Science. 2015;**348**:aaa6090. DOI: 10.1126/science.aaa6090

[119] Giesen C, Wang HA, Schapiro D, Zivanovic N, Jacobs A, Hattendorf B, et al. Highly multiplexed imaging of tumor tissues with subcellular resolution by mass cytometry. Nature Methods. 2014;**11**:417-422. DOI: 10.1038/nmeth.2869

[120] Bendall SC, Nolan GP, Roederer M, Chattopadhyay PK. A deep profiler's guide to cytometry. Trends in Immunology. 2012;**33**:323-332. DOI: 10.1016/j.it.2012.02.010

IntechOpen

IntechOpen
IntechOpen