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Chapter 9

Regulatory Functions of Pax1 and Pax9 in Mammalian Cells

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Abstract

Pax1 and Pax9 are paired-box transcription factors, which play vital roles in axial skeletonogenesis, 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 transcription factors acting on the CREs to determine a gene battery can be mapped into a transcriptional
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) subdomains, 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 “GGAAAC” 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.](image-url)
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 fibrosus (IAF) and outer annulus fibrosus (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<sup>−/−</sup>Mfh1<sup>−/−</sup> mutants show reduced cell proliferation [35]. Noggin (Nog) also induces Pax1 expression in
the absence of Hh signaling (in \textit{Shh}^{−/−} mutants) [44, 45]. Other factors which do not independently induce \textit{Pax1}/\textit{Pax9} expression but can regulate their expression in the somites are \textit{Pbx1}/\textit{Pbx2} and \textit{Meox1}/\textit{Meox2}. In both \textit{Pbx1}/\textit{Pbx2}^{−/−} mutants and \textit{Meox1}/\textit{Meox2}^{−/−} mutants, \textit{Pax1} and \textit{Pax9} expression is diminished in the somites/sclerotome, although \textit{Pax9} to a lesser extent [46, 47]. Furthermore, \textit{Pax1} potentially auto-regulates itself as \textit{Pax1}^{−/−} mutants show reduced \textit{Pax1} mRNA expression. \textit{Pax9} however is independent of \textit{Pax1} in the sclerotome, as \textit{Pax1}^{−/−} mutants do not show any reduction in \textit{Pax9} mRNA [23]. Thus, \textit{Pax1} and \textit{Pax9} can be regulated by different upstream regulators most of which remain to be identified.

5.1.2. Molecular functions of \textit{Pax1} and \textit{Pax9} in axial skeletogenesis

The roles of \textit{Pax1} and \textit{Pax9} in vertebral column development were first identified through spontaneous mouse mutants – \textit{undulated} (\textit{un}) [48], \textit{Undulated short-tail} (\textit{Uns}) [49], \textit{undulated-extensive} (\textit{unr}) [50] and \textit{undulated intermediate} (\textit{un-i}) [51] – which encompass a mutation in \textit{Pax1} or deletion of the loci containing \textit{Pax1} [8]. Subsequent gene-targeted knock-out models of \textit{Pax1} [9] and \textit{Pax9} [30] and generation of compound mutants revealed their synergistic, gene-dosage dependent, redundant roles in axial skeletogenesis [23, 52]. \textit{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 \textit{Pax1}^{−/−} show an overall normal phenotype externally, they possess slight abnormalities in the vertebral column and sternum with varying penetrance, indicating haploinsufficiency of \textit{Pax1} in these structures [9].

Contrary to \textit{Pax1}^{−/−} mice, \textit{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 \textit{Pax9}^{−/−} mutants did not exhibit any overt defects, a hypomorphic allele, \textit{Pax9}^{neo} showed that \textit{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 \textit{Pax1} and \textit{Pax9} were generated [52]. Increasing severity in vertebral column defects was observed with successive loss of \textit{Pax1} and \textit{Pax9} alleles. The most severe phenotype was displayed by \textit{Pax1}^{−/−}\textit{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 \textit{Pax1} and \textit{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, Col2a1, 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, phenotypic 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...
Col2a1, Collagen Type II, alpha 1

1. Expression sites in developing embryo:
   - (1) Sclerotome
   - (2) Vertebral, intervertebral disc, tail, limb and craniofacial cartilage condensations
   - (3) Limb, head and shoulder mesenchyme

2. Function:
   - (1) Major ECM component of cartilage
   - (2) Collagen fibrillogenesis
   - (3) Cartilage development
   - (4) TGF-beta tethering in extracellular matrix (ECM) to modulate its signaling.

3. References:
   - [23, 52, 57, 61-63]

2 Acan, Aggrecan

1. Expression sites in developing embryo:
   - (1) Vertebral, intervertebral disc, tail, limb and craniofacial cartilage condensations
   - (2) Limb, head, nasal mesenchyme

2. Function:
   - (1) Major ECM component of cartilage
   - (2) Cartilage development
   - (3) Water retention and maintain osmotic pressure in cartilage

3. References:
   - [61-63]

3 Sox5, SRY-box-containing gene 5

1. Expression sites in developing embryo:
   - (1) Vertebral, intervertebral disc, tail, limb and craniofacial cartilage condensations
   - (2) Forebrain

2. Function:
   - (1) ECM synthesis
   - (2) Cartilage development
   - (3) Chondrocyte differentiation

3. References:
   - [23, 60-65]

4 Wwp2, WW domain containing E3 ubiquitin

1. Expression sites in developing embryo:
   - (1) Maxilla and mandible
   - (2) Vertebral and intervertebral disc condensations

2. Function:
   - (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

3. References:
   - [23, 62, 67]

5 Bmp4, Bone morphogenetic protein 4

1. Expression sites in developing embryo:
   - (1) Limb and head mesenchyme
   - (2) Nasal pit epithelium
   - (3) Vertebrae and intervertebral disc cartilage condensations
   - (4) Dental and palatal mesenchyme

2. Function:
   - (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.

3. References:
   - [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<sup>−/−</sup>Pax9<sup>−/−</sup> 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.
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

![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 Tgf3 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.](http://dx.doi.org/10.5772/intechopen.71920)
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 dorsal 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
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.

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.
The epithelium and mesenchyme together go through a series of stages from the epithelial thickening (at E11.0) to bud (E13.5), 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
neural crest-specific inactivation of Bmp4 (Bmp4ff;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

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**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.

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