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PAX genes: Roles in development, pathophysiology, and cancer

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ABSTRACT

PAX proteins function as transcription factors and play an essential role in organogenesis during embryonic development in regulating cell proliferation and self-renewal, resistance to apoptosis, migration of embryonic precursor cells, and the coordination of specific differentiation programs. Recent studies have also discovered a role for PAX proteins in specific stem cell or progenitor cell populations, including melanocytes, muscle, and B-cells. The normal functions of the PAX proteins, including apoptosis resistance and repression of terminal differentiation, may be subverted during the progression of a number of specific malignancies. This is supported by the fact that expression of PAX proteins is dysregulated in several different types of tumors, although the precise roles for PAX proteins in cancer are not clearly understood. An emerging hypothesis is that PAX proteins play an essential role in maintaining tissue specific stem cells by inhibiting terminal differentiation and apoptosis and that these functional characteristics may facilitate the development and progression of specific cancers. In this review, we provide a general background to the PAX protein family and focus on specific cells and tissues and the role PAX proteins play within these tissues in terms of development, mature tissue maintenance, and expression in tumors. Understanding the normal developmental pathways regulated by PAX proteins may shed light on potentially parallel pathways shared in tumors, and ultimately result in defining new molecular targets and signaling pathways for the development of novel anti-cancer therapies.

1. Introduction

Somatic stem cells have an extraordinary ability to remodel, maintain, and repair specialized tissues in the adult organism. Tissue specific stem cells have the capacity to self renew as well as generate differentiated daughter cells. While existing in mature tissues, these stem cells maintain embryonic characteristics both in their pluripotental nature and in the genes they differentially express. In essence, these cells recapitulate similar molecular signaling pathways employed during the initial stages of organogenesis in the embryo. The stem cells can "recycle" genes that are essential for embryonic cell maintenance to remain terminally undifferentiated and capable of cell division. PAX proteins may be playing such a role. PAX proteins are essential for normal embryogenesis, and appear to play a critical role in stem cell maintenance. However, *PAX* genes can become subverted during the development of a variety of specific cancers [1].

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In this review, we provide a brief overview of the PAX proteins and focus on their roles in specific tissues in terms of development, pathophysiology, and the progression of specific types of cancer. Due to space restrictions, all of the expression domains for the *PAX* gene family and every disease state in which the expression of human PAX proteins are dysregulated are not described. For example, *PAX* genes play critical roles in the central nervous system and are described elsewhere [2]. We present a model wherein PAX proteins are essential for normal organogenesis, are down-regulated during terminal differentiation in the adult, and are over-expressed and selectively mutated by activating translocations in specific disease states, including cancer. This model is not absolute, as cells within the adult central nervous system and adult neuroendocrine glands such as the thyroid, the thymus and the endocrine pancreas maintain expression of PAX proteins. With these caveats in mind, we propose a general hypothesis that PAX proteins play an essential role in maintaining tissue specific stem cells by inhibiting terminal differentiation and apoptosis and that these molecular qualities are also advantageous in the development or progression of tumors.

2. Overview of the PAX protein family

2.1. *Introduction*

PAX proteins are a family of nine proteins divided into four groups based on which structural domains (Paired and Homeodomain, and the octapeptide) the proteins possess (Fig. 1). PAX proteins are critical during organogenesis, with the expression of *PAX* genes regulated both spatially and temporally. Mutations of the *PAX* genes cause significant developmental abnormalities in a broad spectrum of organisms from flies to humans. While the absence of *PAX* genes has definitive deleterious consequences, little is known regarding the specific mechanisms by which PAX proteins influence organogenesis. Examples of target organs and tissues for the expression of PAX proteins during organogenesis are the skeleton (PAX1 and 9), central nervous system (PAX2, 3, 5, 6, 7, and 8) kidney (PAX2 and 8), B-cells (PAX5), thyroid (PAX8), pancreas (PAX4 and 6) and skeletal muscle (PAX3 and 7). Mouse transgenic models that have mutations disrupting the expression of these specific *Pax* genes exhibit abnormal development or agenesis of the corresponding target tissues [2–10].

2.2. *PAX proteins and their functional domains*

There is a substantial body of evidence demonstrating that the PAX proteins function as specialized transcription factors. All nine family members possess a Paired domain that recognizes specific DNA sequences. Some of the family members have additional whole or partial homeodomains that also interact with DNA. PAX proteins interact with other proteins both through these domains and through motifs outside these regions. The defining characteristic of all PAX proteins is the possession of a Paired domain. The Paired domain was originally identified in the Drosophila Paired (Prd) protein

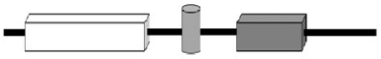
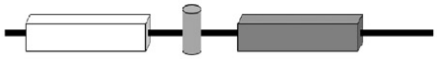
PAX family Group	Protein structure/domains (Paired, octapeptide, homeodomain)	Protein family member	Embryonic Expression Domain	Expression/Mutation in human disease
I		PAX1	Skeleton, thymus 3rd/4th pharyngeal pouch	Klippel-Feil Syndrome, Jarcho-Levin Syndrome
		PAX9	Skeleton, Teeth, Thymus	Jarcho-Levin Syndrome, Oligodentia
II		PAX2	Kidney, CNS	Hyperproliferative dysplastic kidney, Renal hyperplasia, Bladder and renal cancer,Coloboma Syndrome
		PAX5	B-Cells, CNS	Lymphomas
		PAX8	Kidney, Thyroid, CNS	Congenital hypothyroidism, Thyroid carcinomas/adenomas
III		PAX3	Neural Crest, CNS somites/muscle	Waardenburg Syndrome Types I/III, Melanoma, Rhabdomyosarcoma
		PAX7	Neural Crest, CNS somites/muscle	Rhabdomyosarcoma
IV		PAX4	Pancreas, gut	Diabetes
		PAX6	Pancreas, gut, CNS and eye	Aniridia,GI tumors Cataracts/Peter's Anomaly

Fig. 1 – PAX protein family. PAX proteins are divided into groups based on possession or absence of a Paired domain (white rectangle), an octapeptide (grey cylinder), and/or a homeodomain (grey rectangle). Embryonic expression domains are listed; CNS: central nervous system. The third and fourth pharyngeal pouches will give rise to the inferior parathyroid and thymus, and the superior parathyroid and ultimobranchial body, respectively. Neural crest gives rise to a number of tissues, such as the peripheral nervous system, connective tissues, melanocytes and some endocrine glands. Embryonic somites are the precursors to most of the body's skeletal muscle. The human diseases are discussed in text.

[11] and comprises a DNA-binding domain with two structural sub-domains, PAI and RED (PAI + RED = PAIRED) [12]. The N-terminal sub-domain (PAI domain) interacts directly with DNA. The C-terminal RED domain, while highly conserved, does not interact with DNA directly, but may contribute to the overall binding interactions of the Paired domain. The consensus DNA binding site for the Paired domain is (G/T)T(T/C)(C/A)(C/T)(G/C)(G/C), although several PAX family members have different nucleotide preferences, such as PAX6 (TTCACGC) or PAX8 (GTCAC(G/C)C) [12–14]. PAX proteins can also bind to other proteins through the Paired domain. An important example is the Paired domain interaction with the HMG domain of SOX proteins [15]. This interaction leads to synergistic gene activation, such as PAX3 and SOX10 activating both the *Mitf* and *Ret* promoters, and PAX6 and SOX2 promoting the expression of δ-crystallin [16–19]. PAX and SOX complexes can activate transcription from these promoters more robustly than either transcription factor alone.

Several PAX family members contain an additional DNA binding motif, a three helixed homeodomain (PAX3, 4, 6, and 7) or a partial one helixed homeodomain (PAX2, 5 and 8). This homeodomain motif can be found in a large number of proteins, and the amino acid sequence may diverge widely among these proteins; however, these domains all share a conserved helix-turn-helix secondary structure. Homeodomain-containing proteins generally fall into one of two major genetic groups. One subgroup is the Hox genes, which can be further subdivided into four clusters of 13 genes each. These genes are clustered both by their location on the vertebrate genome and in their spatial and temporal expression during embryonic axial patterning. The second homeodomain group is the ''orphan'' homeobox genes. These genes are not in genetic clusters nor do they play a major role in embryonic pattern formation. In general, ''orphan'' homeobox genes tend to be tissue-specific in their expression during embryogenesis. The *PAX* genes fall into this second group along with the *PITX*, *POU*, *SIX*, *OCT*, and *NKX* gene families (a complete list of homeodomain proteins are listed on the website http://research.nhgri.nih.gov/homeodomain/). Most homeodomain proteins function as a sequence-specific DNA binding motif and recognize *cis*-binding elements that contain a core TAAT sequence. The homeodomains found in *PAX* proteins recognize palindromic elements of $TAAT(N)_{2-3}ATTA$ [20].

An additional conserved domain found in all PAX proteins (except PAX4 and 6) is the eight amino acid octapeptide motif. The consensus amino acid sequences are HSIDGIL(G/S) for PAX3 and 7, YSI(N/S)G(I/L)LG for PAX2, 5, and 8, and HSV(S/T)(N/D)ILG for PAX1 and 9 [21]. This octapeptide motif is related to the *eh1* repression domain in engrailed proteins and the Gsc-En homology element in Goosecoid proteins [22]. The octapepetide functions as a transcriptional inhibitory motif. A primary mechanism for this repression is through direct interactions between GRG4 (also known as TLE4 in humans and groucho in Drosophila) to form repressor complexes that inhibit transcription. PAX2, 3 and 5 octapeptide sequences have been found to directly interact with GRG4 [23–25]. PAX5 binds GRG4 through the octapeptide and C-terminal protein tail [24]. Another binding partner for GRG4 is the transcription factor LEF1. PAX5 directly binds to LEF1 as well, and PAX5 and 3 interact with GRG4 and LEF1 in a complex [25,26]. It is through these specific interactions that PAX proteins participate in the canonical Wnt signaling pathway whose dysregulation is important in the development of several types of cancers, notably colorectal cancer [27].

2.3. Functional roles of the PAX proteins

Tissue specific stem cells have been found in a number of different adult tissues, such as blood, muscle, intestine and the skin. The expression of *PAX* genes in tissue specific stem cells is a new finding [25]. However, PAX proteins have been described to promote stem cell characteristics in cell culture models and in embryos by restricting lineage specification, resisting apoptosis and repressing terminal differentiation.

2.3.1. *PAX proteins influence the fate of specific cell lineages*

For example, during the development of the eye, the *PAX6* gene is required for specification of multiple ocular lineages. Loss of PAX6 expression in Drosophila, mice and man results in a complete absence of eye formation because critical developmental programs are never initiated. Conversely, inactivation of *Pax6* at the retinal progenitor stage later in ocular development results in the loss of ability of this committed but undifferentiated cell type to maintain their pluripotency [28]. This results in terminal differentiation into only one retinal cellular subtype, the amacrine interneurons. Additionally, PAX5 is required for determining the B-cell lineage in immature lymphoid precursor cells. In the absence of PAX5 these cells differentiate to other cell types such as T lymphocytes or natural killer cells [5], suggesting that PAX5 represses the choices of cell lineage differentiation toward a specific cell type, in this case B lymphocytes. Furthermore, skeletal muscle in the PAX7 null mouse develops normally (probably because PAX3 can compensate for the loss of PAX7 in the embryonic myoblasts) but mice die shortly after birth due to the inability of skeletal muscle to regenerate [29]. This mortality is due to the lack of maintenance of the satellite cell population that regenerates the muscle tissue, suggesting that tissue specific muscle stem cells terminally differentiate, thereby depleting the stem cell population needed for normal muscle tissue regeneration.

2.3.2. *PAX proteins are anti-apoptotic, a role directly implicated in their relationship with cancer progression*

Inhibition of *PAX2* by siRNA promotes apoptosis in renal carcinoma cells [3,30]. Oligonucleotide inhibition targeting the *PAX3* mRNA translation start site also induces apoptosis in rhabdomyosarcoma cells [31]. Loss of *PAX3* in homozygous *Splotch* mouse (*Sp/Sp*) embryos, which possesses a mutation in the *PAX3* gene leading to an unstable transcript, leads to an increase in cell death during development. This apoptosis can be partially reversed in *Sp/Sp* by also having a loss of function of the p53 gene, which codes for a transcription factor that is a well characterized tumor suppressor protein that inhibits the cell cycle and promotes apoptosis in both normal and tumor cells [32]. PAX3 expression is significantly reduced in the neural tube of embryonic diabetic mice, and the areas of reduced PAX3 expression directly correlate to areas of increased cell apoptosis [33]. PAX3 plays an active role in inhibiting apoptosis by directly regulating transcription of the anti-apoptotic protein Bcl-X_L [34].

PAX2, 5 and 8 demonstrate anti-apoptotic activity in multiple cell types by directly repressing wild type p53 expression through a highly conserved enhancer region [35].

2.3.3. *Down-regulation of PAX genes is important for proper cell differentiation*

For example, PAX3 functions in myogenesis in committed but undifferentiated myoblasts. Genetic studies indicate that PAX3 functions upstream of MyoD [36]. However, PAX3 expression is extinguished prior to activation of the terminal differentiation program [37] and PAX3 protein is not seen at the same time as MyoD or myogenin protein in embryonic myoblasts [38]. PAX3 plays a particularly important role in hypaxial myoblasts that migrate to the limbs since PAX3 deficiency results in loss of limb musculature. Hypaxial myoblasts arise from the lateral somites and are characterized by persistent PAX3 expression during a period of migration. These migrating cells are committed but remain undifferentiated until positively acting external signals allow for definitive myogenesis [38]. PAX3 [39] and closely related PAX7 [29] are both expressed by skeletal muscle stem cells, known as satellite cells, that are committed to the myogenic lineage yet remain undifferentiated at the periphery of adult muscle fibers. PAX3 and PAX7 are down-regulated in these cells following external stimuli, such as muscle injury, that initiate regeneration.

In summary, PAX proteins play roles in proliferation and self-renewal, resistance to apoptosis, migration of precursor cells in the embryo, and the regulation of specific differentiation programs during normal embryological development. While *PAX* genes are essential in organogenesis, most adult cells down regulate the expression of PAX proteins; consequently, persistent expression of particular classes of PAX proteins may have deleterious consequences in the adult organism. The functions of specific PAX proteins are important and potentially permissive for the growth and progression of cancers, and the sustained expression of *PAX* genes may also contribute to the malignant phenotype [1,30,40]. Expression of PAX2, PAX5, and PAX8 have been observed in a variety of different tumor cell lines, including renal cell carcinoma [2,13], and within the kidney PAX2 is expressed in cystic and hyperproliferative dysplastic diseases [3,41,42]. Both PAX3 and 7 are differentially expressed in rhabdomyosarcoma tumors, Ewing sarcomas, melanomas, and neuroblastomas [31,43]. Four of the *PAX* genes (*PAX3*, 5, 7, and 8) are also associated with specific chromosomal translocations in specific tumor types [83–86,114,156,157]. Despite these observations, the differential over-expression or activation of specific PAX genes has not been directly implicated in the initiation or pathogenesis of specific cancers [1]. The role of PAX genes in specific cancers is discussed further in the following sections of this review.

3. Role of PAX genes in specific organ systems in development and disease

3.1. *PAX3 and 7 in normal and pathological neural crest and melanocyte development*

PAX3 and 7 play an essential role in the development of neural crest cells (NCCs), an embryonic cell type that is highly specific in its location within the embryo between the neural plate and the non-neural ectoderm. NCCs also have a high degree of pluripotency [44] and comprise two major groups. The migratory NCCs traveling dorsoventrally near the neural tube within the anterior section of the somites form neural structures. The NCCs migrating mediolaterally dorsal to the somites but under the superficial ectoderm are generally fated to be melanoblasts. PAX3 is expressed in both NCC populations. The importance of PAX3 to the melanocyte population during development is evident by the lack of hair and skin pigmentation in mice and humans with PAX3 gene mutations. Loss of PAX3 expression in NCCs in the mouse embryo leads to a greatly reduced number of melanoblasts. In these mice, although melanoblasts are present, the lack of PAX3 expression inhibits melanoblast precursors from expanding their numbers and providing a pool of committed melanoblasts [45]. PAX3 functions as a transcription factor in these melanoblasts by regulating the expression of melanocyte specific genes including MITF [46]. The basic helix loop helix leucine zipper transcription factor MITF directly activates several melanocyte specific differentiation genes, including Trp1, Trp2/Dct, and tyrosinase [47].

The melanocyte stem cell niche in mature skin has recently been characterized [48]. The bulge region of the hair follicle of adult mice contains multipotential stem cells that can generate a variety of dermal cell types [49-51]. Dct-lacZ transgenic mice have been described to specifically mark the melanocyte stem cells by expressing β-galactosidase in Dct-expressing cells in the hair follicle [48]. Improper maintenance of the melanocyte stem cells, such as through increased apoptosis as a consequence of Bcl2 deficiency, causes stem cell loss and hair graying [52]. PAX3 functions as a nodal point in the melanocyte stem cell differentiation program by activating expression of MITF while simultaneously competing with MITF for occupancy of an enhancer required for expression of dopachrome tautomerase (DCT), an enzyme involved in melanin production [25]. MITF also directly interacts with PAX6, and this leads to inhibition of transcriptional ability of both proteins [53]. The PAX3-mediated repression of *Dct* expression and MITF transactivation is relieved by nuclear β-catenin, a downstream effector of the canonical Wnt signaling pathway [25].

PAX3 and *7* mutations are also found in congenital disease and in neural-crest-derived tumors. Mutations in *PAX3*/*Pax3* are found in both mice and humans. In mice, a single allele mutation in *Pax3* results in the *Splotch* (*Sp*) mouse, named this way due to a white belly spot caused by a melanocyte defect [4,54]. Homozygous mutation in *Sp* results in severe neural crest, neural tube and muscle malformations. The neural crest abnormalities include a severe reduction in the number of melanocytes [45]. Other neural crest related defects include cardiac abnormalities as well as a lack of enteric ganglia due to a loss of PAX3 mediated *Ret* expression [4,18]. In humans, mutation in the *PAX3* gene results in Waardenburg (WS) syndrome types I and III [55,56]. Features of WS include several melanocyte abnormalities such as hair hypopigmentation and deafness. PAX3 and 7 are also expressed in primary melanomas and cell lines [30,57,58]. PAX3 has been detected in the peripheral blood of melanoma patients but not in control subjects [59,60]. The expression of PAX3 in sentinel lymph

nodes also predicts a poor outcome for patients with melanoma [61]. The exact role of *PAX3* in melanoma is not clear. There is evidence that PAX3 promotes melanoma cell survival, since PAX3 promotes the expression of anti-apoptotic gene Bcl-X_L and inhibition of PAX3 in melanoma cell lines triggers melanoma apoptosis [30,34].

3.2. *PAX3 and PAX7 in skeletal muscle development and the progression of sarcomatous tumors*

Non-cranial muscle is derived from embryonic structures called somites, mesodermal segments aligned along the neural tube in the developing embryo. The *PAX3* and *7* genes are expressed throughout the early somite, but as the somite develops this expression becomes restricted to the cells destined to become skeletal muscle in the myotome. During embryonic development both *PAX3* and *7* are expressed in skeletal muscle progenitor cells in the myotome that do not express muscle differentiation markers [62,63]. The expression of *PAX3* and *7* are down-regulated when the cells in the myotome differentiate to lineages expressing myogenic markers [64,65]. Muscle precursor cells lacking both PAX3 and 7 are arrested developmentally and do not leave the myotome [63]. A role for PAX3 in these muscle precursors is the activation of the *c-Met* receptor that is critical for the migration of muscle progenitor cells in the embryo [66,67]. c-Met is a multi-functional proto-oncogene whose dysregulated expression and activation is a hallmark of multiple solid and hematological malignancies and contributes to tumor proliferation, migration/invasion, angiogenesis and resistance to apoptosis [68,69]. These data provide a firm link between PAX3 dysregulation and up-regulation and ligand-independent activation of the c-Met proto-oncogene widely implicated in cancer progression.

PAX3 lies upstream of MyoD, a transcription factor important for muscle specificity. Two parallel pathways for MyoD activation exist in myoblasts, one dependent of PAX3 and the other controlled by the myogenic transcription factor Myf5. *Pax3* and Myf5 double mutant mice have no skeletal muscle formation and lack MyoD expression, whereas the single mutants still express MyoD [36]. The role of PAX3 in skeletal muscle development has been studied using the *Splotch* mouse model, having a characteristic phenotype in which limb muscles are absent [54,64,70]. The *Splotch* phenotype is caused by a mutation in the *Pax3* allele [4,71,72]. PAX7 can compensate for some of the loss of PAX3 expression in the *Splotch* embryos in the neural tube and somites. The *Pax7* gene is up-regulated and its expression area is expanded [73]. This data also suggests that PAX3 represses PAX7 expression in certain cell populations. While PAX7 is unable to completely rescue the function of PAX3 in the development of skeletal muscle, PAX3 can compensate for the loss of PAX7 [74]. Unlike the PAX3 null mice, *Pax7* knockout mouse embryos have normal musculature during gestation and at birth [74,75].

Satellite cells are progenitor cells found in post-natal muscle and are quiescent until they are induced to proliferate and give rise to new muscle tissue in response to injury [76,77]. PAX3 and 7 are co-expressed in satellite cells of adult mice. The number of satellite cells in PAX7 null mice is dramatically reduced. Muscle regeneration may [29,78] or may not [79] be severely affected by the PAX7 null genotype and this variable finding may be due to differences in the mouse strain observed. PAX7 may be necessary both as an anti-apoptotic factor and to impart an undifferentiated state in satellite cells; in contrast, PAX3 is unable to fulfill the anti-apoptotic role of PAX7 in these cells [74]. PAX7 actively promotes a stem cell state in satellite cells by repressing muscle specific genes MyoD and myogenin [80]. Therefore, while PAX3 is crucial for embryonic development of skeletal muscle, PAX7 becomes important for maintenance of adult satellite cells.

Both *PAX3* and *7* mutations are found in diseases of the muscle, including cancers. Waardenburg syndrome (WS) types I and III patients frequently have congenital defects in the *PAX3* gene and can have limb muscle defects, most notably in WS type III [81]. *PAX3* and *7* are also dysregulated in cancers of the skeletal muscle. Over-expression of PAX3 and 7 are commonly seen in embryonal rhabdomyosarcoma (ERMS) [31,43,82]. Rhabdomyosarcomas are malignant tumors commonly effecting a pediatric population. In alveolar rhadbomyosarcoma (ARMS) a chromosomal translocation of *PAX3* t(2;13)(q35;q14) or *PAX7* t(1;13)(p36;q14) is a characteristic feature. These translocations create a fusion protein complex with a Forkhead transcription factor gene (FKHR, also known as FOXO1A) to create PAX3-FKHR or PAX7-FKHR [83-86]. The fusion proteins are functional and act as a more effective transcription factor than wild type PAX3 or 7 [87,88]. Over-expression of these fusion proteins in these tumors confers a resistance to apoptosis and facilitates tumor growth [31]. To further explore the function of the PAX3-FKHR protein product, a mouse model was created that knocked in a PAX3-FKHR gene into the PAX3 locus. These mice show developmental abnormalities, with heterozygotes having limb defects and double knock-in transgenic mice dying during the perinatal period due to cardiac abnormalities [89]. Although these mice have significant developmental abnormalities, the PAX3-FKHR allele was not sufficient by itself to promote sarcoma formation. One probable mechanism for the developmental abnormalities seen in these transgenic mice is the over-expression of c-Met, which leads to abnormal migration of muscle precursor cells [90]. Therefore, the same characteristics of these PAX proteins that are necessary for embryonic muscle development and stem cell viability also facilitate the malignant progression of these rhadbomyosarcomas, although PAX3 translocations are not an initiating or transforming event in this process.

3.3. *PAX2 and PAX8 in normal urogenital development and in carcinomas of the urogenital system*

PAX2 is a known regulator of early urogenital development and is expressed in ductal and mesenchymal cells throughout all three stages of embryonic kidney development, the pro-, meso-, and metanephros [91]. During pronephros, the primary stage of embryonic kidney development, the mesenchymal cells of the intermediate mesoderm transition to epithelial cells to comprise the pronephric duct and *Pax2* is one of the first tissue-specific genes to be expressed during this stage. While PAX2 is expressed early, mice null for PAX2

still form the mesonephric duct [3]; however, the duct then rapidly degenerates [92]. PAX8 is also expressed throughout kidney development but PAX8 null mice have no obvious kidney abnormality, perhaps due to PAX2 compensation [8]. The PAX2 and PAX8 double mutants have a complete lack of kidney formation. PAX2 and 8−/− double mutant embryos do not exhibit mesenchymal–epithelial conversion and, therefore, lack the nephric duct and mesonephric tubules [93]. Experiments with mice with mutations in both *PAX2* and *8* genes suggests that either PAX2 or 8 are necessary and sufficient to specify nephric lineage by facilitating the mesenchymal–epithelial conversions necessary to form the nephric duct, and that PAX2 is critical for later kidney development. In the metanephric mesenchyme, PAX2 has been shown to control the expression of *Wt1* [94] and *Gdnf* [95], genes which are necessary for metanephros initiation and development. PAX2 levels noticeably decrease in the more developed mouse E17 kidney, at which point the tubular epithelium is extensive and major renal structures have differentiated. The down-regulation of PAX2 suggests that it may be involved in the terminal differentiation of renal epithelial components [96].

PAX2 is critical for the survival of fetal collecting ducts and has a primary anti-apoptotic function in embryonic renal cells [97]. Inactivation of endogenous PAX2 increases apoptotic cell death, possibly explaining the eventual degeneration of the nephric duct in PAX2 null mutants [92]. *Pax2* haploinsufficiency increases embryonic collecting duct cell apoptosis in the heterozygous mouse kidney, and does not appear to interfere with the proliferation or mitogenic signals of surviving cells [97]. PAX2 and 8 double mutant embryos, however, display apoptosis of the intermediate mesoderm at E9.5. PAX2 ± heterozygous embryos show an increase in apoptosis during metanephros [93]. PAX2 is expressed in the nuclei of collecting duct cells in normal adult kidneys [98], and in the adult kidney during the regeneration of proximal tubule cells following nephrotoxin damage, playing a possible role in tissue repair. Confirming its role in mesenchymal–epithelial transitions, inhibition of PAX2 expression in adult mouse kidney cultures prevents these transitions of mesenchymal cells [99].

PAX2 is expressed in cystic and hyperproliferative dysplastic diseases of the kidney, potential early stages to renal cell carcinoma [3,41,42]. Genetic abnormalities in the form of point mutations and translocations have been described in renal hypoplasia and coloboma–renal syndrome [100–103]. PAX2 is expressed in a high proportion of primary renal carcinomas, and inhibition of PAX2 expression induces rapid apoptosis in bladder carcinoma cell lines. These data suggest that PAX2 expression is important for the growth and survival of several cancers of urogenital origin [30,40,96,104–106].

3.4. *PAX8 in normal thyroid development and progression of follicular thyroid carcinomas*

The thyroid forms out of the embryonic gut tube, eliciting cells from both the thyroid anlage and ultimobranchial bodies to relocate and merge into the immature gland. The adult thyroid is composed of two cell types: the endoderm-derived thyroid follicular cells (TFCs) and the neural-crest-derived parafollicular C-cells. C-cells are responsible for the production of calcitonin, whereas the much more numerous populations of TFCs synthesize crucial thyroid hormones. PAX8 is expressed upon transition from undifferentiated endoderm cells to TFC-fated cells in the thyroid anlage and continues to be expressed throughout development. In a similar pattern to other *PAX* genes in neuronal cells of the central nervous system and in neuroendocrine cells (such as *PAX4* and 6 in the exocrine pancreas [9,10]), PAX8 expression is maintained in the adult cells of the thyroid. PAX8 is essential for the expression of thyroid specific genes, including thyroglobulin (Tg), thyroperoxidase (TPO), and sodium/iodide symporter (NIS) essential for the synthesis of active thyroid hormone [107]. TFC-knockout mice lacking PAX8 expression die around the time of weaning unless they are treated with thyroid hormones, specifically T_4, demonstrating the crucial role of PAX8 in the biosynthesis of thyroid hormones [108]. The development of the thyroid is orchestrated by PAX8 as well as three other essential transcription factors, thyroid transcription factor-1 (TTF-1), thyroid transcription factor-2 (TTF-2), and Hex. TTF-1 and Hex are homeodomain proteins, while TTF-2 is a forkhead/winged helix domain transcription factor. PAX8, TTF-1, TTF-2, and Hex are all independently expressed throughout the embryo, however, they are only co-expressed in the thyroid anlage, indicating a possible collaborative effort between these proteins for inducing gene expression critical for normal thyroid development.

PAX8 mutations and inactivation are implicated in various thyroid conditions. Congenital hypothyroidism is characterized by inadequate production of thyroid hormones. The condition is most often caused by an absent or underdeveloped thyroid or ectopic placement [109]. Several genetic defects can give rise to this condition; among these are mutations of the *PAX8* gene [131]. A mutated segment in the Paired domain of PAX8 results in hypothyroidism. These cases normally present themselves with varying levels of hypoplasia and can be treated with external sources of thyroid hormones [110,111]. In addition to hypothyroidism, PAX8 plays a role in the progression of follicular thyroid carcinomas and adenomas. PAX8 is expressed in the majority of thyroid cancers, and this expression is correlated with a greater risk of tumor reoccurrence [112,113]. Additionally, *PAX8* is also involved in a translocation mutation t(2;3)(q13;p25) that results in a fusion protein with peroxisome proliferator-activated receptor γ (PPARγ) to form what has been termed the PAX8-PPARγ oncogene [114]. This fusion protein is expressed in both malignant and benign thyroid tumors, but its expression seems to be observed in a significantly greater percentage of follicular thyroid carcinomas [115]. In conclusion, PAX8 plays an extremely important, though still somewhat elusive, role in thyroid development and maintenance. PAX8 mutations contribute to several thyroid disorders, ranging from hypothyroidism due to mutations in the Paired domain, to the progression of follicular thyroid cancer as a result of over-expressed PAX8-PPARγ oncogenic fusion proteins.

3.5. *PAX4 and PAX6 in normal development and cancers of the pancreas and the gastrointestinal tract*

Both PAX4 and 6 are expressed in the embryonic pancreatic bud. In the mouse, the pancreatic anlage begins to bud from

the dorsal side of the primitive gut tube starting at embryonic day 8.5. PAX4 and 6 are expressed initially throughout the early pancreatic bud, but as the pancreas grows and differentiates into exocrine and endocrine compartments this expression becomes restricted to the islet cell precursors. The islets of Langerhans are endocrine cells nested within the exocrine pancreatic gland and are composed of alpha, beta, delta, and gamma cells. In parallel to other endocrine glands (such as PAX8 expression in the thyroid) the expression of PAX4 and 6 is maintained in the pancreatic islets [9,10,116,117]. PAX6 is found in all four islet subtypes while PAX4 expression tends to be more restricted and is not expressed in the alpha cells. Mice lacking PAX6 lack the glucagon producing alpha cells, and the remaining endocrine cells form disorganized islets intermixed with exocrine cells [10]. While distinct islet structures form in *Pax4*−/− mice, there is a failure of differentiation or maintenance of insulin producing beta cells and somatostatin producing delta cells [9]. In mice lacking both PAX4 and 6, endocrine cells fail to differentiate. Absence of PAX4 and/or PAX6 in the developing pancreas may divert development of one specific islet cell lineage to an alternate lineage. This is in parallel to shifting of cells towards an alternative amacrine interneuron phenotype in the *Pax6*−/− retina, and the production of T-lymphocytes and natural killer cells at the expense of a B-cell phenotype in *Pax5*−/− lymphocytes [5,28]. In the pancreas, PAX4 or 6 deficient cells differentiate into other islet cell types or into exocrine cell. In double mutant mice, cells presumed to be fated for islets express the exocrine marker amylase [10]. PAX4 and 6 are also required for endocrine cell development in the gastrointestinal tract. In parallel with the role of PAX6 in the pancreas, absence or dominant negative expression of PAX6 reduces cell types expressing gastrin and somatostatin in the stomach and the proglucagon gene in enteroendocrine cells and duodenal gastric inhibitory peptide (GIP) in the intestine [118,119]. PAX4 loss eliminates basically all duodenal and jejunum endocrine cells.

Dysregulation of PAX4 and 6 is correlated with developmental disorders, diabetes and tumors. A missense mutation in the PAX4 Paired domain (R121W), causing severely reduced PAX4 DNA binding, is found in a subgroup of Japanese type 2 diabetic patients [120]. PAX4 is also mutated in a codon located just after the Paired domain (R133W) in Ketosis-prone type 2 diabetes, found predominately in a West African population [121]. This type of diabetes also has a rare mutation in PAX4 in the amino terminal end of the Paired domain (R37W) that causes a more severe biochemical phenotype than the R133W mutation. The loss of PAX6 in the retina correlates with a reduction in proliferation [28]. An over-expression or lack of PAX6 in the mouse pancreas leads to an increase in ductal epithelium, reduction of islet cells, and diabetes [122,123]. Maintained PAX6 over-expression also leads to cystic adenoma development. PAX6 over-expression also occurs in tumors of the exocrine pancreas and intestine ([124], Lang et al., unpublished findings). The expression of PAX6 in tumor cell types that are not neuroendocrine but are of common embryonic ancestry to PAX6 expressing cells may be explained in part by the tumor arising from a pluripotent tissue specific stem cell.

3.6. *PAX1 and PAX9 in the development of the skeletal system, thymus and parathyroid*

The two highly homologous transcription factors PAX1 and PAX9 are expressed during the development of the skeleton, the thymus and parathyroid glands [125–128]. Unlike other PAX proteins, PAX1 and 9 lack a homeodomain. Both PAX1 and PAX9 have been shown to be important in the developing vertebral column and the third and fourth pharyngeal pouches and their derivatives, and both transcription factors activate the expression of Bapx1, a protein expressed in the embryonic tissue of the sclerotome and in subsequent vertebrae that develop from these structures [129]. PAX1 is involved in the development of the sternum, limb buds, scapula, pectoral and pelvic girdle [127,130,131], while PAX9 is implicated in the development of the tail, head, limb buds, esophagus, teeth, and the larynx of the mouse [127,132]. During tooth development, PAX9 has an overlapping expression pattern with another transcription factor, Msx1, and the two proteins directly interact [133]. Msx1 also binds to PAX3, and this interaction inhibits PAX3 protein DNA binding [134].

Despite their co-expression and highly homologous sequences both PAX1 and PAX9 are necessary for normal embryonic development. Gene inactivation mouse models of *Pax1* and *Pax9* have demonstrated some functional redundancy of PAX1 and PAX9 in the vertebral column. In *Pax1* and *Pax9* double homozygous knockout mice, no vertebral column forms and the phenotype is more severe than single homozygous knockouts of either *Pax1* or *Pax9* [6]. In *PAX1* homozygous knockout mice, there are abnormalities in the tail, abnormal vertebral column, sternum, and scapula compared to wild type [7]. In heterozygous *Pax1* knockout mice, there are less severe malformations in the cervical and lumbar regions of the vertebral column and in the sternum, with no abnormalities in the tail or scapula, when compared to the homozygous *Pax1* knockout mice, indicating that *Pax1* is haploinsufficient [7]. Furthermore, PAX9 cannot fully rescue the phenotype of *Pax1* knockouts [6], perhaps indicating a specific function of PAX1 in the development of the vertebral column. In *Pax9* knockout mice, there are hind-limb abnormalities with missing toes, skull malformations and no teeth. In a *Pax1* heterozygous knockout/*Pax9* knockout there are unique vertebral column malformations when compared to the phenotype of a *Pax1* heterozygous knockout [6,132].

The thymus and parathyroid glands develop from the third pharyngeal pouch and the ultimobranchial bodies from the fourth pharyngeal pouch. Both PAX1 and PAX9 are expressed during development of the third and fourth pharyngeal pouches and their derivatives [125–128,135]. During the development of the thymus, PAX1 and PAX9 are co-expressed, but both perform separate functions necessary for thymus development. PAX1 is required later in thymus development during the maturation of T cells while PAX9 is necessary earlier in these processes. In *Pax1* knockout mice, the thymus is reduced due to the lack of mature thymocytes [128] indicating a key role for PAX1 in thymopoiesis. In *Pax9* homozygous and heterozygous knockout mice, there is an abnormal and undeveloped thymus with low cell numbers, indicating insufficient thymopoiesis compared to wild type [126,132]. PAX1 is unable to compensate for this phenotype and the pups die shortly after birth [132]. Knockout

Pax9 mice are deficient in the development of the thymus, parathyroid glands and ultimobranchial bodies thus indicating that PAX9 is necessary for thymus, parathyroid glands and ultimobranchial bodies development [132]. Double *Pax1*/*Pax9* homozygous knockout mice have no precursor to the thymus, parathyroid glands or ultimobranchial bodies (third and fourth pharyngeal pouches) and resemble the phenotype of the *PAX9* knockout mice [6].

In adult tissues, *Pax1* transcripts can be detected in the developing thymus and then continuing into adult tissue, similar to the retention of PAX8 expression in the thyroid gland. During embryonic development, PAX1 is expressed in the thymic epithelial cells whereas in the adult thymus expression is only seen in a few cortical epithelial cells suggesting the more differentiated cells do not express PAX1 [128]. In adult mice, it has been shown that PAX9 is still detectable in the adult thymus albeit at lower levels than during development.

Both PAX1 and PAX9 are mutated in several human congenital disorders. *PAX1* mutations have been found in congenital vertebral malformation disorders including Klippel–Feil syndrome and Jarcho–Levin syndrome [136,137]. There are several reported *undulated* mutations of PAX1 in mice that show comparable phenotypes to human diseases [138]. However, the *undulated* mutations of PAX1, un^{s} and un^{-s}, demonstrates a different phenotype than the homozygous *PAX1* knockout mouse model [7]. PAX9 mutations have been shown to cause oligodontia in adult humans [139]. In contrast to most of the members of the PAX gene family, there are no known tumors that over-express PAX1 and PAX9. In fact, PAX9 expression actually decreases in a majority of esophageal carcinomas and dysplastic lesions compared to that observed in normal esophageal epithelium [140]. There is some evidence that PAX9 expression may actually be associated with favorable prognoses in specific cancers (see [1]). The underlying reasons for these observations in human cancers are unclear. Whether this has to do with the tissue types that express PAX1 and 9 or their lack of a homeodomain (unlike other PAX family members) is unknown.

3.7. *Role of PAX5 in B-cell development and maturation and the progression of B-cell lymphomas*

B-lymphopoiesis is a highly regulated process by which pluripotent, self-renewing hematopoietic stem cells mature to germinal center B-cells, and later develop into memory B-cells or plasma cells. B-cell maturation is divided into numerous stages that mark the various steps of heavy and light chain antibody arrangement and changes in cell markers. This process starts with a multipotential hematopoeitic stem cell which progresses to a pro-B-cell, which in turn differentiates to a pre-B cell. Terminal differentiation occurs when the Pre-B cell develops into a mature B-cell [5,141]. This process of maturation is first triggered by interactions between E2A, EBF, and common lymphoid progenitors (CLPs). Although the absence of E2A or EBF is enough to arrest B-lymphopoiesis at the earliest stage of development, it is their downstream target transcription factor, PAX5 that is required for B-cell lineage commitment and maintenance of B-cell identity [5].

The PAX5 protein, also called B-cell-specific activator protein (BSAP), is expressed from the pro-B to mature B-cell stages. In the fetal liver, PAX5 plays an important role in commitment. In the adult bone marrow, however, BSAP is vital not only for commitment purposes, but for the progression of B-cells past the early pro-B stage as well [142]. $Pax5^{-/-}$ mice have arrested B-cell development in the bone marrow. Pro-B-cells harvested from these mice do not express CD19, which is a target gene for BSAP [143]. PAX5 null immature B lymphocytes, although unable to mature into an antibody-producing plasma cells, are still able to terminally differentiate. These pluripotent cells can differentiate into a number of alternate hematopoeitic cell types depending on external signals (such as cytokines). This suggests two main functions of PAX5—the ability to activate genes crucial to B-cell development and to repress genes that are B-cell lineage inappropriate [5]. PAX5 inhibits expression of non-hematopoeitic cell factors and represses B-cell specific terminal differentiation markers as well. The former include Notch-1, crucial to T-cell development, and M-CSF, which plays a role in macrophage production [144]. One mechanism for PAX5 transcriptional repression is through GRG4 binding; PAX5 recruits the co-repressor GRG4 by interacting with two separate binding domains that cooperate together in protein binding, causing PAX5 to take on the role of an active repressor [24].

PAX5 also supports differentiation of the earlier pro-B cell to the pre-B cell, which is the direct precursor to the mature B-cell. This pro-B cell maintains a high degree of pluripotency, however, and can differentiate into other hematopoetic cell types [5]. PAX5 removes H3K9 methylation at the V_H locus, allowing for both proximal and distal V-DJ recombination of the heavy chain [145]. It is the influence of PAX5 over V-DJ binding that provides the most crucial aspect of B-cell commitment [146]. PAX5 activates B-cell linker (BLNK) at its proximal promoter region resulting in B-cell proliferation, which in turn allows for an increase in IgG levels, as cell division is necessary for IgH class switching [147]. BLNK also controls signaling of pre-B-cell receptor (pre-BCR) [148]. Pre-BCR is an important checkpoint in B-lymphopoiesis and controls the transition from pro-B to pre-B cell. While PAX5 is important in Pro and Pre-B-cell differentation, there are many examples of PAX5 blocking terminal differentiation. PAX5 inhibits expression of immunoglobulin J chain, and this inhibition is relieved by the exogenous signal of IL-2 [149]. BSAP silences the J chain gene, which is required for assembly of pentamer IgM antibody. The light-chain Igκ 3′ enhancer is another target of PAX5 repression. The Igκ 3′ enhancer contains a motif for PU.1 binding, and a PAX5-PU.1-GRG4 complex actively represses gene expression [150]. PAX5 also activates genes that are involved with B-cell fate. The B-cell lymphocyte kinase, blk, is activated by PAX5 during the pro-B stage of lymphopoiesis and remains active until the mature B-cell begins to differentiate to either a memory or a plasma cell. However, this kinase plays an important role not only in cell differentiation but also in proliferation [151]. BSAP is also capable of binding to the RAG-2 promoter, a gene needed for V(D)J recombination. RAG-2 is not solely dependent upon BSAP, however, and is expressed in BSAP deficient mice [152]. In addition, PAX5, either directly or indirectly, activates the B-cell receptor component Ig-α or represses the cell surface protein PD-1 [153]. During this terminal differentiation, PAX5

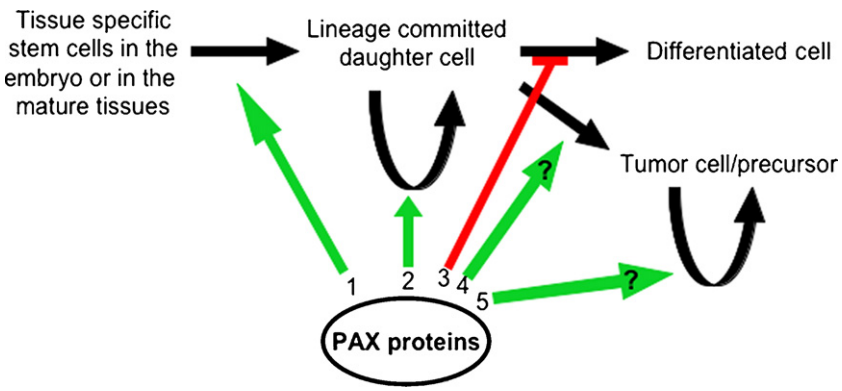


Fig. 2 – Possible roles for PAX proteins in cellular differentiation. Green arrows indicate activation, while red arrows designate inhibition. PAX proteins are expressed in embryonic tissues (see Fig. 1) and possibly in tissue specific stem cells in the mature organism (arrow 1). In mature tissues, PAX proteins have been described in melanocytes, and may be essential for other tissues such as B-cells and muscle [5,25,29]. PAX genes also promote lineage specificity by activating cell type specific genes including Dct and IgJ Chain (arrow 2) [5,25]. PAX proteins inhibit terminal differentiation by repressing expression of terminal differentiation markers, inhibiting apoptosis, and preventing cell cycle arrest (arrow 3). PAX genes are also seen in tumors, but it is unknown if these proteins are involved in initiation (arrow 4) and/or progression (arrow 5). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

is actively down-regulated. During the transition from the Pre-B-cell to the mature B cell, BCL-6 is silenced, and repression of Blimp-1 is lifted. Blimp-1, in turn, down-regulates PAX5 [154].

PAX5 is expressed in a variety of hematological malignancies, including follicular and non-Hodgkin's lymphoma [155]. In a subset of B-cell non-Hodgkin's lymphomas PAX5 is involved in a translocation with the immunoglobulin heavy chain gene t(9;14)(p13;q32) producing an oncogenic fusion protein PAX5-IGH [156,157]. Along these lines, there is evidence that alterations and reduction in PAX5 expression plays a role in B-cell senescence in aging mice [158]. In summary, PAX5 is crucial to the commitment and identity of developing B-cells, represses other hematopoietic cell lineages, and actively blocks terminal differentiation to mature B-cells. Dysregulation of PAX5 through over-expression and the formation of PAX5-IGH oncogenic fusion protein is associated with a subset of non-Hodgkin's lymphoma.

4. PAX genes, stem cells, and cancer—what does the future hold?

There is increasing evidence that specific PAX proteins are beneficial in maintaining stem cells but can be permissive or facilitate the malignant progression of several solid and hematological cancers. With regard to stem cells, PAX proteins may act in parallel signaling pathways to their normal roles in embryonic development in terms of protein partners and regulation of the expression of downstream genes (Fig. 2). The *PAX* genes can provide a tool for stem cell isolation and manipulation, which potentially could be utilized in stem cell therapies. Conversely, dysregulated expression and/or activation of specific members of the PAX family appear to play a major role in the progression of specific cancers arising in those organ systems in which PAX members exert their developmental functions during embryogenesis. Over-expression of these PAX proteins per se does not appear to be an initiating or transforming molecular event in tumor pathogenesis, but facilitate malignant development through their effects on apoptosis resistance, tumor cell proliferation and migration, or repression of terminal differentiation. Further studies of the role of a particular PAX protein in cancer are required to define the utility of specific PAX proteins as prognostic markers and/or potential targets for novel anti-cancer therapies. While there are many unanswered questions in this research arena, the complexities of PAX biology in developmental processes and in specific diseases, including cancer development and progression in the adult, provide fertile ground for basic and translational research.

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