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Self-Regulated *Pax* Gene Expression and Modulation by the TGFβ Superfamily

Victoria Frost* **and Timothy Grocott†**
School of Biological Sciences, University of East Anglia, Norwich NR4 7TJ, UK

Michael R. Eccles
Department of Pathology, University of Otago, PO Box 913, Dunedin 9001, New Zealand

Andrew Chantry
School of Biological Sciences, University of East Anglia, Norwich NR4 7TJ, UK

The mammalian *Pax* gene family encode a set of paired-domain transcription factors which play essential roles in regulating proliferation, differentiation, apoptosis, cell migration, and stem-cell maintenance. *Pax* gene expression is necessarily tightly controlled and is associated with the demarcation of boundaries during tissue development and specification. Auto- and inter-regulation are mechanisms frequently employed to achieve precise control of *Pax* expression domains in a variety of tissues including the eye, central nervous system, kidney, pancreas, skeletal system, muscle, tooth, and thymus. Furthermore, aberrant *Pax* expression is linked to several diseases and causally associated with certain tumors. An increasing number of studies also relate patterns of *Pax* expression to signaling by members of the TGFβ superfamily and, in some instances, this is due to disruption of *Pax* gene auto-regulation. Here, we review the current evidence highlighting functional and mechanistic overlap between TGFβ signaling and *Pax*-mediated gene transcription. We conclude that self-regulation of *Pax* gene expression coupled with modulation by the TGFβ superfamily represents a signaling axis that is frequently employed during development and disease to drive normal tissue growth, differentiation and homeostasis.

Keywords TGFβ, BMP, *Pax* genes, Smads, transcription

INTRODUCTION

A current trend in biological research is the transition from studying individual molecules to larger systems of interactions and the emerging behaviors that result from increasing levels of complexity. One such example is the mammalian *Pax* gene family, which encodes a set of nine transcription factors that are essential for normal development and whose perturbation is associated with several diseases including cancer (for comprehensive reviews refer to Robson *et al*., 2006; Lang *et al*., 2007).

Pax gene expression is generally down-regulated once development is complete, although some *Pax* genes retain functions in adult tissue. We focus this review on *Pax* gene regulation, one aspect of which involves the sequential activation of one family member by another within a particular tissue during development. Another relates to the restriction of individual family members to adjacent but non-overlapping expression domains by sometimes mutually inhibitory interactions. In addition to such "inter-regulation," several examples of "auto-regulation" have also been reported within the *Pax* gene family, giving clues as to how *Pax* gene expression is reinforced at key locations/timepoints, terminated once a given gene function is no longer required, or deregulated in certain pathologies. As the number of such examples increases, it is becoming clear that *Pax* gene self-regulation is an important mechanism for laying down patterns and establishing cell identities within developing tissues.

Address correspondence to Andrew Chantry, School of Biological Sciences, University of East Anglia, Norwich NR4 7TJ, UK. E-mail: a.chantry@uea.ac.uk

*Victoria Frost and Timothy Grocott are joint first authors.

†Present address: Department of Craniofacial Development, King's College London, Guy's Campus, Guy's Tower Floor 27, London SE1 9RT, UK

In addition to reviewing current knowledge of regulation within the *Pax* gene family, we also address the regulation of this family by the Transforming Growth Factor (TGF) β superfamily of signaling molecules. Moreover, we speculate that these interconnections underpin key developmental and disease-specific processes. Before embarking on the main theme of the review, we provide a brief summary of *Pax* gene function, with emphasis on the importance of inter- and auto-regulation in *Pax*-mediated developmental processes. Signaling pathways mediated by TGFb are then considered, followed by a critique of the evidence suggesting that the TGFβ-superfamily directly impacts on *Pax* gene expression by modulating auto- or inter-regulatory mechanisms. We focus on instances where these mechanisms overlap functionally and on the relevance of these observations particularly to development, with some brief examples of how co-ordinate *Pax*/TGFβ signaling might impact on health and disease.

THE PAX FAMILY OF TRANSCRIPTION FACTORS

The nine members of the *Pax* gene family are subdivided into groups based on the domain structures of their protein products (Figure 1). A common feature of all Pax proteins is an N-terminal paired domain (PD) comprising a DNA-binding motif that recognizes a specific consensus sequence, (G/T)T(T/C)(C/A)(C/T)(G/C)(G/C) (reviewed in Lang *et al*., 2007). Members differ in their possession of a central octopeptide domain, and the presence of a DNA-binding paired-type homeodomain. All members except those in Group IV (*Pax4*, *6*), encode the octopeptide domain which is known to fulfill an inhibitory role in gene transcription, and is related to the *eh1* repression domain in engrailed proteins and the Gsc-En homology element present in Goosecoid proteins (Smith and Jaynes, 1996). A full homeodomain is encoded only by Group III (*Pax3*, *7*) and IV members. A partial homeodomain encoding the first helix only is present throughout Group II (*Pax2*, *5*, *8*), while this domain is entirely absent from Group I (*Pax1*, *9*). The intact paired-type homeodomains found in Group III and IV Pax proteins recognize palindromic elements of TAAT$(N)_{2-3}$ ATTA (Wilson *et al*., 1993). Finally, transcriptional activation by Pax proteins bound to DNA is achieved via a C-terminally located transactivation domain (Glaser *et al*., 1994). This proline/serine/threonine-rich region represents a potent transactivation function when fused to a Gal4 DNA-binding domain (Czerny and Busslinger, 1995). In the case of Group IV member Pax6, this region is known to be targeted for regulatory phosphorylation by MAP kinases leading to a "super-activation" of the transcription factor's activity (Mikkola *et al*., 1999).

Consistent with their essential role in embryogenesis, *Pax* genes are intimately involved in the determination of specific cell lineages. Accordingly, *Pax* gene expression patterns are highly restricted both spatially and temporally throughout embryonic development. For example, Group I *Pax* genes are expressed in the developing skeletal system and thymus, with *Pax9* additionally expressed in developing tooth structures. Group II members share embryonic expression in the CNS, while *Pax2* and *Pax8* also have crucial roles in the developing kidney. *Pax8* has additional functions in the thyroid, whereas Pax5 protein is an important transcription factor in B-cells, with a role in antibody class switching. Group III genes (*Pax3*, *7*) are expressed in the CNS, neural crest, somites and regenerating muscle, with important roles in muscle development. Group IV genes (*Pax4*, *6*) are expressed in the pancreas and gut, whereas *Pax6* is additionally expressed in the CNS and ocular tissues, where it plays an essential part in eye development.

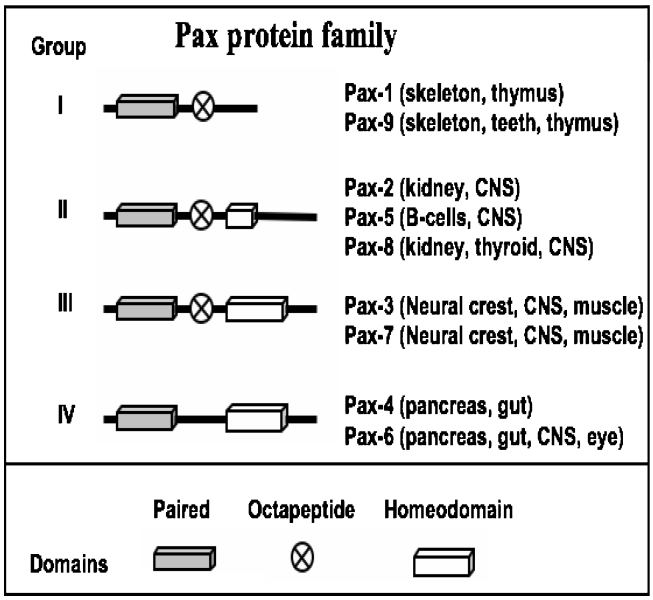

FIG. 1. Domain structure of mammalian Pax proteins. The nine *Pax* family members are subdivided into four groups based on the protein domain structures that they encode. A common feature of all four groups is an N-terminal paired domain (shaded box), which functions as a DNA-binding motif. Groups I–III possess a central octapeptide domain (white crossed circle) which is known to fulfill an inhibitory role in gene transcription. The octapeptide domain is absent from Group IV. Members also differ in their possession of a homeodomain, a highly conserved "helix-loop-helix" DNA-binding motif (white box). A full homeodomain is present throughout Groups III and IV, whereas a partial homeodomain encoding the first helix only is present throughout Group II. The homeodomain is completely absent from the Group I family. Some roles of individual Pax proteins in the development of specific tissues and organs are denoted in brackets (reviewed in Lang *et al*., 2007).

AUTO- AND INTER-REGULATION OF *PAX* GENE EXPRESSION

Group I (*Pax1, 9*)

Inter-Regulation of Pax9 Gene Expression by Pax1

Functional redundancy exists between *Pax1* and *Pax9* with respect to their role in the development of the sclerotome, a

compartment that ultimately forms the skeletal component of the vertebral column and ribs (Peters et al., 1999). The paired binding domains of Pax1 and Pax9 are 98% identical yielding a shared binding affinity for several DNA elements (Chalepakis *et al*., 1991; Czerny *et al*., 1993; Neubüser *et al*., 1995). A severe phenotype is associated with the combined absence of *Pax1* and *Pax9* gene function, characterized by a complete lack of development of ventral elements of the vertebral column along the entire body axis (Peters *et al*., 1999). In contrast, single homozygous mutants of either *Pax1* or *Pax9* show complete or partial rescue compared to double homozygous mutants. Furthermore, as the severity of the phenotype of the double homozygous mutant is greatly reduced by the introduction of heterozygosity in either gene, not only does this show that functional redundancy exists between *Pax1* and *Pax9*, but also that their function in development of the vertebral column is dosage dependent (Peters *et al*., 1999). Furthermore, inter-regulation of *Pax9* by *Pax1* is an integral mechanism during boundary demarcation of the sclerotome. *Pax1* and *Pax9* are the only *Pax* genes expressed in the sclerotome (Figure 2), where *Pax1* expression is initially widespread and precedes that of *Pax9*. As differentiation occurs, the expression of *Pax1* becomes more restricted, showing maximal expression in the posterior, ventral-medial region, while *Pax9* transcription occurs predominantly in the ventral-lateral region of the sclerotome. In *Pax1*-null embryos, *Pax9* is up-regulated and ectopically expressed in the anterior domain of the sclerotome (Peters *et al*., 1999). Thus it appears that a function of *Pax1* in wildtype embryos is to restrict *Pax9* expression to the posterior, ventral domain of the sclerotome. This reflects a second feature of the *Pax* genes, which in addition to displaying functional redundancy can inter-regulate expression of related family members in order to specify boundaries in the developing embryo.

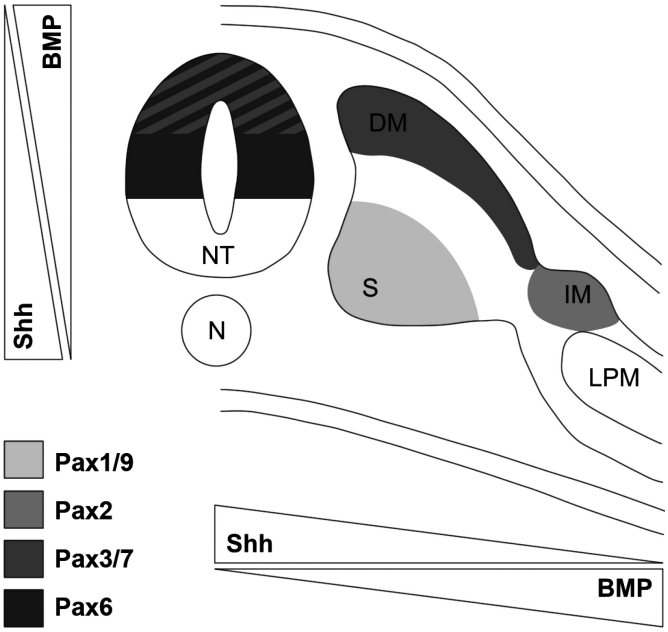

FIG. 2. Schematic representation of *Pax* gene expression domains and TGFβ superfamily (BMP) signaling activity in the developing vertebrate trunk. Transverse view of the vertebrate trunk indicating tissue-specific expression domains of *Pax* family members (shaded areas). The individual expression domains are restricted according to *Pax* gene inter-regulation and BMP signaling activity opposed by Shh (Monsoro-Burq *et al*., 1996; Borycki *et al*., 1999; Peters *et al*., 1999; Timmer *et al*., 2002; James and Schultheiss, 2005). Sources of BMP signals include the dorsal neural tube, dorsal surface ectoderm, and the lateral plate mesoderm. In the ventral somite, Shh signals from the notochord and floor-plate are thought to oppose BMP signaling via a mechanism that depends upon the BMP antagonist Noggin (McMahon *et al*., 2007). Dorsal to the top, medial to the left, lateral to the right. NT — neural tube, N — notochord, DM — dermomyotome, S — sclerotome, IM — intermediate mesoderm, LPM — lateral plate mesoderm.

Group II (*Pax2, 5, 8*)

Inter-Regulation of Pax5 *Expression by* Pax2

A second example of inter-regulation occurs during specification of the midbrain–hindbrain boundary and involves Group II *Pax* members. An organizing center at the midbrain–hindbrain boundary controls the development of the midbrain and cerebellum in vertebrates. Pax and homeodomain transcription factors are essential for the formation and organizer activity of this boundary. In mice, *Pax2* and *Pax5* expression is sequentially activated in this region (Pfeffer *et al*., 2000). Similarly, in zebrafish *Pax2.1* is followed by *Pax5*, *Pax2.2* and finally *Pax8* (Pfeffer *et al*., 1998). A zebrafish mutant lacking a functional *Pax2.1* allele fails to initiate *Pax5* and *Pax8* expression at the midbrain–hindbarin boundary, indicating a dependence of these gene's expression on Pax2.1 protein function (Pfeffer *et al*., 1998). A further study by Pfeffer *et al* (2000) revealed that the mouse *Pax2* gene cooperates with homeodomain transcription factors to regulate a midbrain–hindbrain enhancer of *Pax5* via two distinct elements. In *Pax2* mutant mouse embryos, *Pax5* expression was deficient in the presumptive midbrain–hindbrain region indicating that the latter *Pax* gene is normally subject to inter-regulation by the former (Pfeffer *et al*., 2000). Although the enhancer element contains overlapping consensus binding sites for Group II members, *Pax5* mutant mice revealed that the enhancer's activity does not depend on auto-regulation by Pax5 itself (Pfeffer *et al*., 2000).

Indirect Auto-Regulation of Pax2 *via* Engrailed

A positive auto-regulatory loop may control the expression of *Pax2.1* in some instances. *Pax2.1* auto-regulation is likely to be affected by an indirect mechanism involving engrailed proteins. Comparison of expression from a *Pax2.1*-reporter transgene in wildtype or *Pax2.1* deficient zebrafish embryos revealed a

positive, auto-regulatory loop controlling *Pax2.1* transcription in the development of the midbrain-hindbrain boundary (Picker *et al*., 2002). Feedback occurred cell autonomously and required *Engrailed* family gene products Eng2 and Eng3, known targets of *Pax2.1* regulation (Brand *et al*., 1996; Picker *et al*., 2002). Positive regulation of the *Pax2.1* enhancer is lost in *Eng2*- and *Eng3*-knock-down zebrafish embryos, while four putative Engrailed protein binding sites were identified in the *Pax2.1* enhancer (Picker *et al*., 2002), suggesting that a direct Engrailed-dependent activity might contribute to the feedback loop. Conversely de-repression of factors that normally repress *Pax2* transcription could also contribute to the effect of *Engrailed* gene knock-down. Early activation of *Pax2* transcription is initially *Pax2* independent, however it is suggested that the positive-feedback loop may allow continuous *Pax2* expression to occur independently of the patterning machinery of the gastrula embryo during development of the midbrain-hindbrain organizer (Picker *et al*., 2002).

Inter-Regulation of Pax2 *(and* Pax5 *and* Pax7*) Expression by* Pax6

Precise, localized expression of *Pax* family members is also involved in the definition of the di-mesencephalic boundary of the CNS. During neural development, *Pax6* is expressed in the diencephalon (the future forebrain), while *Engrailed 1* (*En1* in mammals; *Eng* in *Drosophila* and zebrafish) and *Pax2* are expressed in the mesencephalon (the future midbrain). The di-mesencephalic boundary is formed at the site where *Pax6* and *En1*/*Pax2* expression overlap. Matsunaga *et al*. (2000) reported that misexpression of *Pax6* in the mesencephalon caused a caudal shift of the di-mesencephalic boundary, while repressing the expression of *En1*, *Pax2*, *Pax5*, and *Pax7*. It is postulated that *Pax6* represses these genes indirectly, via activation of a transcriptional repressor. Conversely, misexpression of *Pax7* in the diencephalon leads to a loss of *Pax6* expression and the ectopic activation of both *Engrailed 2*, and subsequenctly endogenous *Pax3*/*7* (Matsunaga *et al*., 2001). Interestingly, vertebrate Engrailed proteins are able to interact with Pax6 protein via the paired domain of the latter (Plaza *et al*., 1997; Araki and Nakamura, 1999). Interaction with Engrailed negatively regulates the DNA-binding properties of Pax6 (Plaza *et al*., 1997), potentially severing a positive auto-regulatory loop responsible for driving *Pax6* expression (discussed later). Taken together, it is clear that these various repressive interactions may determine the border of domains expressing *Pax6* and *En1*/*Pax2* and thus help define the di-mesencephalic boundary.

Reciprocal Inter-Regulation of Pax2 *and* Pax6

A reciprocal relationship between the expression of *Pax6* and *Pax2* plays a key role in the patterning of the optic cup. *Pax6* has a crucial and fundamental role in the specification, growth and differentiation of the presumptive eye structures, in addition to its role in the development of the CNS. Of particular interest is the necessity for *Pax6* expression during demarcation of the distal portion of the optic vesicle which forms the neural retina andretinal pigmented epithelium. In contrast, *Pax2* defines the proximal optic primordium, forming the optic fissure and optic stalk. Two members of the Hedgehog family of signaling molecules, Sonic Hedgehog (Shh) and Tiggywinkle Hedgehog (Twhh) may be responsible for generating the complementary domains of *Pax6* and *Pax2* expression in the optic vesicle. Overexpression of either Hedgehog protein in early zebrafish embryos produces an expansion of *Pax2* expression domains at the expense of *Pax6* expression, leading to eye malformation (Ekker *et al*., 1995; Macdonald *et al*., 1995). Furthermore, spatial specification of the optic cup and stalk may result directly from a mechanism of reciprocal transcriptional repression of *Pax2* and *Pax6* (Schwarz *et al*., 2000). Examination of *Pax2* and *Pax6* expression domains in *Pax6* null or *Pax2* null early mouse embryos in this study revealed a corresponding expansion of the opposing *Pax* domain. The presence of Pax2 binding sites on the retina enhancer of the *Pax6* gene, and of Pax6 binding sites on the *Pax2* upstream control region were identified by gel shift assays. Moreover, a reciprocal inhibition of *Pax2* promoter/enhancer activity by *Pax6*, and vice versa, was also demonstrated.

Other signaling molecules in addition to Hedgehog members are required for the morphogenesis of the optic vesicle. A tripartite signaling interaction between Shh, BMP4 and FGF8 has a demonstrable role in this process in chick embryos. Implantation of beads soaked with the TGFβ superfamily member BMP4 in the anterior neuropore of stage 10 chick embryos repressed the expression of both FGF8 and Shh, and this correlated with hypoplasia of the telenchephalic and optic vesicles (Ohkubo *et al*., 2002). This observation is of particular relevance to this review, which seeks to highlight and inter-link common mechanisms in *Pax* gene regulation. As will be discussed in the second part of the review, regulation of *Pax* gene expression is often closely related to the activity of members of the TGFβ superfamily, and evidence for direct regulation of *Pax* gene transcription by TGFβ family members exists. However, during pattern formation of the optic cup, an inverse relationship exists whereby *Pax6* regulates the expression of BMP4 (Reza *et al*., 2007). *Pax6* demonstrates a gradient of high distal and low proximal expression. Ectopic expression of *Pax6* in the optic vesicle of chick embryos resulted in a small eye phenotype with decreased expression of the dorsal marker BMP4 and an increase in FGF8 expression. It is suggested that Pax6 can regulate the balance of FGF8 and BMP4 expression during patterning of the optic cup, and that this is a critical process for retinogenesis. Furthermore, BMP4 expression is complementary to that of Shh and ectopic Shh has been found to suppress expression of BMP4 in the dorsal retina. Conversely, a reduction in endogenous Shh activity results in ventral expansion of BMP4 expression (Zhang and Yang, 2001). Taken together these studies highlight the delicate balancing act of key signaling molecules BMP4, Shh and FGF8 and the reciprocal expression of *Pax6* and *Pax2* which together specify the optic primordium.

Indirect Inter-Regulation of Pax2 *Expression by* Pax8 *via* Wilms Tumor 1

There is evidence suggesting that *Pax2* may be subject to a form of negative regulation by fellow Group II member *Pax8*, but in this case the inter-regulation is indirect and mediated by *Wilms Tumor 1* (*WT1*). The human and mouse *Pax2* promoters, characterized by Stayner *et al*. (1998) and Ryan *et al*. (1995), in contrast to the promoters for *Pax3* and *Pax5*, are GC-rich and lack a consensus TATA or CCAAT box. Instead the *Pax2* promoters resemble those of *WT1* and TGFβ which posess GC boxes in place of TATA and CCAAT motifs, a property more normally associated with house-keeping genes. Interestingly, the *WT1* tumor suppressor has been shown to repress expression from the *Pax2* promoter, while *Pax2* and *Pax8* are also physiological activators of *WT1* expression. Indeed, both *Pax2* and *Pax8* have been shown to transactivate the *WT1* promoter *in vitro* (Dehbi *et al*., 1996; Fraizer *et al*., 1997; McConnell *et al*., 1997). Furthermore, *Pax2* can induce expression of the endogenous *WT1* gene with possible relevance to the role of *Pax2* during mesenchyme–epithelium transition during development of the kidneys. Conversely, the WT1 gene product represses *Pax2* gene transcription via binding to three high affinity sites in the *Pax2* 5′-UTR (Ryan *et al*., 1995). During kidney development *Pax2* expression precedes that of *Pax8*, being present in both the ureteric bud and in the condensing mesenchyme. However, in contrast with *Pax8*, *Pax2* is absent from the S-shaped bodies, and this has been attributed to the expression of *WT1* at this location. More specifically, it is thought that *Pax2* exclusion from this latter structure is due to cross-regulation of *Pax2* by *Pax8*, via *WT1*: *Pax8* enhances *WT1* expression, which in turn represses *Pax2* gene transcription (Fraizer *et al*., 1997).

Group III (*Pax3, 7*)

Inter-Regulation of Pax7 *Gene Expression by* Pax3

Members of the Group III *Pax* family also show a propensity for inter-regulation and the relationship between *Pax3* and *Pax7* is critical for the development of the CNS and muscle. In the developing vertebrate embryo, *Pax3* is expressed in the neural tube, which is the precursor of the CNS, and in the paraxial mesoderm which forms just lateral to the neural tube and gives rise to the somites (Figure 2). Somites are distributed along both sides of the developing neural tube and distinct regions of the somite develop into the dermis (dermatome), muscle (myotome) and vertebrae (sclerotome). The sclerotome forms earlier during development than both the dermatome and myotome, at which stage the collective term for the latter structures is the dermomyotome. The importance of *Pax3* in muscle formation is demonstrated by the absence of limb muscles in Splotch mice that harbour a disruption of the *Pax3* gene. Increased apoptosis is seen in the presomitic mesoderm of Splotch mice embryos, and this is attributed to loss of the survival effects normally associated with *Pax3* (Borycki *et al*., 1999).

Pax7, the closely related member of *Pax* Group III shares a similar expression pattern to *Pax3*, being expressed in the dorsal neural tube and in somites. However, *Pax7* is missing from the presomitic mesoderm, a site of *Pax3* expression, but is induced in late stages of somite maturation (Jostes *et al*., 1991). In a relationship reminiscent of that displayed between *Pax1* and *Pax9* in the developing sclerotome, *Pax3* exerts a repressive effect on *Pax7* expression in somitic cells and neural tube (Borycki *et al*., 1999). *Pax3* shows widespread expression in the early somite, but becomes restricted to the dermomyotome as the somites mature. *Pax7* is absent from early somites, but like *Pax3* is present in the dermomyotome (Figure 2). Later, *Pax3* and *Pax7* are individually expressed in the ventral-lateral and dorsal-medial domains of the dermomyotome respectively, and additionally in a population of myogenic precursor cells that arise from the central dermomyotome (Borycki *et al*., 1999; Relaix *et al*., 2005). In *Splotch* mutant mice, *Pax7* is ectopically expressed in domains of both the dorsal neural tube and somitic mesoderm where only *Pax3* is normally expressed in wild type embryos. Furthermore, expression of *Pax3* in C2C12 myoblasts produces a dramatic down-regulation of endogenous *Pax7* expression (Borycki *et al*., 1999). In *Pax3*/*Pax7* double mutant mice, the myogenic precursor population arising from the central dermomyotome which normally expresses these genes is lost (Relaix *et al*., 2005; Buckingham *et al*., 2006). While during normal development the expression of *Pax3* precludes expression of *Pax7* in certain locations, it is also likely that functional redundancy between *Pax3* and *Pax7* allows compensation for individual disruption of either gene. Evidence for this comes from the more severe phenotype of *Pax3*/*Pax7* mutant embryos in comparison with *Pax3* mutant embryos, and the observation that apoptosis rates are lower in the somites of *Pax3*-deficient embryos that misexpress *Pax7*.

Inter-Regulation of Pax3 *Gene Expression by* Pax7

Pax7 has an essential role in specification of the midbrain and in development of the superior collicular where its graded expression establishes rostral-caudal and dorsal-ventral polarity (Thompson *et al*., 2008). Despite this role, *Pax7* mutant mice lack any gross defects in superior collicular development and it was suggested that this may be due to compensation by the other Group III member, *Pax3*. *Pax3* and *Pax7* expression patterns initially overlap, but become increasingly divergent through development (Thompson *et al*., 2008). *Pax7* mutant embryos exhibit a rostral, caudal and ventral expansion in *Pax3* expression suggesting that *Pax7* normally acts to limit the extent of *Pax3* expression in those regions that normally only express the former. Indeed, it has been shown that exogenous *Pax3* or *Pax7* can act cell-autonomously to down-regulate endogenous expression of the other genes in the midbrain, while in the forebrain, *Pax7* misexpression leads to an up-regulation of *Engrailed 2* and subsequently endogenous *Pax3*/*7* (Matsunaga *et al*., 2001). It has been suggested that the *Pax3*/*7* inter-regulatory mechanism

controls the total expression of Group III *Pax* genes within a cell by activating or inhibiting the expression of one or other gene as appropriate, although the underlying molecular mechanism remains unresolved (Matsunaga *et al*., 2001).

Group IV (*Pax4, 6*)

Auto-Regulation of Pax4 *Gene Expression*

Both members of the Group IV *Pax* family are subject to auto-regulation and this is a key regulatory mechanism during development. In the case of *Pax4* this mechanism is crucial in the developing pancreas where *Pax4* controls the differentiation of pancreatic β cells. Transient expression of *Pax4* in the fetal pancreas plays a fundamental role in pancreatic development, its expression peaking during the period of β- and δ-cell differentiation (Smith *et al*., 1999). The *Pax4* promoter has been reported to include at least two high-affinity binding sites for its own gene product (Smith *et al*., 2000). The negative auto-regulation that ensues is suggested to be an important regulatory mechanism to terminate *Pax4* production once it has reached sufficient levels to specify β-cell and δ-cell fate. The *Pax4* promoter contains consensus sequences for both paired domain and homeodomain motifs. Negative auto-regulation of the *Pax4* promoter was found to depend on the homeodomain, and to a lesser extent on the paired domain of the Pax4 protein. The Pax4 binding sites within the *Pax4* promoter show much greater affinity for Pax4 than its related family member Pax6 *in vitro*.

Auto-Regulation of Pax6 *Gene Expression*

Probably the most extensively analysed example of Pax gene auto-regulation is that of *Pax6*. *Pax6* expression is restricted to the eye, CNS and pancreas, and has been described as a master controller of eye development. Mutations that reduce the level of *Pax6* protein result in eye defects of variable severity. At least two promoters, P0 and P1, control *Pax6* transcription, while a third promoter Pα has been reported in mice. Studies in quail first revealed that *Pax6* protein is able to bind multiple sites in both P0 and P1 (Plaza *et al*., 1993; 1995). This has since been confirmed for the corresponding human promoters (Okladnova *et al*., 1998), as well as several *Pax6* enhancer sequences in mouse (Aota *et al*., 2003; Kleinjan *et al*., 2004). Pax6-responsive sites have been shown to direct auto-regulation of the quail promoters in cultured quail neuroretinal and embryonic cells (Plaza *et al*., 1993), the mouse lens ectodermal enhancer ($\sim$3 kb upstream of P0) in SRA01/04 human lens epithelial cells (Aota *et al*., 2003), the mouse intron 7 enhancer in developing forebrain (Kleinjan *et al*., 2004), and the human *Pax6* promoters in COS-7 cells (Okladnova *et al*., 1998), and FHL124 lens and HEK293 embryonic kidney cells (Grocott *et al*., 2007). In the latter study, Pax6 was unable to auto-regulate a P1-promoter construct that was mutated in a putative paired domain-binding site, highlighting the importance of the paired domain in mediating the interaction between Pax6 and its own promoter. Direct association of Pax6 with a region of the P1 promoter encompassing this paired domain binding site was confirmed by gel mobility shift assay (Grocott *et al*., 2007).

Additional evidence for auto-regulation *in vivo* comes from studies of *Small Eye* mutant phenotypes in mice. One particular *Small Eye* mutant allele, *Sey*, is the product of a single point mutation, and results in a protein that is truncated before the homeodomain (Hill *et al*., 1991). While this is sufficient to disrupt gene function, it does not interfere with the detection of *Pax6* mRNA by *in situ* hybridization. During the initial stages of eye development in $Pax6^{Sey/Sey}$ mice, the mutant mRNA is expressed as in the wild type. Subsequently however, *Pax6* expression is completely lost throughout the head surface ectoderm when it should normally be confined to the developing lens placodes (Ashery-Padan *et al*., 2000). It therefore appears that *Pax6* function is required for maintenance of its own transcription in the presumptive lens ectoderm. The two major isoforms of Pax6 protein, Pax6 and Pax6(5a), have been shown to positively auto-regulate the expression of the endogenous gene when overexpressed in Neuro2A or NIH3T3 cells (Pinson *et al*., 2006).

Pax6 is also important in the developing brain, and numerous studies have shown that mice homozygous for inactivating *Pax6* mutations, as well as lacking eyes and nasal cavities, die at birth with brain abnormalities (for example Hill *et al*., 1991; Grindley *et al*., 1995). *Pax6* has been reported to regulate its own transcription in the caudal diencephalon of developing mouse embryos (Grindley *et al*., 1997). In contrast, a recent study has revealed that in the developing murine cortex *Pax6* is subject to negative auto-regulation (Manuel *et al*., 2007). Embryos of transgenic mice carrying several copies of the human *Pax6* locus including its full regulatory region express *Pax6* in a normal spatial-temporal pattern, with overall levels of Pax6 increasing only 3-fold relative to wild type, an increase not as great as the increase in the number of copies of the gene. The observation that Pax6 protein levels did not increase proportionately with gene copy number led to the hypothesis that negative auto-regulation was restraining the expression of *Pax6*. This was confirmed when these transgenic mice were crossed with a *Pax6* reporter line, revealing that the expression of *Pax6* is indeed limited by negative auto-regulation. Furthermore, artificially increased *Pax6* expression resulted in selectively reduced proliferation of late cortical progenitors in a cell autonomous manner. The authors concluded that negative auto-regulation of *Pax6* expression is a mechanism employed to achieve stabilization of *Pax6* protein levels, and hence define the precise concentration of protein required for cortogenesis. Again, this reinforces the theme of this review that auto- and inter-regulation are often employed to control *Pax* gene expression and hence crucial mechanisms in developmental processes (see Table 1). As we shall see in the following section, a second feature of *Pax* gene expression relates to its frequent correlation with signaling by members of the TGFβ superfamily. Particularly pertinent to this review is the evidence that directly links TGFβ to the auto-regulatory mechanism employed by *Pax6*.

TABLE 1
Inter- and auto-regulation of *Pax* expression

Pax family member	Type of regulation	Biological example	Reference
Pax1/*Pax9*	Inter-	*Pax9* is up-regulated in *Pax1* null embryos and ectopically expressed in the anterior sclerotome	Peters *et al*., 1999
Pax2.1/*Pax5*/*Pax8*	Inter-	Expression of *Pax5* and *Pax8* fails to initiate at the midbrain–hindbrain boundary in *Pax2*.1 mutant zebrafish embryos	
Pax2/*Pax5*	Inter-	Expression of *Pax5* is deficient in the presumptive midbrain–hindbrain region in *Pax2* mutant mouse embryos	Pfeffer *et al*., 2000
Pax2/*Pax5*/*Pax7*/*Pax6*	Inter-	Mis-expression of *Pax6* in the mesencephalon causes caudal shift of the di-mesencephalic boundary while repressing the expression of *Pax2*/*5*/*7* and *En1*	Matsunaga *et al*., 2000
Pax7/*Pax3*/*Pax6*	Inter- and auto-	Mis-expression of *Pax7* in the diencephalon causes loss of *Pax6* and induction of *En2* and endogenous *Pax3*/*7*	Matsunaga *et al*., 2001
Pax2/*Pax8*	Inter-	*Pax8* enhances *WT1* expression which in turn may repress *Pax2* expression in the S-shaped bodies of the developing kidneys	Fraizer *et al*., 1997
Pax3/*Pax7*	Inter-	*Pax3* represses *Pax7* expression in somitic cells and neural tube, and in myoblasts	Borycki *et al*., 1999
Pax2	Auto-	A positive auto-regulatory loop controls *Pax2* transcription at the midbrain–hindbrain boundary. This loop was indirect and involved *engrailed*	Picker *et al*., 2002
Pax4	Auto-	Direct negative auto-regulation in the developing pancreas	Smith *et al*., 2000
Pax6	Auto-	Pax6 is required to maintain its own transcription in the presumptive lens ectoderm	Ashery-Padan *et al*., 2000
Pax6	Auto-	Positive auto-regulation during development of murine caudal diencephalons	Grindley *et al*., 1997
Pax6	Auto-	Positive auto-regulation of endogenous Pax6 isoforms in Neuro2A or NIH3T3 cells	Pinson *et al*., 2006
Pax6	Auto-	Positive auto-regulation of quail promoters in neuroretinal and embryonic cells	Plaza *et al*., 1993
Pax6	Auto-	Positive auto-regulation of human *Pax6* promoter in Cos-7 cells	Okladnova *et al*., 1998
Pax6	Auto-	Positive auto-regulation of human *Pax6* promoter in HEK293 and FHL124 lens cells	Grocott *et al*., 2007
Pax6	Auto-	Negative auto-regulation in the developing murine cortex	Manuel *et al*., 2007

SIGNALING BY THE TGFβ SUPERFAMILY

The TGFβ superfamily comprises a group of cytokines which in mammals approaches 30 members. Aside from the prototype member TGFβ, the superfamily includes activins and inhibins, nodal, bone morphogenic proteins (BMPs), anti-Müllerian hormone (MIS/AMH), and growth/differentiation factors (GDFs) including myostatin. Cellular activation by each of these ligands is mediated by binding to type I and type II receptors in a hetero-tetrameric complex composed of two molecules of each type of receptor. Formation of these various ligand/receptor complexes initiates a process of receptor transphosphorylation, whereby the constitutively active serine/threonine kinase activity of the type II receptor is directed against its type I partner. This unleashes the dormant serine/threonine kinase activity of the type I receptor to initiate a series of signaling events (Figure 3). These events have been extensively reviewed elsewhere (Moustakas *et al*., 2001; Miyazawa *et al*., 2002; ten Dijke and Hill, 2004; Feng and Derynck, 2005).

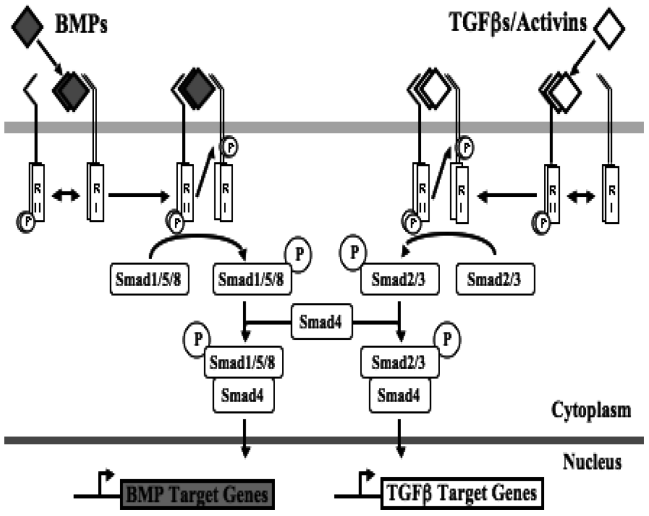

FIG. 3. Smad-mediated signal transduction by the TGFβ superfamily. BMP and TGF-β/activin family signals are mediated by parallel pathways invoking distinct R-Smads, but sharing a single Co-Smad. Binding of ligands of the TGFβ superfamily to their specific combination of type II and type I receptors induces assembly of a receptor complex, which allows the constitutive kinase activity of the type II receptor to phosphorylate the type I receptor. Phosphorylation of the type I receptor occurs in the GS domain of the juxtamembrane, a domain rich in glycine and serine residues, and is the trigger to initiate further signaling events. In the nucleus, R-Smad/Co-Smad complexes work to modulate target gene expression (reviewed in Moustakas *et al*., 2001; Miyazawa *et al*., 2002; ten Dijke and Hill, 2004; Feng and Derynck, 2005).

All TGFβ superfamily ligands share common sequence and structural features including six highly conserved cysteine residues which form a cysteine knot (Sun and Davies, 1995). Signaling specificity in mammalian cells results from selective binding of ligands to a spectrum of seven type I and five type II receptors, with ligand/receptor complexes falling into two distinct groups depending on sequence similarity and the precise signaling pathways they employ (Miyazawa *et al*., 2002). Subsequent signaling events are generally mediated by Smad transcription factors. The Smad family is divided into three groups: Receptor Smads (R-Smads), Co-Smads, and Inhibitory Smads (I-Smads). Features shared by family members include an N-terminal MH1 domain, responsible for nuclear localization and DNA-binding, a central linker region containing a "PY" ubiquitin ligase interaction motif, and a C-terminal MH2 domain which mediates receptor binding and Smad oligomerization, interaction with DNA binding proteins, and transcriptional activation. In all cases, receptor regulated Smads (R-Smads) are directly phosphorylated at a C-terminal -SSXS- motif and thus activated by the type I receptor, but the species of R-Smads targeted for phosphorylation differs between the two groups of receptor complexes. TGFβ, activin and nodal, activate R-Smads 2 and 3 via their type I receptors Alk5 (TβRI), Alk4 (ActR-IB) and Alk7. In contrast, BMPs and MIS/AMH activate R-Smads 1, 5 and 8 via their type I receptors Alk3 (BMPR-IA), Alk6 (BMPR-IB), and Alk2 (Reviewed in Moustakas *et al*., 2001). Unusually, TGFβ and BMP9 in combination with their respective type II receptors share the ability to activate Alk1, a predominantly endothelial cell type receptor, resulting in activation of Smads 1, 5, and 8 (Scharpfenecker *et al*., 2007). Once phosphorylated, R-Smads form homomeric complexes or heteromeric complexes with the Co-Smad, Smad4. These complexes then translocate to the nucleus where they are able to modulate the transcriptional activity of a variety of genes, either via direct DNA binding, or through associations with a range of DNA-binding proteins. For example, Smad3 interacts with the sequence 5′-GTCT-3′ and its reverse complement 5′-AGAC-3′ via a β hairpin in its MH1 domain, whereas Smad2 is unable to bind DNA directly due to an insertion in the β hairpin. Signaling can be antagonized or terminated by the action of the so-called Inhibitory Smads (I-Smads), Smad6 and Smad7. The inhibitory mechanisms employed by I-Smads include the recruitment of ubiquitin ligases to drive ubiquitin-mediated degradation of either receptors or receptor Smads, or interference with R-Smad/Receptor binding.

In spite of their similarities, members of the TGFβ and BMP subfamilies are able to induce diverse cellular responses, and have been implicated in different aspects of development (Smith and Howard, 1992; Graff, 1997; Harland and Gerhart, 1997). Members of the TGFβ/activin/nodal subfamily are required for specification and patterning of mesoderm and endoderm, promoting gastrulation movements, and establishing left–right asymmetry. The BMP/GDF/MIS subfamily positions the neural plate border during primary induction, contributes to dorsal/ventral axis establishment in the neural tube, and patterns ventral and lateral mesoderm. Members of both subfamilies have been shown to act as morphogens (Gurdon *et al*., 1994; Wilson *et al*., 1997).

REGULATION OF *PAX* GENE EXPRESSION BY THE TGFβ SUPERFAMILY

Group I (*Pax1, 9*)

Regulation of Pax1 *(and* Pax3, 9*) by the TGFβ superfamily*

There is strong evidence that members of the TGFβ superfamily can regulate the expression of *Pax1*, thereby impacting upon a variety of developmental processes. In particular, the bone morphogenetic proteins BMP2 and BMP4 are intimately associated with *Pax1* expression during vertebral and limb development (see Figure 2) (Monsoro-Burq *et al*., 1996; Hofmann *et al*., 1998; McMahon *et al*., 2007). Lateral grafting of BMP2-producing cells to the neural tube of chick embryos at embryonic day 2 represses *Pax1* and *Pax3* gene expression in the neighbouring somitic mesenchyme, inhibiting sclerotomal cell growth and differentiation into cartilage. Furthermore, the neural tube becomes dorsalized, showing ectopic *Pax3* expression in the basal

plate contacting the BMP-producing cell graft (Monsoro-Burq *et al*., 1996).

More recently it has been shown that inhibition of BMP signaling by Noggin is required for normal patterning of the vertebrate neural tube and somite (McMahon *et al*., 2007). Noggin binds several BMPs with picomolar affinities, and exhibits a marked preference for BMP2 and BMP4 compared with BMP7. In the murine paraxial mesoderm, Noggin alone is sufficient to induce *Pax1*, while Sonic Hedgehog (Shh) synergistically enhances this effect. In the absence of *Noggin*, *Shh*-dependent ventral cell fates are lost, despite normal expression of *Shh* in the notochord in mutant mouse embryos, highlighting the involvement of *Noggin* in the effects of *Shh* in this system (McMahon *et al*., 2007).

Restricted expression of *Noggin* and *BMP4* are observed in the developing avian sclerotome, and play a role in domain specification; the medial-ventral sclerotome expresses *Pax1* under the control of *Noggin* and *Shh*, whereas the dorsal-lateral sclerotome is exposed to BMP4 and hence does not express *Pax1* (Reviewed in Christ *et al*., 2004). An excess of BMP2 and BMP4 have been shown to block Noggin- and *Shh*-induced *Pax1* expression in the murine presomitic mesoderm (McMahon *et al*., 2007). *Noggin* is also a critical factor in spinal joint formation. The expression of *Noggin* and *Pax1* overlap temporally and spatially in the annulus fibrosus of the developing spine and this specific expression is important for pattern formation of the intervertebral disc (DiPaola *et al*., 2005).

Pax1 expressing cells in chick wing bud give rise to the shoulder girdle. Similarly positioned cells in the mouse forelimb express *Pax1*, while *Pax1* mutant mice display defects in the shoulder girdle. BMP2 and BMP4 repress *Pax1* in developing chick wing, and shoulder girdle defects are associated with increased *BMP* expression in chick embryos (Hofmann *et al*., 1998). However, scapula precursors located within the avian hypaxial somitic domain require BMP signals derived from the adjacent somatopleural cells to embark on the chondrogenic programme. Inhibition of BMP activity is associated with diminished expression of *Pax1* in this compartment and interferes with scapula blade formation (Wang *et al*., 2005; reviewed in Huang *et al*., 2006).

Members of the TGFβ superfamily that signal through the TGFβ type II receptor also regulate skeletal development, as deletion of this receptor results in defects in the axial skeleton, including decreased overall size of vertebral elements in transgenic mice (Baffi *et al* 2006). This may be due to reduced proliferation of sclerotome cells, thereby decreasing the pool of cells available for chondrogenesis. Furthermore, TGFβ Type II receptor is required to maintain boundaries between different compartments of the developing axial skeleton, and deletion of the receptor alters the rostral-caudal boundary of the sclerotome. The disrupted boundary was associated with expansion of *Pax1* and *Pax9* expression to cover the entire sclerotome, rather than their normal restricted expression in only the caudal half of the sclerotome. This suggests that TGFβ Type II receptor is involved in regulating the expression boundaries for *Pax1* and *Pax9* within the sclerotome.

Regulation of Pax9 *by the TGFβ superfamily in Craniofacial Development*

Opposing signals from fibroblast growth factor 8 (FGF8) and BMP2/4 determine *Pax9* expression in the prospective tooth mesenchyme during odontogenesis (Neubüser *et al*., 1997). This is an important step in positioning sites of tooth formation in mouse embryos. *FGF8* and *BMP2/4* have widespread and partially overlapping expression patterns in the oral ectoderm. FGF8 induces *Pax9* expression, while BMP2 and BMP4 prevent this induction. *FGF8* but not *BMP2/4* is produced in the future tooth epithelium, thereby inducing *Pax9* expression in the underlying mesenchyme. However, where *BMP2/4* is expressed in neighboring domains, *Pax9* induction by *FGF8* is repressed, thus defining sites of tooth formation. At later stages in development, *BMP4* no longer prevents *Pax9* expression, instead mediating inductive tissue interactions within the developing tooth.

A critical role for TGFβ3 in the development of the murine palate has been linked to stage-specific expression of *Pax9* (Sasaki *et al*., 2007). *Pax9* expression is induced between embryonic days E13.5 and E15.5 in the developing palate of mice embryos. At E14.5 and E15.5 this expression becomes restricted to the medial edge epithelia of the fusing palate and the immediately adjacent mesenchyme. *TGFβ3* shows a similar but not identical pattern of expression, mimicking the high levels of *Pax9* expression in the fusing medial edge epithelia, but is absent from the palatal mesenchyme. *TGFβ3* is essential for the developmental process of palatal fusion and disruption of the *TGFβ3* gene causes cleft secondary palate. Comparison of wild type and *TGFβ3*-null mice reveals altered expression patterns of *Pax9* and Sonic Hedgehog genes at key stages in development of the palate. In particular, between developmental days E14.5 andE15.5, a time that is critical for palatal fusion, major differences in the pattern of *Pax9* expression are observed between the wild type and *TGFβ3*-null genotypes. At this stage, expression of *Pax9* is greatly down-regulated in the medial edge epithelia and mesenchyme of the palate in the *TGFβ3*-null mice. It is suggested that in normal embryos *TGFβ3* diffusing from the palatal medial edge epithelia positively regulates *Pax9* expression in the underlying palatal mesenchyme, which in turn regulates the processes of medial edge epithelial fusion and mesenchymal condensation during formation of the palate. Furthermore, *Pax9* and *TGFβ3* have recently been linked with susceptibility to nonsyndromic cleft lip with or without cleft palate in a Japanese population (Ichikawa *et al*., 2006).

Group II (*Pax2, 5, 8*)

Regulation of Pax2 by the TGFβ Superfamily in the Intermediate Mesoderm and its Derivative Tissues

The intermediate mesoderm gives rise to the developing urogenital system of vertebrates, and is situated between the

paraxial (pre-somitic) mesoderm and the lateral plate mesoderm which contributes to the circulatory system (Figure 2). The paraxial mesoderm is adjacent to the neural tube, whereas the lateral plate is situated at the periphery of the avian embryo. Chick embryonic mesoderm electroporation with constitutively active receptors for BMP2/4 (Alk3/Alk6) revealed that signaling through these receptors regulates intermediate mesodermal gene expression in a dose dependent manner (James and Schultheiss, 2005). High levels of ectopic expression of BMP receptors promoted lateral mesoderm gene expression, whilst inhibiting the expression of intermediate mesoderm genes including *Pax2*. Therefore, high BMP2/4 signaling converted somite and intermediate mesodermal cells into lateral plate and vascular/hematopoetic cells. Conversely, low level mis-expression of BMP receptors in somites promoted intermediate mesoderm fate in a cell autonomous manner, and this was associated with co-expression of *Pax2* and *Pax7*. It is envisaged that BMP signaling must be strictly controlled across the various mesodermal compartments in order to appropriately regulate *Pax* gene expression and thus normal development of the skeletal, urogenital and circulatory systems. Furthermore, blockade of BMP signaling has been achieved in zebrafish by forced expression of a mutant BMP receptor. This dominant-negative BMP receptor was used to disrupt BMP signaling specifically in lateral mesoderm during somitogenesis in developing zebrafish embryos (Gupta *et al*., 2006). This resulted in expansion of hematopoetic and endothelial cells, while restricting the expression of pronephric marker *Pax2*.1. In this model, normal levels of BMP signaling are postulated to promote pronephric development at the expense of hemato-vascular development. Taken together these studies suggest that BMPs exert important dose-dependent effects on cell lineage decisions in developing somites, and these may be mediated by regulating *Pax* gene expression.

Pax2 is a key factor in the development of various structures of the mammalian urogenital system including the epithelia of the ureter, the Müllerian duct, the Wolffian duct and the nephrogenic mesenchyme. A 0.4 kb fragment of the upstream region of *Pax2* has been identified as the minimal region for gene activation in the Wolffian duct (Kuschert *et al*., 2001). This fragment has consensus binding sites for Smad, homeobox and bHLH transcription factors although these interactions are yet to be confirmed by biochemical analysis. Importantly, *BMP7*-deficient mice described in a study by Luo *et al*. (1995) show aberrant kidney development and die soon after birth. Absence of functional *BMP7* affects the expression of the nephrogenic markers *Pax2* and *Wnt4* between E12.5 and E14.5, highlighting the role of BMP7 in the induction of nephrogenesis.

The nephric duct structure is essential for all further urogenital development. It has been shown that surface ectoderm is essential for the differentiation of cells of the intermediate mesoderm into the nephric duct, and that removal of surface ectoderm decreased levels of *Pax2* and *Sim-1* mRNA expression in mesenchymal nephric duct progenitors and inhibited kidney development (Obara-Ishihara *et al*., 1999). Addition of a BMP4-coated bead to chick embryos lacking the surface ectoderm restored normal expression of *Pax2* and *Sim-1* in nephric duct progenitors, allowing normal formation of the nephric duct. The surface ectoderm expresses *BMP4* and is also required for the maintenance of high levels of *BMP4* expression in the lateral plate mesoderm, highlighting the multitude of interactions between the surface ectoderm, lateral mesoderm and intermediate mesoderm during kidney development.

Other members of the TGFβ superfamily may also regulate kidney development via effects on *Pax* gene expression. Expression of *Pax2* regulates mesenchymal-epithelial transition during kidney tubulogenesis. Treatment of proximal tubule cells with TGFβ1 suppresses *Pax2* expression by reducing *Pax2* mRNA stability (Liu *et al*., 1997). As TGFβ is antiproliferative in these cells this suggests that *Pax2* may have a role in regulating renal cell proliferation. *Pax2* is normally repressed upon maturation of the renal epithelium and continued *Pax2* expression is incompatible with normal kidney development. Dominant gain-of-function mutation of *Pax2* in transgenic mice results in dysfunctional renal epithelium, possibly reflecting reduced differentiation potential of these cells (Dressler *et al*., 1993). Furthermore, up-regulation of components of the TGFβ1 pathway has been implicated in kidney dysplasia, a condition associated with perturbed epithelial-mesenchymal interactions and often responsible for chronic kidney failure in children (Yang *et al*., 2000). In this study, exogenous expression of *TGFβ1* decreased expression of *Pax2* and *Bcl-2* in a dysplastic kidney epithelial-like cell line, and this was associated with decreased proliferation and transition to a mesenchymal phenotype.

Another member of the TGFβ superfamily, activin A, is an endogenous inhibitor of ureteric bud formation, providing a restraining influence on metanephric kidney development. The inhibitory action of activin A must be overcome in order for ureteric bud outgrowth from the Wolffian duct to proceed. The restraining influence of activin A may be related to its inhibitory effect on glial cell-line derived neurotrophic growth factor (GDNF). GDNF provides a critical stimulatory signal for bud formation, and importantly GDNF-induced bud formation is inhibited by activin A in an *in vitro* Wolffian duct culture system (Maeshima *et al*., 2006). Inhibition by activin A was also associated with a block of cell proliferation, reduced expression of *Pax2* and decreased phosphorylation of PI3 kinase and MAP kinase. As described above, *Pax2* is one of the key factors controlling kidney development. The Pax2 transcription factor promotes ureteric bud outgrowth, probably via Pax2-dependent activation of *GDNF*. It is possible that activin A down-regulates *Pax2* gene transcription directly, and in doing so down-regulates *Pax2*-dependent activation of *GDNF*.

Activin A is also negatively implicated in recovery from renal ischemia. Activin A is induced in the tubular cells of ischemic kidneys and is inhibitory to their regeneration. *Pax2* is intimately involved in the regeneration process and is up-regulated following urinary tract obstruction (Cohen *et al*., 2007). In addition,

regenerating tubular cells expressing *Pax2* co-expressed the proliferation marker BrdU, and had progenitor-like properties, perhaps reflecting their regenerative properties (Maeshima *et al*., 2002). Exogenous activin A decreased the number of BrdU/*Pax2* double-positive cells after renal ischemia, and reduced the expression of *Pax2* in a proximal tubular cell line (LLC-PK_1) while inhibiting proliferation. Conversely, inhibition of activin signaling increased *Pax2* expression in LLC-PK_1cells and induced an immature phenotype. This suggests that activin A regulates tubular regeneration via alterations in *Pax2*-dependent processes of growth and differentiation. Activin also inhibits proliferation and enhances apoptosis of a human prostate cancer cell line LNCaP. Interestingly, *Pax2* is one of 12 genes that show highly differential expression during activin-induced apoptosis in these cells, showing strong down-regulation in response to activin treatment (Lin and Ying, 1999). This observation may be linked to potential effects of *Pax2* on the cell cycle. Intracerebral grafting of fetal kidney transplants, a source of *BMP* expression, revealed high levels of *BMP7* mRNA and colocalization of *BMP7* and *Pax2* in the graft (Chang *et al*., 2002). Grafting was associated with a smaller volume of infarction and reduced ischemia-induced Caspase-3 activity, and both effects were reversed by the BMP antagonist Noggin.

BMPs, as the name suggests, play a key role in skeletogenesis and are fundamental to the process of condensation during development of skeletal tissue. Condensation denotes the process whereby a previously dispersed cell population groups together in order to differentiate into a certain single-cell tissue such as bone, cartilage, muscle, tendon or kidney. Condensation represents the earliest stage of tissue-specific gene regulation and has several well-defined events. Condensation size is carefully controlled by the actions of BMP2, BMP4, and BMP7. Overexpression of *BMP2* and *BMP4* in chick embryos results in large increases in the size of skeletal elements, probably as a result of enhanced recruitment of mesenchymal cells to condensations. Noggin antagonizes the growth-promoting effects of BMPs, thus feedback between BMPs and Noggin is central to the regulation of condensation size. *Pax2* and *Hox* gene expression in the condensations is regulated by BMPs in response to Shh, and these transcription factors are key regulators of condensation size (reviewed in Hall and Miyake, 2000).

TGFβ family members also play a role in ear development, which in some cases may be linked to *Pax* gene expression. In particular, BMP signaling is critical for production of hair cells and supporting cells of the inner ear. This occurs via two separate BMP-regulated events: a switch from proliferating sensory epithelium progenitors to differentiating epithelial cells, and promotion of differentiation of hair cells within the sensory epithelia. Inhibition of BMP with Noggin reduces the generation of inner ear cell types in a chicken otocyst culture system, while conversely BMP4 treatment increases hair cell formation. BMP4 also down-regulates *Pax2* expression in sensory epithelial progenitors leading to reduced progenitor cell proliferation (Li *et al*., 2005).

Pax5 and the TGFβ Superfamily

Pax5, also called B cell-specific activator protein (*BSAP*), is expressed in the developing nervous system, testis and B-cell lineages (excluding terminally differentiated plasma cells). Consistent with the latter, Pax5 regulates the transcription of several genes in B-cells and is thought to be involved in the regulation of antibody class switch recombination. Indeed, Pax5 has binding sites 5′ to the switch regions of most Ig heavy chain C region genes. Over-expression of *Pax5* inhibits switching to IgA in a B-cell line stimulated with LPS, TGFβ and nicotinamide (Qiu *et al*., 1998). In contrast, *Pax5* over-expression enhanced switching to IgE in cells stimulated with a combination of LPS, IL-4 and nicotinamide.

Regulation of Pax8 by the TGFβ superfamily

Pax8 plays an essential part in thyroid organogenesis and differentiation, and is the main regulator of thyroid gene transcription. In addition, TGFβ has a major role in the regulation of growth and differentiation of thyroid cells. Perhaps unsurprisingly, considering the intimate association between the TGFβ superfamily and *Pax* transcription factors in other tissues, the TGFβ pathway has been directly linked to *Pax8* expression and activity in thyroid cells. For example, *Pax8* is essential for regulation of the *Thyroglobulin* (*TG*) gene by TGFβ1 (Kang *et al*., 2001). TGFβ1 was shown to decrease *TG* mRNA expression in rat FRTL-5 thyroid cells while reporter assays indicated that this was a result of reduced *TG* promoter activity. Furthermore, TGFβ1 selectively reduced the amount of Pax8 protein able to form complexes with DNA in gel mobility shift assays. The inhibitory action of TGFβ1 on Pax8-DNA complex formation was also partly attributed to a reduction in Pax8 mRNA and protein by TGFβ1. Mutation of the Pax8 binding site in the *TG* promoter abolished sensitivity to TGFβ1 highlighting the key role for Pax8 in regulation of the *TG* gene by TGFβ1. In addition, TGFβ inhibits the transcription of other thyroid specific genes, including the *Sodium/Iodide Symporter* gene (*NIS*) and *Thyroperoxidase* gene (*TPO*), effects that are also mediated by Pax8 protein (Nicolussi *et al*., 2003). In this study a dominant negative mutant of *Smad4* (*Smad4-100T*) was used to disrupt TGFβ signaling in rat FRTL-5 thyroid cells. Smad4-100T protein, which contains a mutation in the MH1 domain, inhibits TGFβ1 action in these cells by blocking nuclear translocation of Smad4 and Smad2. The ability of TGFβ to down-regulate all three thyroid-specific genes *NIS*, *TG* and *TPO* was abrogated in the presence of Smad4-100T. In agreement with the aforementioned study by Kang *et al*. (2001), *Pax8* mRNA levels were strongly decreased by TGFβ, and the authors were able to demonstrate that TGFβ-driven changes in *Pax8* mRNA expression were also abrogated when cells were treated in the presence of Smad4-100T.

The mechanism of action of TGFβ signaling and Pax8 on *NIS* gene expression has been further dissected in a study by Costamanga *et al* (2004). In this study TGFβ was found to

repress the normal transcription of the *NIS* gene that follows Thyroid-Stimulating Hormone (TSH)-stimulation of PC C13 cells. The inhibitory effect of TGFβ occurred via an interaction between Smad3 and Pax8 proteins (Costamagna *et al*., 2004). Both full length and a carboxyl-terminal deleted Pax8 interacted with Smad3 in GST pull-down assays, indicating that the N-terminal part of Pax8 which includes the paired domain was important for the interaction. Band shift assays using an oligonucleotide corresponding to the Pax8 binding site in the *NIS* promoter showed a rapid inhibition of Pax8-DNA complex formation (within 15 min) by TGFβ. Similar assays performed in the presence of recombinant Smad3 showed a progressive decrease in Pax8 DNA binding as GST-Smad3 concentration increased. In accordance with other studies, TGFβ was observed to regulate Pax8 mRNA and protein levels at later time points, although no evidence of direct regulation by Smad proteins at the *Pax8* promoter was discovered. Given recent findings regarding Pax6 regulation by TGFβ signaling (discussed later) it is possible that the observed effect on *Pax8* expression may be explained by Smad-3-mediated disruption of an unidentified *Pax8* auto-regulatory loop.

Studies using mice with a cardiac-specific deletion of *Alk3* have indicated that BMP signaling to *Pax8* (via Alk3) is important for inter-ventricular septum development (Yang *et al*., 2003a; 2003b). Mice with *Alk3* gene knock-out had heart defects involving the interventricular septum. *Pax8* and the platelet-activating factor *acetylhydrolase* were down-regulated in *Alk3* knockout mice, suggesting that both could be important downstream effectors of BMP signaling during interventricular septum development.

Group III (*Pax3,7*)

Regulation of Pax3 *by the TGFβ Superfamily*

Pax3 is a marker gene for dividing muscle precursors of the developing limb. *BMP* expression occurs at sites adjacent to dividing muscle cells and can have opposing effects on the growth of embryonic muscle, either driving expansion of the muscle precursor population or restricting development of this population by apoptosis. The outcome of BMP action is concentration dependent; low *BMP* expression stimulates *Pax3* expression and myogenic cell proliferation whereas higher concentrations induce apoptosis. During chick limb development, precise titration of BMP activity is achieved by a low affinity, reversible interaction of BMP2 and BMP7 with follistatin. Follistatin is a secreted glycoprotein that supports muscle growth, and is known to antagonize many members of the TGFβ superfamily. Interestingly, follistatin enhances BMP7 stimulatory action on muscle growth whilst inhibiting the pro-apoptotic function of BMP7, presumably by titrating BMP in the developing limb to levels that promote proliferation but are insufficient to induce apoptosis (Amthor *et al*., 2002). This is accompanied by an up-regulation of *Pax3* expression, thus promoting muscle cell proliferation and delaying the onset of muscle differentiation.

Due to the inherent propensity of skeletal muscle growth, it is essential that effective growth inhibitors are expressed to counteract excessive and inappropriate muscle expansion, and this role is fulfilled by other members of the TGFβ superfamily including myostatin and activin A. Myostatin is an extremely potent inhibitor of muscle growth. Treatment of chick limb buds with myostatin dramatically down-regulates the expression of transcription factors *Pax3* and *MyoD* (Amthor *et al*., 2004). Follistatin is an antagonist of myostatin action, and both factors are expressed in overlapping locations in developing muscle. Follistatin forms a high affinity interaction with myostatin, resulting in blockade of the inhibitory effect of myostatin on the expression of *Pax3* and *MyoD*. Furthermore, follistatin antagonizes the inhibitory action of myostatin on muscle development and terminal differentiation in chick embryos. Similarly, treatment of limb muscle precursors of chick embryos with activin A produces a transient and incomplete down-regulation of *Pax3* and *MyoD* expression in this system, whereas *Pax7* and *Myf*-5 are largely unaffected (He *et al*., 2005). Activin A also transiently inhibits proliferation and differentiation of these precursors without causing apoptosis. It is likely that activin A and myostatin act in concert to prevent excessive muscle growth.

In addition, there is evidence for myostatin imposing a state of reversible quiescence on embryonic muscle precursors (Amthor *et al*., 2006). In this study *Pax7*, unlike *Pax3* and *MyoD*, was shown to be resistant to the inhibitory effect of myostatin. However, although myostatin is unable to down-regulate *Pax7* gene expression in the muscle precursors of chick embryo limb buds, it reversibly inhibits the proliferation of *Pax7*-expressing precursor cells (Amthor *et al*., 2006). Two populations of *Pax7*-expressing cells, dividing and non-dividing, were identified during muscle development, with the proliferating population becoming less abundant as development proceeded. The authors concluded that an important function of myostatin is to implement a progressive program of cell quiescence in myogenic precursor cells of the developing embryo. The continued expression of *Pax7* might contribute to the memory of myogenic precursor cells which allows them to resume development when inhibitory signals are removed.

Regulation of TGFβ2 by Pax3

An interesting twist in the interplay between the TGFβ superfamily and Pax transcription factors is the observation that Pax3 can regulate *TGFβ2* expression (Mayanil *et al*., 2006). Chromatin immunoprecipitation, bandshift and reporter assays indicated that Pax3 directly regulates *TGFβ2* transcription by binding directly to *cis*-regulatory elements in the *TGFβ2* promoter. Furthermore, the *cis*-regulatory elements were revealed to bind acetylated Pax3, and were associated with p300/CBP and histone deacetylases. *Pax3*$^{-/-}$ mouse embryos, which display neural tube defects, had reduced levels of *TGFβ2* transcripts compared to their wild-type litter mates, possibly contributing to the observed phenotype.

Group IV (*Pax4*, 6)

Regulation of Pax4 *by the TGFβ Superfamily*

Consistent with its key role in the developing pancreas, lack of Pax4 prevents the formation of insulin-producing β-cells, produces a marked decrease in δ-cells, and an increase in α-cells (Smith *et al*., 1999; Sosa-Pineda *et al*., 2004). The highly related Pax6 protein is also required for pancreatic development, contributing to the generation of all of the four endocrine cell lineages of the pancreas (Sander *et al*., 1997). The importance of the combined action of Pax4 and Pax6 in pancreatic endocrine cell differentiation is underlined by a complete failure to produce mature pancreatic endocrine cells in corresponding double null mutants (St-Onge *et al*., 1997). One function of *Pax4* is to regulate transcription from the human insulin promoter in a concentration-dependent manner; high concentrations of *Pax4* repress transcription whereas low concentrations exert an opposite effect, promoting transcription of the insulin gene (Ueda, 2000). In the same study, activin A was found to increase expression of the *Pax4* gene and promote insulin production and β-cell differentiation. Similarly, Brun *et al*. (2004) reported that treatment of islets with activin A produced a concomitant increase in *Pax4* mRNA levels and cell proliferation. In this study, Pax4 was shown to stimulate the promoter activities of both c-Myc and Bcl-xL genes. Interestingly a type 2 diabetes linked mutation in the paired domain of *Pax4* reduces the transactivation of both genes (Brun *et al*., 2004). It was proposed that by regulating both apoptosis (via the survival factor *Bcl-xL*), and proliferation (via *c-Myc*), *Pax4* is ideally placed to determine the population of pancreatic β cells and thus control islet mass. The mechanism by which activin A induces expression of Pax4 in pancreatic β cells has been found to involve transactivation of the basic helix-loop-helix transcription factors E47/E12 (Kanno *et al*., 2006). These factors, in combination with hepatocyte nuclear factor 1α (HNF-1α) activate the *Pax4* gene in response to activin A via a cluster of binding sites present in the *Pax4* promoter.

The use of human stem cells to produce insulin-producing β cells for transplantation is a promising strategy in the combat of type 1 diabetes. FH-B-TPN cells are human fetal liver cells engineered to express the transcription factor PDX-1. This factor is considered a defining marker of the very early pancreatic lineage, and consequently FH-B-TPN cells display many features of the β-cell phenotype. However, these cells exhibit differential expression of many genes compared with β-cells, and this is coupled to reduced insulin content. Interestingly, culture of FH-B-TPN cells with activin A up-regulates expression of β-cell associated genes and down-regulates the expression of *Pax6*, while increasing insulin content to approximately 60% of that of normal β-cells (Zalzman *et al*., 2005). This offers promise for the stable differentiation of cells into a β-cell phenotype for cell replacement in type 1 diabetes.

Regulation of Pax6 *by the* TGFβ *superfamily*

As previously mentioned, *Pax6* is an essential factor for lens determination and morphogenesis. Misexpression of the *Drosophila Pax6* homologue *Eyeless* is sufficient to induce ectopic eye structures on the legs, wings and antennae of this organism (Halder *et al*., 1995), and ectopic lenses in whole embryos and animal cap explants of *Xenopus* (Altmann *et al*., 1997). *Small Eye* mice homozygous for mutations in *Pax6* have no lenses or nasal cavities (Grindley *et al*., 1995). It has been suggested that upstream regulation of *Pax6* is conserved through evolution (Callaerts *et al*., 1997; Xu *et al*., 1999; Onuma *et al*., 2002), and several signaling pathways have been implicated.

An increasing body of evidence is accumulating in support of a functional relationship between BMPs and *Pax6*, particularly with respect to the normal patterning of the developing eye and neural tube. Studies of lens induction in the mouse have implicated *BMP7* in the normal maintenance of *Pax6* expression in presumptive lens ectoderm (Wawersik *et al*., 1999). In *BMP7* deficient mice, *Pax6* expression is lost just prior to the time when the lens placode should appear, and this is around the same time that *Pax6* auto-regulation is required in this tissue. Furthermore, treatment of the optic rudiment with the activin/BMP inhibitor follistatin leads to a marked decrease in the frequency of lens formation (Wawersik *et al*., 1999). Consistent with this role in lens formation, *BMP7* is known to be expressed in the presumptive lens ectoderm prior to and during lens formation, as are BMP type I and type II receptors (Wawersik *et al*., 1999; Faber *et al*., 2002). Inhibition of the fibroblast growth factor receptor (Fgfr) also results in diminished *Pax6* expression in the presumptive lens ectoderm of mice (Faber *et al*., 2002), and it has been suggested that there is a genetic interaction between *Fgfr* and *BMP7* signaling (Wawersik *et al*., 1999), converging on *Pax6* expression during lens induction. A key role for *BMP13* in neural and eye development has also been demonstrated in *Xenopus* embryos (Hanel and Hensey, 2006) where knock-down of *BMP13* resulted in decreased eye size, loss of laminar structure and a reduction in differentiated neural cells within the retina. These effects were correlated with a reduced phosphorylation of BMP-specific Smads (1, 5, and 8), and a reduced *Pax6* expression domain at early optic vesicle stages.

In addition, activin A has been linked to the differentiation of the retina. Activin A is reported to stimulate the differentiation of chick embryo amacrine cells in primary retina cell cultures (Belecky-Adams *et al*., 1999). Furthermore, overexpression of follistatin in chick embryo retina leads to reduced *Pax6* expression and corresponding loss of amacrine cell differentiation (Moreira and Adler, 2006). This effect was attributed to the ability of follistatin to inhibit activin signaling, as activin subunits and receptors are expressed near the vitreal surface of the retina where amacrine and ganglion cells differentiate. It also appears that activin derived from extraocular tissues may be important to the differentiation of the pigment cells of the retinal

pigmented epithelium (RPE). Extraocular tissues are essential for normal growth and differentiation of the eye. Studies performed in explant cultures of chick optic vesicles have shown that extraocular mesenchyme is sufficient to promote the expression of genes specific to RPE, while inhibiting the expression of *Pax6*, a marker of the neural retina (Fuhrmann *et al*., 2000). Activin can substitute for the extraocular mesenchyme by promoting the expression of RPE-specific genes (*Mitf* and *Wnt13*) while down-regulating expression of *Pax6*. Thus, it appears that activin performs dual roles in the neural retina and the retinal pigmented epithelium, the first of which is linked to maintained expression of *Pax6*, whilst the second correlates with a decrease in *Pax6* expression.

In addition to the important contribution of BMPs and activin in normal eye development, inappropriate signaling by TGFβs has an established role in eye disease. In lens tissue, TGFβ can induce the development of fibrotic plaques that resemble subcapsular cataracts. Furthermore, TGFβ-induced anterior subcapsular cataract formation may depend on a reduction in *Pax6* expression (Lovicu *et al*., 2004). Using transgenic mice that overexpressed *TGFβ* in the lens, it was found that the resulting subcapsular plaques were composed of a heterogenous cell population including myofibroblastic cells and fiber cells. Cells expressing lens epithelial markers including *Pax6* and *Connexin-43* were lost from the plaques, suggesting that *in vivo* TGFβ induces phenotypic changes to the lens epithelium, and that this pathological process is mediated by a reduction in *Pax6* expression levels.

A further link between BMP signaling and *Pax6* activity has been demonstrated in the regulation of *Neural Cell Adhesion Molecule L1*. *L*1 modulates neuron–neuron and neuron–glia interactions during development of the central and peripheral nervous systems, and thereby controls axonal guidance and fasciculation. Consequently, the expression of *L*1 is dynamic and tightly regulated, which has led to an investigation of the regulatory region of the *L*1 gene (Meech *et al*., 1999). A DNA element termed the HPD that contains binding motifs for Pax proteins (including Pax6) and homeodomain proteins was identified in the *L*1 regulatory region. Whereas an ATTA sequence was required for binding to homeodomain protein Barx2, a separate paired domain recognition motif mediated interaction of the HPD with Pax6. Furthermore, reporter constructs containing the HPD were found to respond positively to BMP2 and BMP4 ligands, with similar levels of induction as that observed with *Pax6* over-expression (Meech *et al*., 1999).

Activin A has been reported to inhibit *Pax6* expression in the developing neural tube. Treatment of the chick neural plate with activin A results in a partial to complete loss of *Pax6* expression in the neural tube at later stages in a dose dependent manner (Pituello *et al*., 1995), suggesting that region specific expression of *Pax6* in this tissue (Figure 2) is under the control of activin-like molecules. In this study, chick neural plate explants were cultured for 24 hours in the presence of activin A ligand at concentrations ranging from 5 ng ml^{-1} up to 600 ng ml^{-1} (Pituello *et al*., 1995). Subsequent analysis of *Pax6* expression by *in situ* hybridization revealed that doses of activin A below 50 ng ml^{-1} had no specific effect on *Pax6* expression normally located either side of the ventral midline. However, concentrations from 50 ng ml^{-1} to 150 ng ml^{-1} yielded a dose-dependent inhibition of *Pax6* expression, while concentrations above 150 ng ml^{-1} abolished all *Pax6* expression in these explants. Significantly, this response was found to function independently of the intervening ventral midline cells, suggesting a direct action of activin A on the *Pax6* expressing population (Pituello *et al*., 1995).

The borders of *Pax6* expression in the chick neural tube are also thought to be determined by the combined influence of dorsal BMP and ventral Sonic Hedgehog signals (Figure 2) (Timmer *et al*., 2002). The absence of *Pax6* expression in roof plate correlates with the expression of several TGFβ family members, particularly *BMPs*, in both the roof plate and surrounding tissues. At this developmental stage, dorsal and ventral intermediate cells of the caudal neural tube express *Pax6* at low and high levels respectively, and a distinct step in expression level is observable at the boundary of these discontinuous regions. It has been reported that chick neural tube which has been electroporated with the constitutively activated BMP Type I receptor Alk6 (caAlk6) exhibits a silencing of *Pax6* expression in all transfected cells (Timmer *et al*., 2002). This finding suggests that high levels of *BMP* expression in the roof plate may be responsible for the lack of dorsal *Pax6* expression. Moreover, following electroporation of lower concentrations of caAlk6, or with a less potent constitutively active Alk3, moderately transfected embryos exhibited a ventral shift of the border between the low expressing dorsal intermediate cells and the high expressing ventral intermediate cells (Timmer *et al*., 2002). This suggests that a BMP gradient may act to set the dorsal border of the higher level *Pax6* expression domain. Consistent with these findings is the recent observation of elevated *Pax6* expression levels in the neural tubes of phenotypic *Smad1*$^{+/-}$ and *Smad8*$^{3loxP/3loxP}$ knock-out mouse embryos (Hester *et al*., 2005). This most recent evidence clearly implicates the receptor regulated R-Smads from the BMP pathway, in addition to the upstream BMP Type I receptors Alk3 and Alk6.

The involvement of Smad proteins in *Pax6* regulation is further supported by RNA interference experiments in P19 murine embryonic carcinoma cells (Carpenter and Zernicka-Goetz, 2004). siRNAs targeted against *Smad4* were shown to effectively inhibit *Pax6* expression within 48 hours of transfection. Interestingly, it was reported that this inhibition of *Smad4* expression correlated with an increase in the level of subsequent *Pax6* expression and a decrease in the mesodermal marker Brachyury (Carpenter and Zernicka-Goetz, 2004). While, it is important to note that BMP4 is known to promote mesoderm formation at the expense of neuroectoderm, *Pax6* being a primarily ectodermal marker, a direct influence of Smad4 on *Pax6* expression cannot be ruled out.

A clue to how members of the TGFβ superfamily might regulate *Pax6* expression directly in certain tissues relates to the

TABLE 2
These tables summarize regulation of *Pax* gene expression by members of the TGFβ superfamily

Pax family member	Type of regulation	Biological example	Reference
		(a) TGFβ/activin/myostatin-dependent regulation of *Pax* expression	
Pax1/*Pax9*	TGFβ	Deletion of the TGFβIIR leads to expanded *Pax1* and *Pax9* expression and disruption of the rostral–caudal boundary of the sclerotome	Baffi *et al*., 2006
Pax9	TGFβ	*Pax9* is down-regulated in the developing palate of *TGFβ3-null* mice	Sasaki *et al*., 2007
Pax2	TGFβ	TGFβ1 reduces *Pax2* mRNA stability and expression in proximal tubule cells	Liu *et al*., 1997
Pax2	TGFβ	TGFβ1 reduces *Pax2* expression in dysplastic kidney cells	Yang *et al*., 2000
Pax2	Activin	Activin A reduced *Pax2* expression and blocked uteric bud formation	Maeshima *et al*., 2006
Pax2	Activin	Activin A decreased recovery of BrdU/*Pax2* positive cells after renal ischemia	Maeshima *et al*., 2002
Pax2	Activin	*Pax2* is down-regulated in human prostate cancer cells undergoing apoptosis due to activin	Lin and Ying, 1999
Pax5	TGFβ family	Co-incident changes in activity of Smads and *Pax5* in a model of Parkinson's Disease	Xu *et al*., 2005
Pax5	TGFβ family	Co-incident changes in expression of Smads, TGFβR and *Pax5* in schizophrenic or bipolar hippocampi	Benes *et al*., 2007
Pax8	TGFβ	TGFβ1 reduced *Pax8*/DNA binding and regulation of the thyroglobulin promoter	Kang *et al*., 2001
Pax8	TGFβ	dnSmad4 abrogates TGFβ-driven down-regulation of *Pax8* expression and inhibition of thyroid-specific gene expression	Nicolussi *et al*., 2003
Pax8	TGFβ	Pax8/Smad3 interact and decrease Pax-8/DNA binding to the sodium/iodide symporter promoter	Costamagna *et al*., 2004
Pax3	Myostatin	Myostatin inhibits the expression of *Pax3* in chick limb buds	Amthor *et al*., 2004
Pax3	Activin A	Activin A downregulates *Pax3* in chick limb muscle precursors	He *et al*., 2005
Pax4	Activin A	Activin A increases expression of *Pax4* and promotes β-cell differentiation	Ueda, 2000
Pax4	Activin A	Activin A increases expression of *Pax4* and islet cell proliferation	Brun *et al*., 2004
Pax4	Activin A	Activin A activates *Pax4* expression via transactivation of E47/E12.	Kanno *et al*., 2006
Pax6	Activin A	Activin A down-regulates the expression of *Pax6* in human stem cells engineered towards the β-cell phenotype	Zalzman *et al*., 2005
Pax6	Activin	Over-expression of follistatin in chick embryo retina leads to reduced *Pax6* expression and reduced amacrine differentiation.	Moreira & Adler, 2006
Pax6	Activin	Activin can substitute for extraocular mesenchyme in down-regulating *Pax6* and promoting differentiation of retinal pigmented epithelium	Fuhrmann *et al*., 2000
Pax6	TGFβ	TGFβ-induced subcapsular cataract formation may depend on a reduction in *Pax6*	Lovicu *et al*., 2004
Pax6	Activin A	Activin A down-regulates *Pax6* expression in the neural tube	Pituello *et al*., 1995
Pax6	TGFβ family	siRNA knockdown of Smad4 correlated with increased *Pax6* expression in murine embryonic carcinoma cells	Carpenter *et al*., 2004
Pax6	TGFβ	TGFβ-activated Smad3 binds to Pax6 and reduces the formation of Pax6/P1 promoter complexes, hence inhibiting positive *Pax6* autoregulation	Grocott *et al*., 2007

(Continued on next page)

TABLE 2
These tables summarize regulation of *Pax* gene expression by members of the TGFβ superfamily *(Continued)*

Pax family member	Type of regulation	Biological example	Reference
		(b) BMP-dependent regulation of Pax expression	
Pax1/*Pax3*	*BMP*	Lateral grafting of BMP2-producing cells to the neural tube of chick embryos represses *Pax1* and *Pax3* expression	Monsoro-Burq *et al*., 1996
Pax1	*BMP*	Noggin induces *Pax1* in the murine paraxial mesoderm, while BMP2 and BMP4 block this effect	McMahon *et al*., 2007
Pax1	*BMP*	Overlapping expression of *Noggin* and *Pax1* in the annulus fibrosus of the developing spine	DiPaola *et al*., 2005
Pax1	*BMP*	BMP2 and BMP4 repress *Pax1* in developing chick wing	Hofmann *et al*., 1998
Pax1	*BMP*	Inhibition of BMP inhibits *Pax1* expression in scapula precursors	Wang *et al*., 2005
Pax9	*BMP*	BMP2 and BMP4 prevent induction of *Pax9* by FGF8 in prospective tooth mesenchyme	Neubüser *et al*., 1997
Pax2	*BMP*	*BMP7*-deficient mice show defective *Pax2* expression during nephrogenesis	Luo *et al*., 1995
Pax2/*Pax7*	*BMP*	Dose-dependent regulation of *Pax2* and *Pax7* in somites with ectopic Alk3/6 activity	James and Schultheiss, 2005
Pax2	*BMP*	Dominant negative BMPR restricted expression of *Pax2.1*during somitogenesis in zebrafish	Gupta *et al*., 2006
Pax2	*BMP*	BMP4 can replace surface ectoderm in driving *Pax2* expression in nephric duct progenitors	Obara-Ishihara *et al*., 1999
Pax2	*BMP*	*BMP7* and *Pax2* co-localise in intracerebral grafts during ischemic injury of the cerebral cortex	Chang *et al*., 2002
Pax2	*BMP*	*Pax2* expression is regulated by BMPs to determine condensation size	Hall and Miyake, 2000
Pax2	*BMP*	BMP4 down-regulates *Pax2* expression in proliferating sensory epithelial progenitors	Li *et al*., 2005
Pax8	*BMP*	Cardiac-specific deletion of *Alk3* results in down-regulation of *Pax8* and defects of the inter-ventricular septum	Yang *et al*., 2033a, 2003b
Pax3	*BMP*	Follistatin up-regulates *Pax3* expression in muscle precursors	Amthor *et al*., 2002
Pax3	*BMP*	*BMP2* expressing cells implanted adjacent to the paraxial mesoderm inhibited expression of *Pax3* and impaired somite formation	Andrée *et al*., 1998
Pax6	*BMP*	In *BMP7*-deficient mice *Pax6* expression is lost just prior to the time when lens placode should appear	Wawersik *et al*., 1999
Pax6	*BMP*	Knock-down of *BMP13* correlated with reduced *Pax6* expression in early optic vesicles of *Xenopus*	Hanel and Hensey, 2006
Pax6	*BMP*	BMP2 and BMP4 up-regulate the *Pax6*-responsive promoter of the *L1 cell adhesion molecule* gene	Meech *et al*., 1999
Pax6	*BMP*	Electroporation of chick neural tube with constitutively active Alk6 silences *Pax6* expression	Timmer *et al*., 2002
Pax6	*BMP*	*Pax6* expression is elevated in neural tubes of $Smad1^{+/-}$ or $Smad8^{3lox P/3lox P}$ knockout mouse embryos	Hester *et al*., 2005

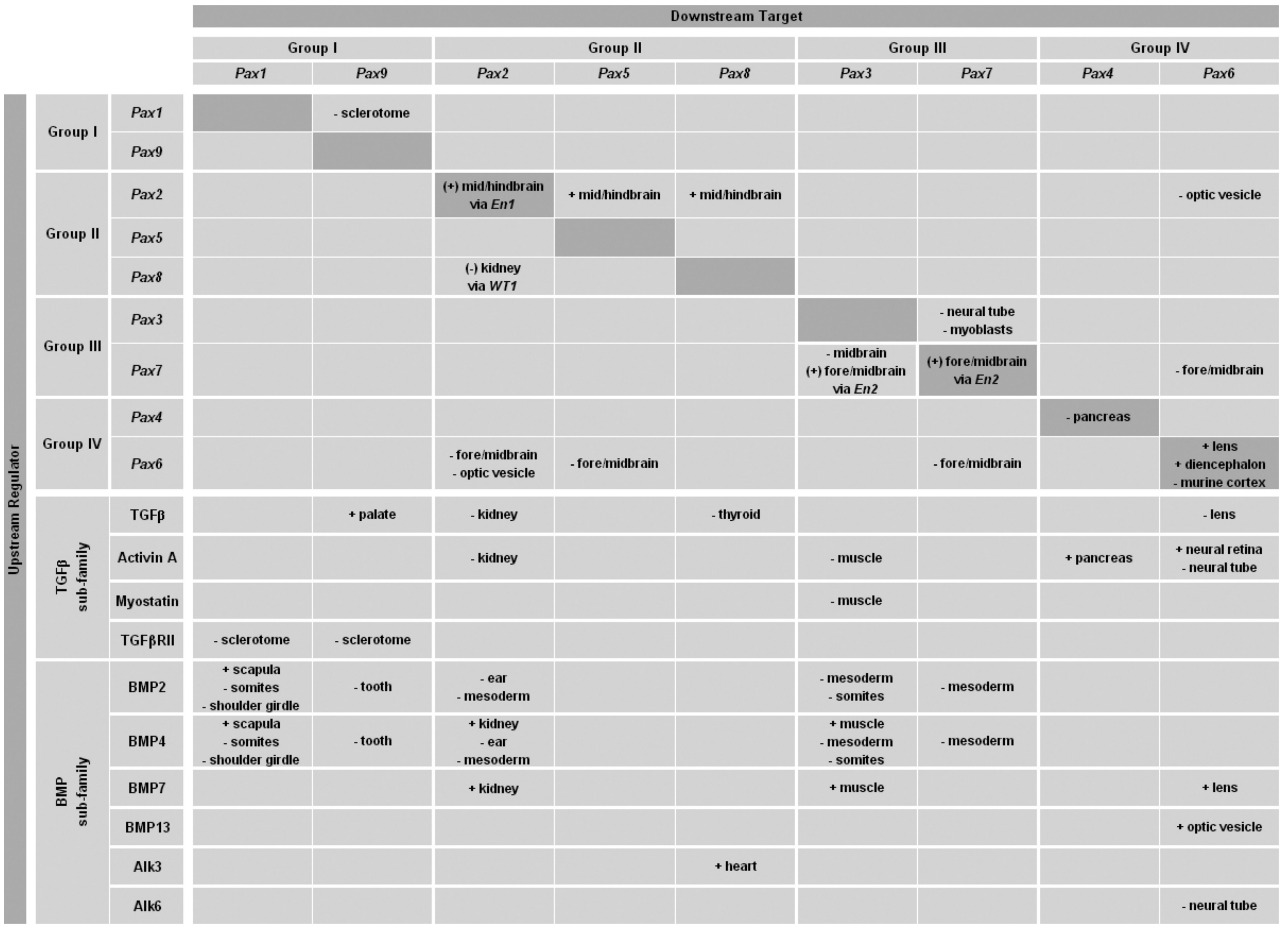

FIG. 4. Summary of self-regulation currently reported within the *Pax* gene family and modulation by TGFβ superfamily signaling molecules. Shaded boxes indicate auto-regulatory events. Activations are indicated by +, inhibitions by –, while indirect regulations are shown in parentheses. Refer to the tables for further details and references and to the appropriate sections of the main text for a discussion of biological context.

observation that TGFβ signaling may disrupt *Pax* auto-regulatory processes. Specifically the MH1 domain of Smad3 has been observed to interact with Pax6 and represses auto-regulation of the *Pax6* P1 promoter (Grocott *et al*., 2007). As mentioned previously, Pax6 protein is able to directly regulate its own promoters, and this is partly mediated by an interaction between the paired domain of Pax6 with a corresponding binding site in the *Pax6* P1 promoter. Selective interaction between Smad3 protein (but not Smad2 or Smad4) with Pax6 protein *in vivo* was found to be signaling-dependent, and was only detected following activation of the TGFβ-pathway by co-transfection of constitutively activated TGFβ Type I receptor. Furthermore, an endogenous interaction between Pax6 and Smad3 was demonstrated in lens epithelial cells following stimulation with TGFβ ligand. GST pull-down assays revealed a strong interaction between the isolated paired domain of Pax6 and the MH1 domain of Smad3, and further investigation revealed that the Smad3 MH1 domain binds to the RED sub-region of the Pax6 paired domain. A mechanism to explain the repressive effect of TGFβ signaling on Pax6 auto-regulation was suggested by the observation that Smad3 could out-compete the binding of a biotinylated aptamer of the paired domain binding site in DNA absorption assays. These observations indicate a mutually exclusive interaction between the Pax6 paired-domain with either Smad3 or DNA that may harness Pax6-DNA binding at the P1 promoter and hence the positive auto-regulation of the *Pax6* gene (Grocott *et al*., 2007). Further published evidence that the expression of a multitude of *Pax* genes can be controlled by several TGFβ superfamily members suggests that this regulatory mechanism may not only be restricted to *Pax6* (see Table 2).

CONCLUDING REMARKS

It is clear that recurring themes exist in the regulation of *Pax* gene expression. Auto-regulation is a frequent mechanism employed to achieve either reinforcement or termination of *Pax* gene expression, by positive or negative regulation, respectively. The ability of certain *Pax* genes to inter-regulate the expression of other *Pax* family members in precise locations is exemplified

during embryogenesis, where *Pax* genes often display spatially and temporally regulated expression which can be critical for patterning events. For example, where the combined action of two or more *Pax* family members is required for cell specification, positive inter-regulation of *Pax* expression can drive coincident expression of multiple *Pax* gene products. Conversely negative inter-regulation of *Pax* family members is a tactic employed to define developmental boundaries where the non-overlapping expression of individual *Pax* gene products is key to the demarcation of developing tissues (summarized in Figure 4). Another recurring theme in *Pax* gene regulation is the intimate association between *Pax* gene expression and signaling by members of the TGFβ superfamily. TGFβ, BMPs and activin A are central players in numerous developmental processes and their effects are often linked to the regulation of *Pax* gene expression (Figure 4). The sometimes antagonistic effects of individual TGFβ superfamily members during development may be related to the opposing effects on *Pax* gene expression in some instances. A further layer of complexity arises in a few examples whereby Pax transcription factors may regulate signaling by the TGFβ superfamily. Further work will be necessary to unravel the complexities of *Pax* gene self-regulation and to fully understand the general applicability of all TGFβ superfamily members in coordinating the expression of all *Pax* genes in both development and human disease.

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