Pax1 and Pax9 synergistically regulate vertebral column development

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SUMMARY

The paralogous genes Pax1 and Pax9 constitute one group within the vertebrate Pax gene family. They encode closely related transcription factors and are expressed in similar patterns during mouse embryogenesis, suggesting that Pax1 and Pax9 act in similar developmental pathways. We have recently shown that mice homozygous for a defined Pax1 null allele exhibit morphological abnormalities of the axial skeleton, which is not affected in homozygous Pax9 mutants. To investigate a potential interaction of the two genes, we analysed Pax1/Pax9 double mutant mice. These mutants completely lack the medial derivatives of the sclerotomes, the vertebral bodies, intervertebral discs and the proximal parts of the ribs. This phenotype is much more severe than that of Pax1 single homozygous mutants. In contrast, the neural arches, which are derived from the lateral regions of the sclerotomes, are formed. The analysis of Pax9 expression in compound mutants indicates that both spatial expansion and upregulation of Pax9 expression account for its compensatory function during sclerotome development in the absence of Pax1.

In Pax1/Pax9 double homozygous mutants, formation and anteroposterior polarity of sclerotomes, as well as induction of a chondrocyte-specific cell lineage, appear normal. However, instead of a segmental arrangement of vertebrae and intervertebral disc anlagen, a loose mesenchyme surrounding the notochord is formed. The gradual loss of Sox9 and Collagen II expression in this mesenchyme indicates that the sclerotomes are prevented from undergoing chondrogenesis. The first detectable defect is a low rate of cell proliferation in the ventromedial regions of the sclerotomes after sclerotome formation but before mesenchymal condensation normally occurs. At later stages, an increased number of cells undergoing apoptosis further reduces the area normally forming vertebrae and intervertebral discs. Our results reveal functional redundancy between Pax1 and Pax9 during vertebral column development and identify an early role of Pax1 and Pax9 in the control of cell proliferation during early sclerotome development. In addition, our data indicate that the development of medial and lateral elements of vertebrae is regulated by distinct genetic pathways.

Key words: Pax1, Pax9, Vertebral column, Chondrogenesis, Proliferation, Apoptosis, Mouse

INTRODUCTION

The vertebrate body is supported by the vertebral column, a series of segmental skeletal elements that provide both stability and mobility. The development of the axial skeleton is a multi-step process starting with the formation of somites from the unsegmented paraxial mesoderm on both sides of the neural tube. Shortly after formation, the somites compartmentalise to generate dermomyotomes and sclerotomes, the latter forming skeletal elements of the vertebral column and ribs. Vertebral column development requires the co-ordination of a series of cellular events. These include de-epithelialization of somites, proliferation and migration of sclerotomal cells, and establishment of anteroposterior polarity of the sclerotome (reviewed in Keynes and Stern, 1988; Christ and Wilting, 1992; Christ and Ordahl, 1995). In mammals, a single vertebra is composed of a variety of components that perform different functions, depending on the level of the body axis. Thus, a fine-tuned regulation is required to co-ordinate the prepatterning of individual skeletal elements by region-specific mesenchymal growth and condensations that are finally replaced by cartilage and bone. Although in the recent years much progress has been made in the understanding of somite patterning and sclerotome formation (Tajbakhsh and Spörle, 1998), most aspects of the genetic control that regulates the complex development from the sclerotomal mesenchymes to the vertebral column remain to be elucidated.

Pax1 and Pax9 form one group within the family of nine vertebrate Pax genes, which are unified by the presence of the paired box that encodes a DNA-binding domain (Waltner et al., 1991; Noll, 1993). Analyses of mouse mutants of all Pax genes have demonstrated multiple roles in the genetic control of mammalian organogenesis (reviewed in Dahl et al., 1997). The 128 amino acid long DNA-binding paired domains of Pax1 and Pax9 are almost identical (98%). DNA-binding studies have shown that the e5 site, a DNA element present in the
Drosophila even-skipped promoter, as well as CD19-2 (A-ins) and H2A-17C, two elements originally identified as recognition sites for Pax5, are bound by Pax1 and Pax9 (Chalepakis et al., 1991; Czerny et al., 1993; Neubüser et al., 1995; H. P. and R. B., unpublished results). These findings suggest that Pax1 and Pax9 may have similar functions in tissues in which they are co-expressed.

During mouse embryogenesis, Pax1 and Pax9 are expressed in similar patterns in the pharyngeal pouches and the developing limbs (Neubüser et al., 1995). Pax9-specific expression domains in craniofacial regions are correlated with its essential role for secondary palate and tooth formation (Peters et al., 1998). Pax1 and Pax9 are the only Pax genes expressed in the sclerotomes, the ventromedial compartments of somites. The expression of Pax1 precedes that of Pax9 and is initially found in all sclerotomal cells. At later stages of sclerotome differentiation, the expression of Pax1 acquires a maximum in the posterior, ventromedial compartment. On the contrary, transcription of Pax9 is predominantly detectable in the posterior, ventrolateral region of the sclerotomes. In the region of the future vertebral bodies, expression of Pax1 and Pax9 is downregulated when sclerotomal cells start to chondrify, but their expression is maintained in the perichondrium of vertebrae and in the intervertebral disc anlagen (Deutsch et al., 1988; Neubüser et al., 1995; Peters et al., 1995; Ebensperger et al., 1995).

Previous analyses of three natural Pax1 mutant alleles in mice, the undulated series (un, unex, UnS), have demonstrated an important role for Pax1 during sclerotome development (Grüneberg, 1954; Balling et al., 1988; Wallin et al., 1994; Dietrich and Gruss, 1995). Using a gene targeting approach, we have recently shown that homozygous mutant mice of a defined Pax1 null allele are viable and exhibit morphological abnormalities of vertebrae and intervertebral discs similar to those seen in the recessive alleles un and unex (Wilm et al., 1998). In contrast, homozygous mutants of the semidominant UnS allele, which has a deletion including the Pax1 gene, die at birth and exhibit a severely affected...
vertebral column (Wallin et al., 1994). Thus, the deletion in the Un allele interferes with vertebral column development not only through the loss of Pax1 function. Homozygous Pax9 mutants have skeletal defects in the limbs and in the skull, but exhibit no obvious defects in the axial skeleton (Peters et al., 1998).

There is accumulating evidence that closely related transcription factors perform redundant functions during development. This has been shown for paralogous Hox genes (Rijli and Chambon, 1997, and references therein), the zinc finger protein encoding genes Gli2 and Gli3 (Mo et al., 1997), for the basic helix-loop-helix genes MyoD and MRF4 (Rawls et al., 1998), and the homeobox-containing genes Prx1 and Prx2 (ten Berge et al., 1998; Lu et al., 1999), and Alx1 and Cart1 (Qu et al., 1999). The overlapping expression patterns during mouse embryogenesis and the high sequence conservation of the DNA-binding paired domain of members within each group of the Pax gene family suggest redundancy at sites where these genes are co-expressed.

To investigate a possible genetic interaction between Pax1 and Pax9 during mammalian development, we analysed double mutant mice. These mutants reveal a gene dosage-dependent co-operation of Pax1 and Pax9 during sclerotome development. In the absence of Pax1 and Pax9, no vertebral bodies and intervertebral discs are formed, a phenotype that is not found in either single homozygous mutant, whereas mice lacking three functional gene copies exhibit intermediate phenotypes. Notably, the formation of neural arches is not affected in the double mutants, demonstrating a different genetic control for the development of these vertebral structures. Pax1 can fully compensate for the absence of Pax9 whereas Pax9 function incompletely rescues vertebral column formation in Pax1-deficient mice. We show that Pax1 and Pax9 are required to maintain chondrocyte-specific gene expression in the sclerotome, but their earliest function during vertebral column development may be involved in the control of cell proliferation during a specific and relatively short phase of sclerotome development.

**MATERIALS AND METHODS**

**Animals**

Pax1<sup>fl/fl</sup>/Pax9<sup>lacZ</sup> double heterozygous mutant mice were generated by crossing mice carrying null alleles of Pax1 (Pax1<sup>fl/fl</sup>) and Pax9 (Pax9<sup>lacZ</sup>), both of which have been described previously (Wilm et al., 1998; Peters et al., 1998). Genotyping of mice and embryos was performed as described previously (Wilm et al., 1998; Peters et al., 1998).
Phenotype analyses
Skeletal staining of newborn mice was performed as described by Kessel et al. (1990). To visualize Pax9lacZ expression, mutant mouse embryos were stained with X-gal and cleared with benzylbenzoate/benzylalcohol as described in Gossler and Zachgo (1993). Whole-mount in situ hybridizations with digoxigenin-labeled RNA probes specific for Sox9 (Wright et al., 1995), the α1 subunit (Metsaranta et al., 1991) of Collagen II (Col2α1), and Uncx4.1 (Mansouri et al., 1997) were performed according to Wilkinson (1992). In situ hybridization on paraffin sections was carried out as described in Neubüser et al. (1995).

BrdU labeling and TUNEL staining
Cell proliferation was analysed by incorporation of BrdU into embryos and subsequent immunochemical detection of BrdU on paraffin sections according to the protocol of the ‘In Situ Cell Proliferation Kit’ (Boehringer-Mannheim). Numbers from at least six sections were combined in each group for determination of the standard deviation. Differences, standard deviations were calculated from numbers around the notochord (Fig. 7). To demonstrate statistically significant

RESULTS
Pax1 and Pax9 are required for vertebral column development in a gene dosage-dependent manner
To reveal a potential interaction between Pax1 and Pax9 during development, we crossed double heterozygous mutant mice carrying the previously described Pax1null and Pax9lacZ alleles (Wilm et al., 1998; Peters et al., 1998), which we refer to in the following as Pax1 and Pax9 mutants, respectively. At the newborn stage, all nine possible genotypes were obtained at the absence of either Pax1 or Pax9. These include abnormalities of the shoulder skeleton in Pax1-deficient mice (Wilm et al., 1998), and preaxial polydactyly and craniofacial abnormalities in the absence of Pax9. Furthermore, double heterozygous mutant mice lack the derivatives of the third and fourth pharyngeal pouches, a phenotype that is also seen in Pax9-deficient mice (Peters et al., 1998, and data not shown).

The vertebral columns of heterozygous and homozygous Pax9 mutants appear normal, as revealed by skeletal staining of newborn mice (Fig. 1B,C). However, in mice that are deficient for one functional copy of Pax1, heterozygosity and homozygosity of the Pax9 mutation result in vertebral malformations that are not seen in single Pax1 heterozygous mutants. In the lumbar region, fused vertebrae, split vertebrae, as well as ossified fusions between vertebrae and neural arches are found (Fig. 1E,F). Thus, Pax1 and Pax9 interact during vertebral column development. This result is more clearly demonstrated in mutants that lack both copies of Pax1: in Pax1lacZ; Pax9lacZ double homozygous mutants, no vertebral bodies and intervertebral discs are formed (Fig. 1I), thereby dramatically decreasing the overall length of the body axis. Furthermore, the proximal parts of most ribs, all skeletal elements of the tail, as well as the connection between sacrum and pelvic girdle are missing (Figs 1I, 2B,D). A similar, but less severe, phenotype was detectable in the vertebral column of Pax1lacZ; Pax9lacZ mutants. In these mice, an irregular, cartilaginous rod that is interrupted by ventral extensions of the neural arches replaces the vertebral bodies and intervertebral discs of the lumbar region (Fig. 1H). These results identify a role for Pax9 in vertebral column formation and reveal a gene dosage-dependent manner of Pax1/Pax9 function during vertebral column development. The findings also indicate that Pax1 and Pax9 may have similar or identical functions that are redundant at many steps during sclerotome development.

Interestingly, the lateral derivatives of the sclerotomes, the neural arches, are formed in the absence of Pax1 and Pax9, although they were found to have an abnormal shape (Fig. 2D). Moreover, in the lumbar region, ectopic cartilage formation is detectable in the dorsal region between adjacent neural arches (Fig. 2D). The disturbed development of the neural arches might be a direct consequence of Pax1 and Pax9 deficiency. However, secondary effects of the complete absence of ventral vertebral elements could also contribute to this phenotype. While our analysis does not distinguish between these two possibilities, the presence of neural arches in Pax1/Pax9 double homozygous mutants rules out an essential role for Pax1 and Pax9 in their formation. Furthermore, the development of neural arches indicates that sclerotome formation can not be completely abolished in the absence of Pax1 and Pax9.

Upregulation and spatial expansion of sclerotomal Pax9 expression in the absence of Pax1
To explore the basis of the functional redundancy between Pax1 and Pax9 during vertebral column development and to test whether Pax1 and Pax9 functions are required to maintain Pax9 promoter activity, we analysed lacZ expression of Pax1/Pax9 compound mutants. Previous studies have shown that the expression pattern obtained by X-gal staining of Pax9lacZ mice in our breeding colony, indicating that this genotype causes early postnatal lethality. Except for the vertebral column (see below), all other skeletal phenotypes observed in the double homozygous mutants were specific to the absence of either Pax1 or Pax9. These include abnormalities of the shoulder skeleton in Pax1-deficient mice (Wilm et al., 1998), and preaxial polydactyly and craniofacial abnormalities in the absence of Pax9. Furthermore, double heterozygous mutant mice lack the derivatives of the third and fourth pharyngeal pouches, a phenotype that is also seen in Pax9-deficient mice (Peters et al., 1998, and data not shown).

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restriction of Pax9 expression to the posterior, ventral domain of the sclerotomes. Therefore, spatial expansion and upregulation of Pax9 in the sclerotomes is a likely mechanism for the partial rescue of vertebral column development in Pax1 mutant mice. Furthermore, the maintenance of lacZ expression in the double homozygous mutant shows that Pax9 transcription during early sclerotome development does not require the function of Pax1, or of Pax9 itself (Fig. 3C,F).

**Anteroposterior polarity of somites and chondrogenesis**

To identify and characterise the onset of phenotypic abnormalities in Pax1/Pax9 double mutants, we performed in situ hybridisation of genes that are expressed during early sclerotome development. The expression of Uncx4.1, a paired-type homeobox-containing gene, is induced in the posterior half of newly formed somites and thereafter is maintained in the posterior halves of the sclerotomes (Mansouri et al., 1997). At E10.5 (around 30 somites), the expression patterns of Uncx4.1 were indistinguishable among all possible allelic combinations of Pax1 and Pax9 mutations (Fig. 4A, B, and data not shown). This result shows that Pax1 and Pax9 are not necessary to maintain the expression of Uncx4.1 and indicates that the anteroposterior polarity of the sclerotomes is not disturbed in Pax1/Pax9 double homozygous mutants.

To investigate the induction and fate of the chondrogenic cell lineage in the sclerotomes of Pax1/Pax9 double mutants, expressions of Sox9, encoding a HMG-box-containing transcription factor, and Collagen II (Col2α1) were analysed. Recent studies have shown that Sox9 is required for cartilage formation (Bi et al., 1999). Col2α1, a direct target of Sox9 in precartilaginous mesenchymes (Bell et al., 1997), encodes a major component of the extracellular matrix of cartilage and was shown to be required for normal vertebral column development (Li et al., 1995). At E10.5, both genes are transcribed in a similar, segmented pattern along the body axis of normal embryos (Fig. 5A,D). This pattern was detectable in all Pax1/Pax9 compound mutants including double homozygous Pax1/Pax9 mutants (Fig. 5B,C,E,F, and data not shown). In addition to the segmented expression pattern, a continuous expression of Col2α1 is normally detectable in the ventral domain within the somites, between forelimb and hindlimb of E10.5 embryos (Fig. 5D,E,G). In contrast, this domain was barely detectable in double homozygous mutants (Fig. 5F,H). Cross sections through this region revealed that the reduction of the Col2α1 expression domain in double homozygous mutants is caused by a size reduction of the ventromedial region of the sclerotome, rather than by the inability of the sclerotome to express Col2α1 (Fig. 5I). These results confirm that the absence of Pax1 and Pax9 predominantly affects the ventromedial region of the sclerotomes, whereas the lateral sclerotome appears to develop normally at this stage.

The presence of Col2α1 expression in double homozygous mutants at E10.5 indicates that a chondrogenic cell fate is induced in the ventromedial regions of the sclerotomes in the absence of Pax1 and Pax9 and that chondrogenesis must be arrested at a later stage of embryonic development. Chondrogenesis of the vertebral column starts in the cervical region around E12.5 of mouse development. At this stage, the sclerotomes have formed chondrifying rudiments of the vertebræ that express Col2α1 and alternate with yet undifferentiated mesenchymes of the future intervertebral discs (Fig. 6A,E). In the absence of Pax1 and Pax9, a loose mesenchyme surrounding the notochord has developed that lacks signs of cartilage formation and is completely devoid of Col2α1 transcripts (Fig. 6D,H). In Pax1 single homozygous mutants, the overall size of vertebral precursors is reduced (Fig. 6B,F), whereas in Pax1+/−; Pax9+/− embryos, cartilage formation was found in the ventral sclerotome only. In the latter mutants, the cartilaginous rudiments exhibit an irregular spacing (Fig. 6G). Expression patterns in the sclerotomes similar to those shown for Col2α1 have been obtained using a Sox9-specific probe (data not shown). Together, these findings demonstrate that the control of normal size, shape and spacing of the cartilaginous precursors of the vertebræ and intervertebral discs require Pax1 and Pax9 in a gene-dosage-dependent manner.

**Programmed cell death and cell proliferation in Pax1/Pax9-deficient sclerotomes**

To investigate the mechanisms that cause the reduced size of the ventromedial sclerotome in Pax1/Pax9 double homozygous mutants, the number of cells undergoing apoptosis and the number of proliferating cells were determined during early sclerotome development. We analysed serial transverse sections of tail somites of E12.5 embryos in these experiments because, at this level of the body axis, the formation of skeletal elements is entirely dependent on Pax1/Pax9 function.

The histological analysis of caudalmost somites revealed no obvious defects of the sclerotomes. Both formation as well as ventromedial migration of sclerotomal cells occur in the absence of Pax1 and Pax9 (Fig. 7A,C). However, as somites mature, the sclerotomes of double homozygous mutant embryos become less condensed, as compared to the wild-type control (Fig. 7I,K). Notably, Tunel-labeling revealed that the number of cells undergoing programmed cell death in the sclerotomes of this region was not increased in Pax1/Pax9 double homozygous mutant embryos (data not shown). On the contrary, when the sclerotomes of wild-type embryos have formed precartilaginous condensations, the proportion of apoptotic cells decreases almost completely (Fig. 7O) whereas, at the same axial levels of Pax1/Pax9 double homozygous mutant embryo, dying cells were abundant (Fig. 7P). Thus, prevention of apoptosis in the sclerotomes may require the functions of Pax1/Pax9.

To determine whether cell proliferation is affected in the developing Pax1/Pax9-deficient sclerotomes, we analysed incorporation of BrdU in dividing cells of E12.5 embryos. Starting shortly after sclerotome formation, we analysed approximately 20 successive somites in 50 μm intervals (Fig. 7M). The comparison of BrdU-labelling profiles of wild-type and Pax1/Pax9 mutant sclerotomes reveals two phases. In the first phase (intervals 1-7, Fig. 7N), no differences in the number of BrdU-positive cells were found in wild-type and Pax1/Pax9 mutant embryos (84±1.5 in wild type versus 82±1.7 in Pax1/Pax9 double homozygous mutants). In the second phase, however, the number of dividing cells decreases in the mutant sclerotomes (65.7±7.7) whereas significantly higher numbers of labelled cells were found in the wild-type sclerotomes (94.7±8.7). Conversely, a higher number of proliferating cells were found in the developing neural tube of Pax1/Pax9 double
Fig. 5. Induction of a chondrogenic cell fate in Pax1/Pax9 mutant embryos, visualised by whole mount in situ hybridization of Sox9 (A-C) and Col2α1 (D-H) at E10.5. In the somites, a segmented expression pattern of Sox9 is detectable in wild-type (A), Pax1+/−, Pax9+/+ (B), and Pax1+/−, Pax9−/− embryos. (D-F) In embryos of the same genotypes, a similar pattern is found for Col2α1 expression. (G) Higher magnification of a control embryo showing a continuous band of Col2α1 expression (arrows) between forelimb (fl) and hindlimb (hl). (H) This expression domain is absent in Pax1/Pax9 double homozygous mutant. (I) Transverse section of a wild-type embryo at E10.5 stained for Col2α1 expression in the developing sclerotomes at the prospective trunk level. The regions of the future vertebral bodies and neural arches are outlined by Col2α1 expression. (J) Col2α1 expression is also detectable in the Pax1+/−, Pax9−/− mutant embryo, however, note the dorsoventral reduction of the sclerotome size in the Pax1/Pax9 double homozygous mutant as compared to the wild-type control (vertical bars).

Fig. 6. Normal formation of cartilaginous vertebral precursors is Pax1/Pax9 gene dosage dependent. (A-D) Sagittal sections of the cervical region of E12.5 embryos stained with Hematoxylin/Eosin (HE). Genotypes are indicated on top of the panels. (E-H) In situ hybridization of Col2α1 to sections adjacent to those shown in A-D. (A,E) In wild-type embryos, Col2α1 is strongly expressed in the chondrifying anlagen of the vertebral bodies (vb), in the notochord (nc), and weakly in the future intervertebral discs (id). (B,F) In the absence of Pax1, vertebral bodies are smaller. (C,G) In Pax1+/−, Pax9+/− embryos, remnants of the vertebral bodies exhibit an irregular spacing. In addition, expression of Col2α1 in the region dorsal to the notochord is almost absent (arrowheads). (D,H) In Pax1/Pax9 double homozygous mutants, the mesenchyme surrounding the notochord is not condensed and lacks Col2α1 expression.
homozygous mutants (Fig. 7N), a finding that is consistent with the successive increase of the size of the neural tube in the mutant embryo (Fig. 7G,K).

These results show that in the absence of Pax1 and Pax9 the sclerotomal cell population is reduced by a decrease in cell proliferation, followed by an abnormally high rate of apoptosis that later contributes to the substantial loss of the sclerotome size.

**DISCUSSION**

**Redundant functions of Pax1 and Pax9 during vertebral column development**

In this report, we demonstrate that the closely related transcription factors Pax1 and Pax9 have conserved biological functions during the development of the sclerotome. In the absence of Pax1 and Pax9, no ventral elements of the vertebral column develop along the entire body axis. The comparison of this phenotype to that of either single homozygous mutants reveals that Pax1 can completely compensate for the absence of Pax9, whereas loss of Pax9 is rescued by Pax1 to a considerable extent (Fig. 1). In addition, the severity of the phenotype in the absence of either gene is significantly exacerbated by introducing heterozygosity of the other gene. Thus Pax1 and Pax9 functions are redundant and their requirement for normal formation of the vertebral column is gene dosage dependent. Functional redundancy has recently been identified for Pax2 and Pax5, two paralogous genes of the Pax2/Pax5/Pax8 subgroup, which have overlapping functions during midbrain and cerebellum development (Schwarz et al., 1997). Similarly, earlier onset of embryonic lethality than in homozygous Pax3 mutants was found in mouse embryos lacking both Pax3 and Pax7, which together constitute another class within the Pax gene family (Mansouri and Gruss, 1998). Together, these data suggest that functional redundancy between paralogous Pax genes is a general mechanism that can act in those tissues in which members of a given subgroup have overlapping expression patterns.
In the absence of Pax1, Pax9 expression is upregulated and is ectopically induced in the anterior domain of the sclerotomes (Fig. 3), thereby providing a basis for functional redundancy between Pax1 and Pax9 in the developing vertebral column. The molecular mechanism by which upregulation of Pax9 is achieved is presently unclear. Our data indicate that, in wild-type embryos, Pax1 is involved in the suppression of Pax9 expression in the anterior sclerotome. Interestingly, a similar repression between paralogous Pax genes has recently been identified during the development of the dermomyotomes, which generate dermis and skeletal muscles. In Pax3 mutant embryos, expression of Pax7 is ectopically activated in the anterior half of the dermomyotome and in vitro data suggest a direct role of Pax3 in the suppression of Pax7 in cultured myoblasts (Borycki et al., 1999). Together, these results strongly suggest that Pax1 and Pax3 functions contribute to the control of the normal expression patterns of Pax9 and Pax7, respectively, in the sclerotomes and dermomyotomes of the developing somite. However, in the posterior sclerotomes of wild-type embryos, Pax1 and Pax9 are co-expressed, indicating that high levels of Pax1 do not necessarily result in the downregulation of Pax9 and suggesting that Pax9-activating signals are more potent in the posterior half of the sclerotome.

Cell proliferation and prevention of apoptosis

Complete failure of organ formation in Pax mutants has demonstrated essential roles of Pax genes during organogenesis. Although localised cell proliferation is commonly observed in tissues participating in organogenesis, the role of Pax genes in cell survival during embryogenesis is not well understood. Reduced proliferation rates have been found in the developing diencephalon of Small-eye (Pax6) mutant embryos (Warren and Price, 1997). In addition, it was shown that Pax5 is required to stimulate B cell proliferation in vitro (Wakatsuki et al., 1994) and cells overexpressing Pax genes can form tumours in nude mice (Maulbecker and Gruss, 1993). Moreover, growth inhibition as well as induction of apoptosis has been observed by inactivating Pax genes in vitro systems (Gnarra and Dressler, 1995; Rossi et al., 1995; Bernasconi et al., 1996; Hewitt et al., 1997). Increased apoptosis in the neural tube and somites was recently described in homozygous Pax3 mutant embryos (Borycki et al., 1999). Likewise, results of the present work show that sclerotomal cells of Pax1/Pax9-deficient mice undergo increased programmed cell death (Fig. 7). However, we noted an increase in apoptosis after a reduced sclerotome size has already manifested in Pax1/Pax9 double homozygous mutants. Thus, although programmed cell death clearly contributes to the elimination of sclerotome cells in the mutants, prevention of apoptosis is unlikely the primary function of Pax1 and Pax9.

Previous studies have revealed that Pax1 and Pax9 expression is typically found in tissues that exhibit high levels of cell proliferation (Müller et al., 1996). Our analysis has shown that Pax1 and Pax9 are indeed required to maintain a high rate of cell proliferation during a restricted phase of sclerotome development (Fig. 7). At the end of this phase, mesenchymal condensations are formed in wild-type, but not in the double mutant, sclerotomes. Thus, the precursor pool in the sclerotomes of Pax1/Pax9-deficient embryos might not reach a critical size, a process that could be essential to continue the sclerotome-specific developmental program.

Mesenchymal condensations and cartilage formation

Accumulation of mesenchymal cells precedes the formation of skeletal elements. Initially, a high mitotic activity is required to produce enough cells for the process of condensation, which subsequently is characterised by dramatic changes in gene expression, reorganisation of extracellular matrix and concentration of cells towards the centre of the future skeletal element (for review, see Hall and Miyake, 1995). Copies of Col2α1 mRNAs were found to increase one hundred-fold in the developing chick limb bud as condensation occurs (Kravis and Upholt, 1985). The sclerotomes of Pax1/Pax9 double homozygous mutants initially express Col2α1 and its transcriptional activator Sox9, but the enhancement of their transcription does not occur (Fig. 5 and Fig. 6). Instead, the expression of Sox9 and Col2α1 is gradually lost and mesenchymal condensations do not form.

Disturbed mesenchymal condensations have been identified in all alleles of the undulated series, which carry different mutations in the Pax1 gene (Grüneberg, 1954; Wallin et al., 1994; Dietrich and Gruss, 1995). Our analysis indicates that reduced cell proliferation and increased apoptosis in the sclerotomes of Pax1/Pax9 double mutants are causally related to the absence of mesenchymal condensations. Thus, in the compound mutants, partial loss of Pax1 and Pax9 could result in a small, but significant reduction of proliferating rates, which in turn would cause a partial size reduction of the mesenchymal condensation. The gradual increase in the severity of the phenotype observed in Pax1/Pax9 compound mutants would be compatible with this idea. However, it should be noted that the absence of Pax1 and Pax9 might also simultaneously affect the expression of cell adhesion molecules and/or other molecules required for the process of condensation.

Heterogeneity in the development of vertebral elements

During embryonic development of vertebrates, the somites are differentially influenced by neighbouring structures such as the notochord, neural tube, the intermediate mesoderm and surface ectoderm (reviewed in Christ et al., 1998). A central role for vertebral formation, ventralization of somites, as well as for the formation and survival of sclerotomes has been identified for the notochord, which is required for the sclerotomal expression of Pax1 and Pax9 (Watterson et al., 1954; Koseki et al., 1993; Brand-Saberi et al., 1993; Pourquie et al., 1993; Dietrich et al., 1993; Goulding et al., 1994; Neubüser et al., 1995). SHH, which is secreted by the notochord has been shown to be the key molecule regulating these processes (Johnson et al., 1994; Fan and Tessier-Lavigne, 1994; Teillet et al., 1998). Indeed, Pax1 expression is rapidly lost and no skeletal elements of the vertebral column form in Shh-deficient mice (Chiang et al., 1996). The ventromedially restricted vertebral defects in Pax1 mutants have raised the possibility that Pax9, which is expressed in the lateral regions of the sclerotomes, can partly rescue the consequences of Pax1 deficiency. Thus, the presence of neural arches, though malformed, in Pax1/Pax9 double homozygous mutants was an unexpected result of this study. Nonetheless, this result shows that different genetic pathways regulate the ventromedial and mediolateral regions of the sclerotomes. Whereas the former requires Pax1 and Pax9 function, Pax gene expression in the
lateral region of the sclerotome appears to be dispensable for skeletal development. In addition to the mediolateral differences, the development of the dorsoventral regions of the sclerotomes have also been shown to be regulated by distinct molecular pathways: experimental evidence in chick demonstrated that, shortly after sclerotome formation, the dorsalmost sclerotome cells become independent of SHH signalling. These cells develop under the control of dorsally expressed Bmp and Msx genes to form the spinous process, a mechanism that involves downregulation of Pax1 expression in the dorsal cells (Monsoro-Burq et al., 1994, 1996). Although recent data indicated that at later stages also the development of the ventral sclerotome requires signalling by Bmps (Murtaugh et al., 1999), these findings support the idea that each vertebra is assembled from multiple elements that are regulated by different developmental pathways. Among those, the Pax1/Pax9-dependent pathway is only required for the development of ventromedial structures. Moreover, the ectopic dorsal cartilage formed in the lumbar region of Pax1/Pax9 double homozygous mutants (Fig. 2) could indicate that dorsal and ventral regulatory pathways compete for a common pool of sclerotome cells.

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