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Vertebrate aristaless-related genes

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ABSTRACT Aristaless-related genes, a subset of the Paired-related homeobox genes, have in the past few years emerged as a group of regulators of essential events during vertebrate embryogenesis. One group of aristaless-related genes has been linked to the morphogenesis of the craniofacial and appendicular skeleton by their expression patterns and by the phenotypes of natural and artificial mouse mutants. Expression and function in the nervous system characterise a second group, and a third group, the *Pitx* genes, have been shown to have many different roles, including functions in the pituitary, left-right determination and limb development.

KEY WORDS: *homeobox genes, skeletogenesis, craniofacial development, limb development*

Introduction

It is now an established fact that homeobox genes have crucial roles in the molecular mechanisms underlying many developmental decisions. While *Hox* genes, related in structure and function to the clustered homeotic genes of *Drosophila*, remain the most intensively studied homeobox gene family, several other families have been described (Bürglin, 1994). Paired-class homeobox genes carrying a homeobox related to that of the *Drosophila* segmentation gene, *paired,* constitute a second major class of homeobox genes (see for a recent review and definition Galliot *et al.*, 1999). Paired itself belongs to the Pax family of transcription factors defined by the presence of a paired domain; only a subset of Pax proteins carries in addition a homeodomain. Pax-homeodomains, and no other homeodomains, are of the Paired/S50 type, indicating the presence of a serine at position 50. This amino acid is of crucial significance for specificity of DNA interaction (Hanes and Brent, 1989; Treisman *et al.*, 1989; also Wilson *et al.*, 1996). Other Paired-class homeodomain proteins carry a Q50 or K50 homeodomain.

Aristaless-related proteins are a specific subset of *Paired*-class homeodomain proteins, structurally defined by the presence of, in addition to a Paired/Q50 or Paired/K50 homeodomain, a conserved domain of unknown function that is always located near the C-terminus. As *aristaless* (Schneitz *et al.*, 1993) was the first of presently four known *Drosophila melanogaster* genes (*aristaless, D- Pitx, D-Otp, and D-Rx*) encoding such factors, we choose its name as eponym for this gene subfamily and for the characteristic conserved domain. The aristaless domain has been named by others OAR-domain, C-peptide or (in spite of its absence in the paired protein) 'Paired tail'. The *Pax-37* gene of the ascidian *Halocynthia roretzi* is the only known example of a gene encoding a Pax protein carrying also an aristaless domain (see *Galliot et al.,* 1999). The structural resemblances mentioned above indicate phylogenetic relation and potential for similar biochemical interactions. Even if, as it seems the case, these genes are involved in very different biological processes they may have analogous molecular roles in the underlying mechanisms.

This review deals with aristaless-related genes and their roles in vertebrate development. These genes are currently emerging as key players in vertebrate embryology. We give an overview of the three groups of vertebrate aristaless-related genes that we distinguish, and pay some attention to characteristic and relevant molecular properties of their products. Group-I genes receive in this review most emphasis, as they have been studied more as a group than the other two, but have remained relatively underexposed in the literature. More specifically, we go into some aspects of mechanisms involved in the way some of these genes may play a role in the morphogenesis of the craniofacial and appendicular skeleton.

Molecular properties of aristaless-related homeodomain proteins

Defining the aristaless-related family of transcription factors based on the presence of two conserved domains raises the question of what is the molecular significance of these structural features. The function of the aristaless domain remains largely

Abbreviations used in this paper: E*n*, embryonic day *n*; AER, apical ectodermal ridge; ZPA, zone of polarising activity.

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obscure. A function as activation domain (suggested by Simeone *et al.,* 1994) is not excluded, but is highly unlikely in view of work of Hudson *et al.* (1998). The restriction to a specific set of proteins carrying similar homeodomains might suggest a function directly related to the way these homeodomains exert their function. The occurrence of genes encoding alternative splice forms leading to proteins with or without aristaless domain (Kern *et al.*, 1992; Rao *et al.*, 1997) could be a basis for research into the functional significance of the aristaless domain.

The Paired-type homeodomain, obviously mediating DNA binding, has been linked to a number of distinctive functions, based mostly upon work *in vitro*. It should be noted that these functions are not exclusive to the aristaless-related homeodomains, since they apply also to Pax proteins.

MADS-factor interaction

Grueneberg *et al.* (1992, 1995) found evidence for potential of the Prx1 homeodomain to recruit MADS transcription factors like MCM1 and SRF to their target sequences. Formation of stable ternary complexes consisting of Prx1, SRF and the target DNA depends on the initiator binding protein TFII-I (Grueneberg, 1997). Cserjesi *et al.* (1994) also found evidence for convergence of *Prx1* and a MADS factor, MEF2), but the *in vivo* significance of these interactions remains to be demonstrated.

Rb interactions

As will be discussed later, Wiggan *et al.* (1998), found evidence for interactions between Paired-type homeodomains with the N-terminal portion of retinoblastoma family members.

Dimerisation

Paired-type homeodomains have been shown to mediate cooperative dimeric binding to sites consisting of two palindromic TAAT sequences separated by two or three less-significant nucleotides (P2 sites and P3 sites, respectively; Wilson *et al.,* 1993). Pax-type (S50) homeodomains bind slightly better to P2 sites than to P3 sites, while the other Paired-type homeodomains, including those of the aristaless-related (Q50 and K50) homeodomains prefer P3 sites (TAATNNNATTA). A further distinction was made for particular combinations of the three nucleotides separating the two TAAT sequences; Q50 favours YNR and K50 CCG (Y= C or T; R= A or G; note that the latter consensus is a subset of the former). High-resolution crystal structure analysis of Paired- class homeodomain dimers has offered an excellent explanation for the difference with other classes of homeodomains, which do not display this type of dimerisation. Wilson *et al.* (1995) have demonstrated the involvement of amino acids 28 and 43 in the homeodomain-homeodomain interaction. The presence of arginine residues at either or both of these positions, as is found in homeobox classes like Antennapedia (Hox), engrailed and many others would be incompatible with the intimate association of two homeodomains. Support for homodimerisation occurring *in vivo* has been provided by several groups of authors who have demonstrated reporter gene activation through P3 sites in cell transfection experiments (Cai, 1998; Hudson *et al.*, 1998; Qu *et al.*,1999). Wilson *et al.* (1995) and Sheng *et al.* (1997) have presented strong evidence that *rhodopsin 1* and possibly other eye-specific genes are directly regulated by the Pax protein eyeless through cooperative binding to P3 sites. A potential for heterodimerisation would obviously be of tremendous consequence for the way these regulators might exert their functions, and for the interpretation of their expression patterns. Heterodimerisation *in vitro* has been demonstrated by Qu *et al.* (1999), in band shift experiments using purified homeodomains of the aristaless-related factors Alx4 and Cart1. The same authors used transient transfection experiments in fibroblasts to demonstrate that Cart1 and Alx4 act as transcriptional activators far more efficiently through P3 sites than through half sites, P2 or P4 sites. Since activation by a mixture of Cart1 and Alx4 was simply additive, and not synergistic, these experiments do not prove that part of the gene activation involves heterodimers. Therefore, heterodimerisation has been proved to occur *in vitro*, but in living cells only homodimerisation has been demonstrated. Mead *et al.* (1998) designed an *in vitro* technique to clone Paired-typed homeodomain proteins based on potential for heterodimerisation between the Paired-type homeodomain protein Mix1 and partners. The observation that only *Mix*-related genes (*Mix2, Mix3* and *Mix4*) were cloned indicates preferential heterodimerisation between highly related Paired-class homeodomains.

Classification

A combination of structural and biological features suggest categorising of aristaless-related genes in three groups.

Group I

Genes expressed predominantly in mesenchyme and mesoderm that are implicated in functions in morphogenesis of the skeleton.

The emphasis in this review is on the group-I genes, which have been studied more as a group than the other two and have been shown, directly or indirectly, to have functions that are consequential to morphogenesis of skeleton.

Group II

Genes predominantly expressed in the central and/or peripheral nervous system.

Group III

The *Pitx* genes (Pitx1-3) that encode Paired/K50 homeodomain proteins.

Named for the pituitary where the first two members discovered are expressed, they are now known to have complex expression patterns and a variety of biological functions.

Table 1 gives an overview of vertebrate aristaless-related genes and presently available evidence for their function. In addition, their many alternative names are indicated; these are not at all used in the text. For clarity, we concentrate on the mouse genes, and do not give homologous genes from other species; however, in two cases a gene has been described only for another species. For a complete listing of aristaless-related and other Paired-related homeobox genes, see Galliot *et al.* (1999).

Group-II aristaless-related genes: functions in the nervous system

At least two of the Group-II genes have important functions in the eye. *Rx* is expressed in the anterior neural fold and developing retina (Furukawa *et al.*, 1997). It is involved in the earliest steps of eye development: mice homozygous for a targeted mutation of *Rx*

do not form optic cups, and consequently no eyes. Moreover, *Rx* RNA injected in *Xenopus* embryos leads to induction of ectopic retinal pigment epithelium and to hyperproliferation of the neuroretina (Mathers *et al.,* 1997). *Chx10* is expressed in the developing and mature neuroretina, including the earliest retinal neuroepithelial cells of the optic vesicle (Liu *et al.*, 1994). The gene has been shown to be allelic to the *ocular retardation (or)* mutation. Mice homozygous for this mutation have microphtalmia, optic-nerve aplasia and cataract. The disease is caused by reduced cell proliferation in the neural retina followed by progressive retardation of eye size (Burmeister *et al.*, 1996). Phenocopies of this mutation have been produced by injection of anti-sense Chx10 RNA in zebrafish (Barabino *et al.*,1997).

Orthopedia, (*Otp),* has been cloned from mouse, chicken, sea urchin and *Drosophila* (Simeone *et al.*, 1994). It was sequence conservation between these homologous genes that first indicated the existence of the aristaless domain as a conserved domain. *Otp* is expressed in the nervous system of mouse as well as *Drosophila*. Earliest expression is found at mouse E9.5 in spinal cord. After that, *Otp* expression remains restricted to the nervous system. From extensive analysis of the complex expression patterns in diencephalon, hindbrain and spinal cord, the authors conclude that *Otp* may play a role in regionalisation of the ventral diencephalon.

Drg11 has been described by Saito *et al.* (1995). It is expressed in sensory neurones as well as a subset of their targets in the CNS. Expression was not found prior to rat E12.5.

Arx is expressed in neuromeric patterns in the telencephalon, diencephalon, and in the midbrain/hindbrain border and floor plate of the mouse and zebrafish (Miura *et al.*, 1997). Arx has unusual high sequence similarity with the *Drosophila* aristaless protein, with a near-identical aristaless domain and 85% identities in the homeodomain. It shares with DRG11 sequence similarity outside homeo- and aristaless-domain.

No mutant phenotypes have been described for *Orthopedia, DRG11* or *Arx.*

TABLE 1

OVERVIEW OF VERTEBRATE ARISTALESS-RELATED GENES

	Classical Mouse Mutant	Human Syndrome Described	Targeted Mutant	Alias
Group I				
Prx1			+	Mhox, Phox1, K2a/b, Pmx, Rhox
Prx2			+	S8
Alx3			-	
Alx4	*Strong's luxoid (lst)*		+	B4
Cart1			+	
Prx3		C. de Lange?[1]	-	Shot; Og-12
Shox[2]		Turner Leri-Weill[3]		
Group II				
Drg11[4]			-	
Arx			-	
Chx10	*ocular retardation (or)*		-	Vsx-2, Alx
Otp			-	
Rx			+	Rax, Xrx-1
Group III				
Pitx1		TCFS?[5]	+	Ptx1, Otlx1, Bft, Brx2, P-Otx
Pitx2		Rieger	-	Ptx2, Otlx2, Brx1, RIEG, Solurshin, ARP1
Pitx3	*Aphakia*?	ASMD[6]; cataract	-	Ptx3

[1] Cornelia de Lange or Brachmann-De Lange Syndrome.
[2] Only human gene described.
[3] Leri-Weill dyschondrosteosis syndrome.
[4] Only rat gene described.
[5] Treacher Collins Franceschetti syndrome.
[6] anterior segment mesenchymal dysgenesis.

Group-III aristaless-related genes: *Pitx* genes

Pitx homeodomain proteins are usually referred to as '*bicoid-(bcd-)* like', which is merely to point out the presence of a lysine residue at position 50 of the homeodomain, and its consequential binding to a 'bcd-site', TAATCC. *Bicoid* is a maternal gene involved in the earliest specification of antero-posterior identity in the *Drosophila* egg. As the product of a recent duplication of the *zerknüllt* gene it is related to the *Hox* genes (Stauber *et al.*, 1999), and has no homologue in vertebrates. The Pitx1 homeodomain shares 22 (32%) identical amino acids with bicoid and 36 (60%) and 35 (58%) with aristaless and paired, respectively. There is therefore more structural basis to assign *Pitx* genes to the Paired-class, and, combined with the presence of aristaless domains in aristaless and Pitx and its absence in bicoid, more specifically as 'aristaless-like'. Paired/K50 homeodomains bind cooperatively to the palindromic version of the bicoid site called a 'P3K' site, TAATCCGATTA, but this cooperativity is 25-fold, which is about twofold lower than found for Paired/Q50 and Paired/S50 homeodomains (Wilson *et al.,* 1996). No *Pitx*–target interactions *in vivo* through P3K sites have been described.

The roles of *Pitx* genes in general and specifically of *Pitx1* and *Pitx2* in the pituitary gland have recently been reviewed (Drouin *et al.*,1998; Gage *et al.*,1999).

Pitx1 has been identified by Lamonerie *et al.* (1996) as an activator of the pro-opiomelanocortin *(pomc)* gene through a TAATCC containing site in its promoter. It has also been isolated in a two-hybrid screen, by virtue of its interaction with the N-terminal portion of the POU-domain factor Pit1 (Szeto *et al.*, 1996), and shown to synergistically regulate *pomc* and other pituitary-specific target gene promoters in cell transfection experiments (Szeto *et al.*, 1996; Tremblay *et al.*,1998). *Pitx1* is expressed at mid to late streak stages in extraembryonic mesoderm, and at E8 in the stomodeal epithelium and adjacent foregut endoderm (Lanctôt *et al.*, 1997). Subsequently *Pitx1* expression continues to be expressed in derivatives of the stomodeum including two placodal invaginations that result in formation of the olfactory organ and the pituitary. The stomodeal domain defined by *Pitx1* is designated the 'stomodeal ectomere' by these authors, referring to the neuromeric model of brain development of Puelles and Rubenstein (1993). It is also expressed in proximal mandibular-arch mesenchyme, and subsequently both in mesodermal (tongue) and ectomesenchymal (Meckel's cartilage) derivatives of the arch. *Pitx1* is expressed in hind limb, but not forelimb mesoderm. Recently, *Pitx1* mutants have been analysed (Lanctôt *et al.*, 1999; Logan and Tabin, 1999; Szeto *et al.*, 1999). Interestingly, the hind limbs of these loss-of-function mutants display features that makes them morphologically more similar, although not identical to fore limbs. The expression of another hind-limb specific factor, *Tbx4,* was reduced. Further evidence that *Pitx1* is involved in the mechanism responsible for the differential morphology of fore- and hind- limbs was obtained by ectopic expression of *Pitx1* in chick wing bud. This

resulted in altered morphology of the limb, interpreted as more leg-like; in addition, *Tbx4* was induced. The most conspicuous craniofacial defect is the short mandible (Lanctôt *et al.*, 1999). As it appears that this is due to the loss of proximal/lateral structures (the region containing the incisors is normal) the phenotype relates with the mesenchymal rather than the epithelial expression. In humans, a subset of cases of the Treacher Collins Franceschetti Syndrome, consisting of craniofacial abnormalities, may be caused by mutations in *Pitx1* (Crawford *et al.*, 1997).

Pitx2 has been cloned by positional cloning in a search for the molecular basis of the human Rieger's Syndrome (Semina *et al.*, 1996). Mutations in *Pitx2* cause this dominant congenital disorder that includes anomalies of the eye, dentition and umbilicus, as well as at least one related syndrome, iridogoniodysgenesis (Kulak *et al.*, 1998). In cell transfection experiments similar to those performed with *Pitx1* (see above), *Pitx2* is also capable of activating a target promoter in synergism with the gene encoding the POU domain factor Pit1. Interestingly, one of the *Pitx2* mutations found in Rieger patients results in a protein that does not display this *Pit1* synergism in spite of retained capacity to bind DNA (Amendt *et al.*, 1998). *Pitx2* is expressed in the pituitary gland, cranial mesenchyme, limb muscle precursors. In addition, its expression displays a-symmetric patterns in lateral plate mesoderm, heart and gut (see below). Although no targeted mutants of *Pitx2* in the mouse have been described, important functional data have been obtained through other means. A series of six papers (Logan *et al.*, 1998; Meno *et al.*, 1998; Piedra *et al.*, 1998; Ryan *et al.*, 1998; St. Amand *et al.*, 1998; Yoshioka *et al.*, 1998) have documented the function of *Pitx2* in the determination of left-right asymmetry. These data have been extensively reviewed by Harvey (1998) and Beddington and Robertson (1999). In short, it appears that the Tgf-β-like molecules like nodal, lefty-1 and 2 have crucial upstream roles in determining the left side of the body. Downstream from these factors *Pitx2* has been described as a global executor of the L-R pattern. It is induced by ectopic expression of nodal and lefty in chick embryos, and ectopic expression of *Pitx2* itself in the right side of chick embryos leads to isomerisation of the heart and gut derivatives. Involvement in typically asymmetry-related phenomena like heart looping and gut looping in different species have been convincingly demonstrated by the authors cited in Harvey (1998), Beddington and Robertson (1999), and by Campione *et al.* (1999). Other aspects of the *Pitx2* expression pattern notably concerning tooth formation have been described by Mucchielli *et al.*, 1997. Like *Pitx1*, *Pitx2* is expressed in stomodeum and oral epithelium, and expression in the latter has been shown to depend on signals from dental mesenchyme (Mucchielli *et al.*, 1997). An obvious conjecture from the expression patterns of *Pitx1* and *Pitx2* is that they share redundant functions in this region that should become evident in the phenotype of *Pitx1/Pitx2* double mutants. Interestingly, *Pitx2* has also been picked up in a differential-display screen for genes regulated by the mouse *MLL*/*ALL1* trithorax homologue that is directly involved in human acute leukaemia (Arakawa *et al.*, 1998). These authors found *Ptx2* to be expressed in bone marrow, but not in six different leukaemia lines. It has not yet been reported whether *Ptx2* is abnormally expressed in *MLL* mutants (Yu *et al.*, 1995).

Pitx3 has been cloned based on sequence similarity to *Pitx2* (Semina *et al.*, 1997) and by a general homeobox-directed PCR screen (Smidt *et al.*, 1997). It has a quite specific expression pattern in the brain starting at rat embryonic day 11.5, that appears to be linked to the mesencephalic dopaminergic system. Consistent with this is a decrease in the number of *Pitx3* expressing neurones in Parkinson patients, and a complete absence in an animal model for this disease, 6-hydroxydopamine-lesioned rats (Smidt *et al.*, 1997). Mouse *Pitx3* is also expressed in E10 embryos in the eye and subsequently in the forming lens pit throughout the stages of lens development. In this connection, it is important that *Pitx3* has been shown to be mutated in human patients for the anterior segment mesenchymal dysgenesis and congenital cataracts (Semina *et al.*, 1998a). In addition, it has been linked to the mouse *aphakia* mutation, characterised by absence of the lens, which fails to develop beyond E11 (Semina *et al.,* 1997).

Group-I aristaless-related genes

To this group belong the four *Prx* genes (*Prx1-3* as well as an additional human *Prx3*-like gene, *Shox*), *Alx3*, *Alx4* and *Cart1*. Of these seven genes, six have been linked to functions in the development of the craniofacial and/or appendicular skeleton, either through mouse artificial mutants or human syndromes. We have preliminary evidence that the seventh gene, *Alx3*, is no exception (ABe, ABr and FM, unpublished). These genes are characterised by predominantly mesenchymal expression patterns in stages from late gastrulation through at least mid-gestation. Expression patterns always include neural-crest-derived ectomesenchyme of the craniofacial region and lateral plate derived mesoderm sometimes including cardiac mesoderm. Most of these genes are also expressed at moderate to high levels in mesonephric (intermediate) mesoderm.

Prx 1 and 2

Cloning and expression

Prx1 and *2* encode structurally highly related proteins that share, in addition to almost identical homeodomains and aristaless-domains, a conserved 16-amino acid peptide near the N-terminus. This 'Prx-domain' is not found in Prx3 or any other protein, which underscores the close phylogenetic and presumably biochemical relation between Prx1 and Prx2 (Ten Berge *et al.,* 1998b).

Prx2, the first aristaless-related homeobox gene described, was cloned in a screen for homeobox genes potentially involved in hematopoiesis as Kongsuwan *et al.* (1988) isolated a small cDNA fragment from a spleen cDNA library. Further data on structure and expression were published by Opstelten *et al.* (1991), De Jong and Meijlink (1993), Leussink *et al.* (1995) and Ten Berge *et al.* (1998b). *Prx2* is expressed from late primitive streak stages on, diffusely in mesoderm and later transiently in the somites. Subsequently a complex expression pattern evolves in lateral-plate mesoderm derivatives including limb buds, genital eminence, tail and cardiac mesoderm, as well as in much of the neural-crest derived mesenchyme of the frontonasal mass and pharyngeal arches that contributes to the cranial and pharyngeal skeleton and the meninges. Expression is high in a number of sites like the developing teeth and hair follicles where interactions between ectodermal epithelia and mesenchyme are known to occur. At later stages, expression is seen at many locations where connective tissues are forming.

Prx1 has been cloned in various laboratories through quite different strategies. Cserjesi *et al.* (1992) identified mouse Prx1 as a factor binding to an AT-rich sequence near the muscle

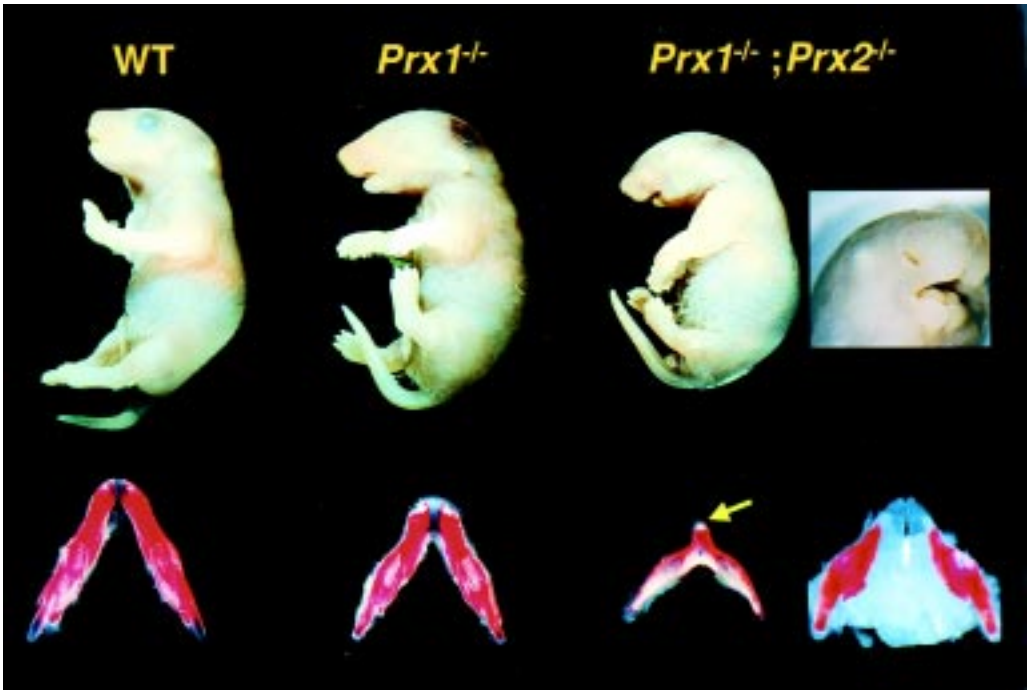

Fig. 1. Craniofacial phenotype of *Prx1, Prx2* double mutants. *On top a newborn wild-type mouse (WT), a Prx1 single mutant (Prx1-/-), and a double mutant (Prx1$^{-/-}$, Prx2$^{-/-}$) are shown. At the right, a frontal view of a rare example of strong expression of the phenotype, leading to split mandible and tongue. Underneath, alizarin red (bone)/alcian blue (cartilage) staining of mandibles from mice as shown on top. Yellow arrow indicates fused incisor in double mutant.*

creatine kinase promoter and Kern *et al.* (1992) isolated it from a heart cDNA library. Nohno *et al.* (1993) cloned a chicken homologue from a limb bud library using a PCR approach aimed at identifying homeobox genes. Grueneberg *et al.* (1992, 1994) cloned a human homologue by virtue of its capability to recruit MADS transcription factors like MCM1 and SRF to their target sequences. Several of these authors reported expression patterns similar to that of *Prx2* (Cserjesi *et al.,* 1992; Kern *et al.,* 1992; Nohno *et al.,* 1993; Kuratani *et al.*, 1994). Leussink *et al.* (1995), directly comparing expression of *Prx1* and *Prx2,* reported a high degree of overlap between these patterns, but nevertheless a number of clear differences. Most notably, *Prx1* is expressed in the brain and in the dermomyotome, whereas Prx2 is expressed in the spleen. At the late-streak stage, only Prx1 is expressed in the ectoderm. There are also subtle differences in the limits of the expression domains in the cranial mesenchyme and in the differentiating sclerotome (Leussink *et al.,* 1995). *Prx1* and *2* have distinct expression patterns in the cardio-vascular system. *Prx2* is expressed throughout the myocardium, and also in the endocardial cushions and their descendants, the valves. In addition, it is one of the earliest markers of ductus arteriosus differentiation (Leussink *et al.,* 1995). Expression of *Prx1* is even more distinctly expressed in the endocardial cushions and valves. Vascular expression of *Prx1* tends to suggest a relation with connective tissues rather than to smooth-muscle tissue, as was shown by Bergwerff *et al.* (1998) in a detailed study in the chicken, a more suitable system to make such distinctions. These authors observed absence of overlap with muscle actin and location to vascular fibroblasts of the adventitia and non-muscular cells of the media and intima but also with certain smooth cells of low-profile contractile properties (Bergwerff *et al.,* 1998).

Functions

In view of the similar expression and structure of *Prx1* and *2*, it is hardly surprising that mutant studies reveal strongly 'redundant' functions. Furthermore, comparison of different composite genotypes indicates that the phenotypes depend on the dose of intact *Prx1/2* alleles, and do not suggest basic qualitative differences. Nevertheless, deleting one allele of *Prx1* has more impact than deleting a *Prx2* allele.

It is intriguing that defects observed in *Prx1/2* mutants always occur at locations where both genes are expressed and that no abnormalities are found related to tissues where only one of these genes is expressed (see above; Leussink *et al.*, 1995; Bergwerff *et al.*, 1998).

Prx2 null mice have been generated but no abnormalities in these mice have been observed (Ten Berge *et al.*, 1998b). *Prx1* null mutants display various skeletal defects, most notably in the craniofacial region and including cleft palate. The squamosal, zygomatic, alisphenoid and tympanic are deleted or hypoplastic. In addition, a number of ectopic cartilages and inappropriate fusions are observed, involving Meckel's and Reichert's cartilages and other elements. The basioccipital is deleted and spina bifida occurs as a consequence of vertebral defects in a minority of mice (Martin *et al.,* 1995). Endochondral as well as dermal bones were affected, of either neural crest or mesodermal origin.

The phenotypes of double mutants clearly demonstrate the overlapping functions of these two genes (Ten Berge *et al.,* 1998b; Lu *et al.*, 1999a,b), as many craniofacial and limb defects are observed in the double mutant that are either aggravations of the *Prx1* phenotype or completely absent from the single mutants.

Craniofacial defects in Prx*1/2 mutants*

Prx1/2 double mutants are born with, amongst other defects, a severe reduction of the lower jaw (micrognathia). While in *Prx1* mutants Meckel's cartilage is only shortened and distorted, most of it is deleted in (newborn) double mutants, only a malformed proximal and a distal portion remaining. The dentaries are reduced in size, and, apparently because of mis-orientation during growth, have fused at a more proximal position than normal. They contain

only one central incisor, or none at all. About 8% of the newborn double mutants have a cleft lower jaw and tongue (Ten Berge *et al.*, 1998b; see Fig. 1).

While *Prx1* mutants survive up to 24 h, double mutants die within an hour after birth presumably due to almost complete inability to breath, as the airway appears to be obstructed by the tongue passing through the cleft palate into the nasal cavity. Presumably, this is caused by the reduction of the lower jaw leaving no space to contain the tongue, and the complete absence of the palatal processes of the maxillae in double mutants. This is a further example of a moderate defect in the *Prx1* mutant that is aggravated in the double mutant, as the *Prx1* mutants have hypoplastic remnants of the palatal processes.

In the double mutant, the otic capsule is much smaller than normal and the lateral semicircular canal is absent, whereas in *Prx1* mutants only a small reduction in the size of the optic capsule is seen (Ten Berge *et al.*, 1998b). Interestingly, outgrowth of the epithelial semicircular canal from the otic vesicle appears to be dependent on *Prx1*, that is only expressed in adjacent mesenchyme. Although *Prx2* is expressed in the epithelium of the otocyst (Opstelten *et al.*, 1991) *Prx2* mutants develop normal inner ears. This indicates involvement of *Prx* genes in the epithelio-mesenchymal interactions underlying this induction (Ten Berge *et al.*, 1998b).

Limb defects in Prx*1/2 mutants*

While *Prx1* mutants have only mildly abnormal limbs, with shortened zeugopods and bending of the anterior (preaxial) elements (Martin *et al.*, 1995), the phenotype in the double mutant is again far more severe, leading to very short zeugopods in which both elements (ulna/radius and fibula/tibia) are strongly bent. The autopods were also affected, showing preaxial as well as postaxial polydactyly. In addition, a variety of defects like deletion, bifurcation or abnormal placement of the autopod bones is found. As in many other aspects of the phenotype, variety between animals was considerable, presumably mainly due to genetic variance. This also caused differences in the polydactyly types observed by the two groups that described *Prx1/2* double mutants using mice in a different genetic background. Both groups observed postaxial polydactyly in the forelimbs, but Lu *et al.* (1999b) observed preaxial polydactyly in the hind limbs, whereas Ten Berge *et al.* (1998b)did not observe hind limb polydactyly, except for a single ectopic cartilage between the metatarsals. A role of non-genetic variance is suggested by the occurrence of random left/right differences.

Other defects in Prx1/2 mutants

Other skeletal defects present in *Prx1/2* double mutants included failure of the pubic symphysis to form and abnormalities of the scapula (Ten Berge *et al.*, 1998b). Outside the skeleton, abnormalities in the curvature of the aortic arch and a misdirected and elongated ductus arteriosus have been observed by Bergwerff *et al.* (1999). In spite of the highly specific expression in the heart, no cardiac defects have been reported. We have observed haemorrhages in the developing jaw of *Prx1/2* double mutants that occur at a low frequency, but were never seen in wild type embryos (DtB, ABr and FM, unpublished). The cause of these bleedings remains presently unclear, but it is conceivable that it is related to the vascular expression of *Prx1* and *2* (see above).

Cart1, Alx3, Alx4

Cloning and expression

Cart1, Alx3, Alx4 constitute a structurally and most likely functionally highly related group, encoding proteins with, in addition to near-identical homeodomains, similar aristaless-domains and significantly related sequences in between; only the sequences towards the N-terminus are almost completely divergent.

Alx3 was first identified by Rudnick *et al.* (1994), who cloned it in a search for potential regulators of the insulin gene from the insulinoma cell line T15 M2.2.2 that resembles beta cells in its insulin production but not in some other aspects. These authors reported expression in adult pancreas and testis, and in one exocrine pancreas cell line. Developmental expression was investigated by Ten Berge *et al.* (1998a), and is found in cranial neural crest derived mesenchyme, bodywall mesoderm, and, similarly to *Alx4* (see below) anteriorly in the limb bud. The only non-mesenchymal expression found was from around day E8.0, in amnion and ectodermal epithelia near the tail bud. Expression in pancreas could not be confirmed. A mutant phenotype for this gene has not yet been published (ABe, ABr and FM, in progress).

Cart1 has been isolated in a PCR screen for homeobox genes in a rat chondrosarcoma tumour (Zhao *et al.*, 1993). It is highly expressed in the frontonasal processes, and in pharyngeal arches, lung buds, mesonephros and tendons. A feature that strikingly contrasts with the other group-I aristaless-related genes, is its persistent expression at many sites in mesenchymal cells differentiating into cartilage. *Prx1, -2*, and *Alx4* are frequently seen to be expressed in mesenchyme prior to condensation, but they are apparently down-regulated inside the condensations prior to cartilage differentiation, their expression persisting in perichondrium and periosteum. *Alx3* expression patterns do not suggest a direct relation with chondrogenesis or bone formation. In view of its expression pattern, *Cart1* was initially suggested to have role in chondrogenesis (Zhao *et al.*, 1994).

Alx4 was fortuitously cloned as a non-significant result of a two-hybrid screen (Qu *et al.*, 1997a). It was also picked up in a two-hybrid screen using the retinoblastoma-related protein p130 as bait (Wiggan *et al.*, 1998). In the same screen, two other aristaless-related proteins, Prx1 and Chx10, and the Pax protein Pax3 were shown to interact similarly with p130. The common structural feature of these four proteins, the Paired-like homeodomain, mediates this interaction. Interestingly, binding occurred exclusively to the non-phosphorylated form of pRB, which is the form known to actively control progression of cells into S phase. Furthermore, pRB was shown to repress activation by Pax3 in cell transfection experiments. It has been proposed that the skeletal phenotype seen in *p130/p107* double mutants (Cobrinik *et al.*, 1996) may be linked to deregulated activity of Paired-like homeodomain proteins capable of interacting with these pRB family members (Wiggan *et al.*, 1998). *Alx4*'s mesenchymal expression pattern is characteristic of the members of this group. In addition, it has a striking anterior location in limb mesoderm (Qu *et al.*, 1997a,b).

Functions

Inactivation of *Cart1* through targeted mutagenesis (Zhao *et al.*, 1996) causes a decreased viability in a subset of forebrain mesenchymal cells, which is already evident at E8.75, when abundant abnormal apoptosis occurs in *Cart1* null embryos. This early defect subsequently results in failure of neural tube closure at the fore-

brain/midbrain border, and during gestation evolves further in increasingly dramatic defects. Eventually, *Cart1* null mice die perinatally from meroanencephaly and exencephaly. This phenotype has a 63-100% penetrance depending on genetic backgrounds and, interestingly, could be suppressed by prenatal treatment with the B-vitamin folic acid, an established food supplement recommended to pregnant women to prevent neural-tube closure defects (spina bifida) of the foetus. Many other parts of the cranium are affected in *Cart1* null mice, but these are in many cases, for instance the missing frontal and parietal bones, secondary to the exencephaly. Mandibular, maxillary and zygomatic bones are smaller than normal and supraoccipital, squamosal, palatine and alisphenoid were severely affected. Those animals that do have a closed neural tube also die at birth, and have abnormally shaped heads, and irregular folding and thickening of neural tissue, possibly due to retarded closure of the neural tube. Newborns die with apparent inability to breathe, which is apparently not due to cleft palate (see Qu *et al.,* 1999).

Alx4 has been shown to be allelic with the classical mutant *Strong's luxoid* (*lst*; Forsthoefel, 1963; Qu *et al*., 1998; Takahashi *et al.,* 1998), as all three available *lst* alleles were shown to carry mutations in the *Alx4* gene. These mutants display a severe and complex but variable phenotype including craniofacial defects, defects in abdominal wall closure (gastroschesis), strong preaxial polydactyly (see below) and absence of tibia, abnormalities of the phallus and cryptorchidism (failure of the testes to descend); the small percentage of mice that survives birth long enough display dorsal baldness. The mutation is semidominant since in heterozygotes a weaker degree of polydactyly, dependent on genetic background, is usually observed in the hind limbs.

Overlapping functions of Cart1 and Alx4

Functions of *Cart1* and *Alx4* overlap significantly, as is evident from observations on double mutants. The polydactyly seen in *Alx4* heterozygotes and homozygotes was exacerbated in the double mutants, although expression of *Cart1* in the developing limb is only strong in the precartilaginous condensations and does not display the location to anterior limb mesoderm characteristic for *Alx4* and also *Alx3* (Zhao *et al.,* 1994). At the molecular level, one explanation in addition to a merely additive effect, would be a possible qualitative importance of heterodimerisation between Alx4 and Cart1 (see section on Molecular Properties).

The exencephaly typical of the *Cart1* null mice does not appear to be exacerbated by *Alx4* mutations. As secondary effects of the $Cart1^{-/-}$ phenotype impede the analysis of double mutants, Qu *et al.* (1999) mainly used $Alx4^{-/-}$; $Cart1^{+/-}$ mice to study the craniofacial phenotype of *Cart/Alx4* double mutants. This is reasonable in view of the dose dependency of the limb phenotype. The major novel defect seen in $Alx4^{-/-}$;$Cart1^{+/-}$ mice was cleft face (including split nasal septum) and palate. Other defects include reduction of the mandible distally from the alveolar process, and malformation of the basisphenoid. The molecular basis of the split face phenotype remains presently unclear. Hedgehog signalling has been largely excluded by Qu *et al.* (1999), since expression of both Sonic hedgehog and the general hedgehog target *patched* is normal in *Cart1/Alx4* double mutants.

Prx3/Shot and Shox

Rovescalli *et al.* (1996) first isolated mouse *Prx3* by PCR from a genomic library. The expression pattern in rodents has been described in several groups. The gene is expressed in foetal and adult brain, initially in broad areas that develop in the dorsal thalamus, pretectum and tectum. In the adult the expression is most prominently expressed in nuclei that are part of the subcortical visual system (Van Schaick *et al.,* 1997). In addition, it is expressed in the peripheral nervous system, the nasal process, the mandibular arch and other craniofacial structures, and in the proximal developing limb. It is also expressed in muscles and it has an intriguing expression pattern in the cardiovascular system in inflow- as well as outflow tract (Van Schaick *et al.,* 1997; Blaschke *et al.,* 1998).

Human *Prx3* is a candidate gene for the Cornelia de Lange syndrome (De Lange, 1933; Blaschke *et al*., 1998; Semina *et al*., 1998b). This syndrome is characterised by a combination of mental retardation (especially evident in verbal communication), craniofacial features, eye defects and limb defects. These aspects of the syndrome correlate well with expression pattern in rodents, but allelism of the syndrome with *Prx3* has not yet been proven.

SHOX is highly related to *Prx3*, and no homologues in other organisms than Homo sapiens have been described. For this reason, and because expression levels are very low, knowledge on its expression pattern is limited. Differential splicing gives rise to at least two mRNAs, *SHOXa* and *SHOXb*, of which only the former encodes an aristaless domain. RT-PCR shows that both mRNAs are expressed in skeletal muscle and bone marrow fibroblasts, *SHOXa* also in placenta, heart and pancreas, and *SHOXb* also in foetal kidney (Rao *et al*., 1997). *SHOX* is a pseudoautosomal gene that has been linked to idiopathic short stature and Turner syndrome, as it was found to be present in a 170 kb region that is deleted in patients with these syndromes (Rao *et al.,* 1997; Shanske *et al.,* 1999). In addition, *Prx3* is deleted in the similar Leri-Weill dyschondrosteosis syndrome (Belin *et al.,* 1998; Shears *et al*., 1998). This disease is associated with Madelung's deformity, a shortening and bowing of the radius, not unlike the phenotype of *Prx1/2* double mutants.

Group-I genes and craniofacial development

In the mouse, the first pharyngeal (mandibular) arch is formed from two lateral processes that are first detectable in mouse embryos around E8.5, and that grow out medially to fuse around E10.5. An anterior prominence of the mandibular arch, the maxillary process, gives rise to the upper jaw, whereas the lower jaw is formed from the remaining part of the first pharyngeal arch, the much larger mandibular component. The arches consist of a mesodermal core in a jacket of neural-crest derived mesenchyme (Le Douarin *et al*., 1993; Osumi-Yamashita *et al*., 1994; Trainor and Tam, 1995). This ectomesenchyme gives rise to skeleton and other connective tissues and is responsible for patterning the arch mesoderm that gives rise to the muscles. In the first arch of primitive gnathostomes, the upper and lower jaws derive from two main cartilage elements, the palatoquadrate and Meckel's cartilage, respectively. The cartilages seen during early mammalian development in the first arch are homologous to these elements, but have been highly modified in form and function during evolution. In mammals, most of Meckel's cartilage only exists as a transient structure that never ossifies, except for the most proximal part that produces the malleus, one of the middle-ear ossicles, and a distal portion that contributes to the mental ossicle. The major bone of the lower jaw is the dentary, a dermal bone carrying the

teeth. The palatoquadrate has contributed to the alisphenoid and the incus, the second middle-ear ossicle. (See Thorogood, 1997; Smith and Schneider, 1998, and references therein).

During the early stages of mandibular arch development, the group-I aristaless-related genes are typically expressed distally in the processes, and subsequently medially in the resulting arch. The differences between these expression patterns are mainly in the proximal extent of the expression domain (Ten Berge *et al.*, 1998a). This type of expression is already seen at E8.5 for the *Prx1* and *2* genes, but not until a day later for *Alx3*. Similar distal/medial expression in mandibular processes has also been reported for a number of other genes, including the homeobox genes *Msx1* and *2, goosecoid* (Gaunt *et al.*, 1993), the Endothelin-receptor A (Clouthier *et al.*, 1998), and the bHLH transcription factors *dHAND* and *eHAND* (Srivastava *et al.*,1995). Compared to these genes, *Dlx1*, *2, 3* and *5* are expressed more proximally in the arch including the maxillary processes and less extreme distally in the processes (Robinson and Mahon, 1994; Qiu *et al.*, 1997). Expression patterns as well as mutant studies are consistent with *Dlx* and group-I aristaless-related genes having complementary functions in proximo-distal patterning of different areas the mandibular arch (Qiu *et al.*, 1995, 1997). In *Drosophila*, *Distal-less* functions upstream from aristaless and both genes are involved in proximo-distal patterning of appendages (Campbell and Tomlinson, 1998). It is conceivable that there is a relation between the distal expression in the mandibular processes of vertebrate group-I aristaless-related genes and the expression of *aristaless* in the distal part of the insect appendages.

Prx1 and 2 and morphogenesis of the lower jaw

The earliest signs of abnormal morphology of the mandibular processes of *Prx1/2* double mutants are seen around E10.5. Interestingly, in spite of the absence of *Prx* expression in branchial epithelium, the most conspicuous abnormality at this stage is an abnormal, thickened medial epithelium that tends to detach from the underlying mesenchyme. This observation indicates that *Prx* genes have functions related to mesenchyme-to-epithelium signalling.

In normal embryos the condensations of Meckel's cartilage are first seen at E12.5, but in *Prx1/2* double mutants only the proximal- and distal-most parts are present. Therefore, the newborn phenotype is already foreshadowed at this early stage, emphasising the fundamental and early function of *Prx* genes in the morphogenesis of the lower jaw. Obviously, there is no reason to exclude other later functions of the *Prx* genes in the craniofacial morphogenesis. It is noteworthy that *Prx* expression is correlated at many sites with bone formation, and is found in perichondrium and periosteum. At later stages *Prx1* is also expressed in bone (Leussink *et al.*, 1995; Ten Berge *et al.*, unpublished).

At stages when the morphological abnormalities of the mandibular arch are barely visible, the expression of a number of genes is already markedly changed. *Pax9* has a crucial role in tooth development (Peters *et al.*, 1998) and its expression foreshadows sites of future odontogenesis. In the normal developing mandibular arch, it is accordingly expressed in two paired spots, corresponding to the future lower incisors. In the *Prx* mutants at E11.0 the mandibular processes are deformed in such a way that the Pax9 spots are abnormally close to each other, and have at E11.5 fused (Ten Berge *et al.*, 1998b). *Pax9* expression in the mandibular-arch mesenchyme is known to be induced by the growth factor FGF8 that is expressed in the branchial epithelium, which is antagonised by two other epithelial signalling factors, BMP2 and 4 (Neubüser *et al.*, 1997). *Fgf8* expression and to some extent *Bmp4* expression are displaced in mandibular arches of *Prx1/2* double mutants, in a way that is still consistent with the antagonistic relation between these factors and *Pax9*. The mechanism that makes epithelial growth factor expression dependent on mesenchymal expression of *Prx* genes remains presently unclear.

Depending on genetic background and perhaps stochastic events, the lower-jaw phenotype is occasionally weaker, resulting in a fragment of a second tooth or stronger, resulting in no tooth at all being formed. The single tooth usually found appears normal by itself and we have concluded, therefore, that *Prx* is not involved in the differentiation process of the teeth per se.

In contrast, Lu *et al.* (1999a) have apparently only encountered mice lacking lower incisors and concluded that the teeth are specifically blocked in the bud stage. They report accordingly a down-regulation of *Pax9*. It seems likely that this aspect of the phenotype is stronger in the genetic background of the animals studied in this group.

We hold the view that in *Prx1/2* null mice the basis of the mandibular arch defect is a deficiency of medial tissues caused by locally reduced outgrowth of the mandibular processes. This is illustrated most imposingly by extreme expression of the phenotype, observed in a minority of the mutants, the split mandible and tongue. Here the integration of the mandibular processes has failed to an extent where it has led to their persistence as two separate structures (see Ten Berge *et al.*, 1998b). This phenotype is also observed in targeted mutants for *Endothelin-1* (Kurihara *et al.*, 1994).

Group-I genes and limb development

Limb development is one of the favourite themes of developmental biology. Intense research in this field has focused on the mechanisms that determine primary events of limb field location and initial limb outgrowth, and on the molecular and cellular basis of the subsequent growth and patterning events that generate the highly a-symmetrical limb. Essential roles in the initial phase of limb bud formation have been demonstrated for FGF10, retinoid signalling and presumably *Hoxb* genes. Crucial areas that are responsible for three-dimensional organisation of the limb include the apical ectodermal ridge (AER), the zone of polarising activity (ZPA), and the dorsal and ventral ectoderm. A distal transient population of mesenchymal cells called 'progress zone' and expressing the homeobox gene *Msx1*, remain in a proliferating state under the influence of FGFs and other growth factors expressed in the AER. Removal of the apical ectoderm at specific developmental stages induces limb truncations in a way that indicates that the point in time when cells leave the progress zone determines their proximo-distal identity. Antero-posterior identity depends on the location and action of the posteriorly located zone of polarising activity (ZPA) that functions in part in interaction with the AER. The ZPA is marked by expression of *Sonic Hedgehog* (*Shh)* which by itself is capable of inducing the polarising activity. FGF4 is thought to play a role in the integration between proximo-distal and antero-posterior patterning; it is expressed on the posterior part of the AER and is part of a positive feedback loop

with *Shh* that involves the product of the *limb deformity (ld)* gene, formin. Dorso-ventral patterning depends on the action of genes like *en-1*, *Lmx1,* and *Wnt7a*; the gene product of the latter is thought to interact with Shh to integrate dorso-ventral and antero-posterior patterning. Much of currently ongoing research focuses on questions like how the patterning processes along these three axes are integrated, and what determines the initial location of the signalling centres mentioned above. For reviews and further references, see Cohn and Tickle, 1996; Johnson and Tabin, 1997; Zeller and Duboule, 1997.

Prx1/2 –related limb defects

The occurrence of both pre-axial and post-axial polydactyly in *Prx1/2* double mutants appears to exclude a fundamental role in antero-posterior patterning by Prx genes. A relation to disturbed proximo-distal patterning seems more logical. In accordance with this, Lu *et al.* (1999b) report normal expression of *Shh* and *Hoxd11* in double-mutant limb buds, but abnormalities in expression of two signalling factors that are expressed in the AER, although at relatively late stages. *Fgf8* is normally expressed throughout the AER, but its expression domain is abnormally truncated in the posterior forelimb at E10.5. This is not observed in the hind limb, where no postaxial polydactyly was observed. An AER-defect would offer another example of an epithelial function apparently dependant of *Prx* expression in adjacent mesenchyme, since *Prx1* and *2* are not expressed in limb bud epithelium. This abnormality is, however, weak and not 100% penetrant, which contrasts with the severity and reproducibility of especially the zeugopod phenotype. It appears likely that the primary defect in autopod formation is not reflected in the AER-abnormality, but must be related to other mechanisms (Lu *et al.*, 1999b). *Bmp4* is reported to be down regulated in the AER at E11.5, as are two of its alleged targets, *Msx1* and *Msx2* in the underlying mesenchyme at E12.5. The meaning of these observations is not directly interpretable, in view of the complexities involved in the way BMP4 exerts its defects (see Zou *et al.*, 1997), and requires further investigation.

Alx4 –related polydactyly

In the past several years it has emerged that a negative regulatory cascade is involved in the proper localisation of the ZPA (see Johnson and Tabin, 1997; Zuniga and Zeller, 1999, and references therein). Preaxial polydactyly in a number of mouse and chick mutants and in experimentally induced situations has been associated with an ectopic anterior ZPA marked by expression of *Shh* (Charité *et al.,* 1994; Chan *et al.*, 1995; Masuya *et al.*, 1995). One such mutant is *Strong's luxoid (lst)* that was intensively studied in the sixties by Forsthoefel. He found that heterozygotes usually have mild hind limb polydactyly and homozygotes very strong polydactyly in all four limbs. Interestingly, he concluded on histological grounds that the AER had duplicated (Forsthoefel, 1963). Chan *et al.* demonstrated in 1995 the presence of an anterior ZPA and an anterior AER with posterior characteristics in the limbs of *lst* mutants by two criteria: (1) transplantation of anterior mesoderm from day 11.5 *lst* limb buds into chick limb buds giving rise to digit duplication and anterior *Hoxd11* expression; and (2) ectopic anterior expression of *Shh* and *Hoxd11* in mesoderm and of *Fgf4* in the anterior AER in *lst* limb buds. These authors suggest that the *lst* product has an early function in localisation of the ZPA expression either in a positive way or negatively, by suppressing ZPA induction anteriorly. The latter proved to be the case, as it was subsequently demonstrated that (1) *lst* and *Alx4* are allelic, (2) *Alx4* is expressed anteriorly in the limb bud, and (3) *lst* is a loss-of-function mutation (Qu *et al.,* 1998; Takahashi *et al.*, 1998). The presence of the ectopic ZPA has also been shown independently for the *Alx4* targeted mutant (Qu *et al.*, 1997b). The availability of a molecular marker for the *lst* allele allows the analysis of activity of this locus in the context of other mutations. Dunn *et al.* (1997), have reported that *Alx4* mutation increases penetrance and expressivity of polydactyly in BMP4 loss-of-functions mutants. These authors favour the explanation that this is due to an additive effect of two different mechanisms, namely low Shh-mediated induction of mitosis as a consequence of the *Alx4* mutation, and reduced cell death caused by the BMP4 mutation; this would result in allowing the population of anterior cells to 'exceed the threshold for making extra digits' (Dunn *et al.*, 1997).

Takahashi *et al.* (1998) analysed in more detail the role of *Alx4* in the development of both the mouse and chick limb bud. Expression of *Alx4* and *Shh* were shown to be mutually exclusive, Shh expression being linked to a posterior identity of limb bud mesoderm and *Alx4* to anterior identity. This is true not only in the natural situation, but also in the context of mutant and manipulated embryos. Application of Shh-soaked beads in the anterior margin of chick limb buds at concentrations known to lead to mirror-image-duplications resulted in rapid (within 6 h) down-regulation of *Alx4.* This down-regulation is much faster than, and therefore independent from, down-regulation of *Gli3*, a known negative target of *Shh* whose expression is also mutually exclusive with *Shh*. In addition, *Alx4* expression is unaffected in the *Gli3* mutant extra toes *(Xt).* Conversely, *Gli3* is anteriorly down regulated in *lst* mutants, but only when and at the site where Shh becomes ectopically activated. Therefore, the 'negative feedback loop' (Takahashi *et al.*, 1998) between Shh and *Alx4* does not seem to involve Gli3.

The significance of *Alx4* downregulation by Shh in the natural situation cannot be related to initial positioning of the ZPA, since *Alx4* is expressed prior to *Shh* during development of normal and artificially induced limbs. FGF2 beads applied to the presumptive flank region of chick embryos are known to induce limbs with reversed antero-posterior polarity (Cohn *et al.*, 1995). In accordance with this, in these ectopic limbs *Shh* is expressed on the anterior side (with respect to the embryo), and *Alx4* on the opposite side (Takahashi *et al.*, 1998). In this experiment the FGF-bead is implanted in flank mesoderm anteriorly from the hind limb, where *Alx4* is normally expressed. Between 12 and 18 h *Alx4* expression was downregulated, in accordance with these cells acquiring a 'posterior limb identity' the induced limb bud. Induction of Shh is seen only at 24 h. The obvious experiment of overexpressing *Alx4* has not yet been reported but should show whether potential for downregulation between FGF4 and Shh is mutual.

The chick *diplopodia4* mutant is one of the few mutants with preaxial polydactyly in which no anterior ectopic *Shh* expression is observed, but its molecular genetic basis is not known. Interestingly, down-regulation of *Alx4* does occur, and several posterior markers (*Bmp2*, *Fgf4*, and *Hoxd*) are expressed anteriorly. While this observation may seem to unhook the relation between *Shh* and *Alx4*, it remains possible that a downstream factor of the hedgehog pathway is affected in the mutant, or that another member of the hedgehog family is ectopically expressed, as has been shown for

Indian hedgehog in the *double foot* mouse mutation (Yang *et al.*, 1998). Recently a negative limb-specific regulatory element of *Shh* as been identified by Sharpe *et al.* (1999), which is a promising basis for further studies on the mechanism that restricts ZPA localisation.

Dependence of *lst* polydactyly upon a AER-linked function is indirectly suggested by the histological AER abnormality mentioned above (Forsthoefel, 1963), and further by the observation that double-mutants mice heterozygous for both *lst* and *ld* do not have polydactyly (Vogt and Leder, 1996); the defect of the *ld* mice consists of a malfunctioning AER caused by defective FGF4-Shh feedback loop (Haramis *et al.*, 1995). Consistent with this, Takahashi *et al.* (1998) showed that *Alx4* expression is down-regulated within 24 h after removal of the anterior, but not the posterior half of the AER at stage 18. Apparently, this dependence on the AER has disappeared at stage 20 (Takahashi *et al.*, 1998).

Perspective

Research involving aristaless-related genes is at a stage when it has become clear that these genes have important functions in developmental processes, whereas understanding of the mechanisms involved remains poor. As is true for many other gene families, aspects of gene functions may not be uncovered until complex genotypes have been generated to offset the consequences of functional overlap; so far this has been demonstrated for the group-I genes and will undoubtedly be for the *Pitx* genes and certain combinations of group-III genes. At this moment, not much is understood of the interactions linking aristaless-related genes to the other players in the network of developmental regulators. Study of the regulation of aristaless-related genes has hardly started. Even more important is the notoriously difficult task of identifying the target genes that are significant for the way the aristaless-related genes exert their functions. Instrumental in this will be the biological material produced by the targeted mutants.

An example of a question that deserves direct attention is the way *Prx* genes are involved in epithelio-mesenchymal interactions. Phenotypic analysis of the inner ear, the mandibular arch and the limbs, as well as numerous aspects of the expression patterns, provide evidence that group-I *Prx* genes regulate factors that signal from mesenchyme to epithelium, but the nature of these factors remains totally obscure. Interestingly, *Pitx* genes are among the genes in the mandibular epithelium that respond to signals from the mesenchyme. In addition, at the molecular level, a number of potentially important issues need to be verified and pursued. These include the molecular role of the aristaless domain, the proposed link with cell cycle regulators through retinoblastoma-related proteins, the link with MADS transcription factors and the role of heterodimerisation *in vivo*.

Note added in proof: The phenotype of *Pitx2* mutant mice has been described recently and confirms functions of *Pitx2* in left-right determination, craniofacial development, and determination and proliferation of the anterior pituitary gland. Lu *et al.* (1999; *Nature 401:* 276-278) and Lin *et al.* (1999, *Nature 401*: 279-282).

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