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Dosage requirement of *Pitx2* for development of multiple organs

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SUMMARY

Pitx2 is a homeodomain transcription factor that is mutated in Rieger syndrome, a haploinsufficiency disorder affecting eyes and teeth. Pitx2 also has a postulated role in left-right axis determination. We assessed the requirements for Pitx2 directly by generating hypomorphic and null alleles. Heterozygotes for either allele have eye abnormalities consistent with Rieger syndrome. The ventral body wall fails to close in embryos homozygous for the null allele, leaving the heart and abdominal organs externalized and the body axis contorted. In homozygotes for either allele, the heart tube undergoes normal, rightward looping and the stomach is positioned normally. In contrast, homozygotes for both alleles exhibit right isomerization of the lungs. Thus, Pitx2 is required for left-right asymmetry of the lungs but not other organs. Homozygotes for either allele exhibit septal and valve defects, and null homozygotes have a single atrium proving that a threshold level of Pitx2 is required for normal heart development. Null homozygotes exhibit arrest of pituitary gland development at the committed Rathke pouch stage and eye defects including optic nerve coloboma and absence of ocular muscles. This allelic series establishes that Pitx2 is required for the development of mulitple organs in a dosage-sensitive manner.

Key words: *Pitx2*, Rieger syndrome, Mouse, Multiple organs, Asymmetry

INTRODUCTION

The *bicoid*-like homeobox gene *Pitx2* is a member of a multigene family with overlapping and distinctive expression patterns. Multiple members of this gene family have been identified in vertebrates including amphibians, fish, birds and mammals (Gage et al., 1999). Identification of *Pitx* in *Drosophila* implied an important evolutionary role for the Pitx gene family, yet *Drosophila* mutants have no obvious anomalies (Vorbruggen et al., 1997). Recent experiments in chick and frog have indicated a role for *Pitx2* downstream of *sonic hedgehog* and *nodal* in a genetic pathway regulating laterality of heart, gut and other asymmetric organs (Logan et al., 1998; Piedra et al., 1998; Ryan et al., 1998; Yoshioka et al., 1998). Pitx2 expression is altered in mouse mutants with laterality defects, including *iv*, *inv* and a targeted mutant in *Lefty*-2, suggesting that *Pitx2* participates in left-right determination in mammals as well (Meno et al., 1998; Piedra et al., 1998; Ryan et al., 1998).

The importance of each member of the *Pitx* gene family is emerging through analysis of human patients and targeted mutations in mice. *PITX2* mutations cause Rieger syndrome, type I (RGS1, MIM 180500) (Semina et al., 1996), an autosomal dominant haploinsufficiency syndrome with defects in the anterior segment of the eye (cornea, trabecular meshwork and iris stroma), and other features of varying severity including cataracts, glaucoma, missing or misplaced teeth (hypodontia), umbilical abnormalities and cardiac defects (Jorgenson et al., 1978; Semina et al., 1996). *PITX3* mutations result in autosomal dominant cataracts and anterior segment mesenchymal dysgenesis (MIM 107250) (Semina et al., 1998). In contrast to *PITX2* and *PITX3*, *Pitx1* mutations in mice are recessive, affecting development of the palate, mandible and limbs (Lanctot et al., 1999; Szeto et al., 1999).

Pitx2 is expressed in many tissues during development, including the left lateral plate mesoderm, derivatives of the first branchial arch, the eye, brain, pituitary gland, mandible, heart and limbs (Arakawa et al., 1998; Gage and Camper, 1997; Kitamura et al., 1997; Muccielli et al., 1996; Semina et al., 1996). The phenotypes of RGS patients with null alleles establish an essential role for this gene in eye, tooth and umbilical development, and glaucoma (Amendt et al., 1998). The occasional occurrence of isolated growth insufficiency and aortic arch defects in RGS patients suggests that *PITX2* may also play a role in pituitary gland and heart development (Mammi et al., 1998; Sadeghi-Nedjad and Senior, 1974). However, the importance of PITX2 is still enigmatic because of the one remaining functional allele in these heterozygous patients.

We used gene targeting in embryonic stem (ES) cells, combined with cre-loxP technology to generate an allelic series of *Pitx2* mutations of varying severity. Heterozygotes for hypomorphic (neo) or null (–) alleles for *Pitx2* mimic some aspects of RGS, including eye and tooth defects. The phenotypes of homozygotes for both alleles establish that *Pitx2* is essential for positioning and development of the heart and for

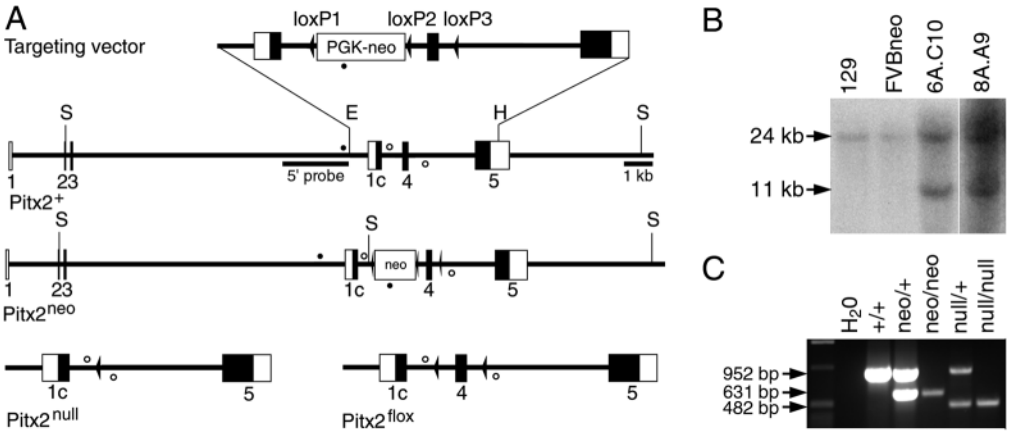

Fig. 1. *Pitx2* allelic series. (A) The structure of the targeting vector is shown enlarged in relationship to *Pitx2*$^{+}$. The complete structure of *Pitx2*^{neo} and abbreviated structures for *Pitx2*^{null} and *Pitx2*^{flox} are also shown at the same scale as *Pitx2*$^{+}$. Exon (boxes) and coding sequence (black boxes). (B) Southern analysis illustrating homologous recombination 5′ of the neo^{R} cassette in two cell lines heterozygous for *Pitx2*^{neo}. A 5′ probe located outside of the targeting vector detected the expected 24 and 11 kb *Spe*I fragments (S) from the wild-type and targeted *Pitx2* alleles, respectively. (C) Genotyping wild-type(+/+) embryos, and embryos heterozygous and heterozygous for *Pitx2*^{neo} (*neo*/+ and *neo/neo*) or *Pitx2*^{null} (–/+ and –/–) by multiplex PCR. *Pitx2* genomic primers (◦ in A) located 5′ of loxP1 and 3′ of loxP3 amplify 952 bp and 482 bp products from *Pitx2*$^{+}$ and *Pitx2*^{null}, respectively. The 5′ *Pitx2* primer and an antisense neoR primer (• in B) amplifies a 631 bp product. The *Pitx2* genomic primer pair does not readily amplify across *Pitx2*^{neo}.

lung asymmetry. While pituitary and eye development are grossly normal in neo/neo fetuses, –/– mice eye exhibit an early arrest in pituitary development and multiple eye defects. The differing sensitivity of various organs to *Pitx2* deficiency demonstrates a tissue-specific dosage-dependence on *Pitx2* for both laterality and organogenesis.

MATERIALS AND METHODS

Targeting of *Pitx2* gene

A P1 clone containing *Pitx2* was obtained from a 129/OLA genomic library by screening with *Pitx2* exon 5 PCR primers (Sternberg, 1992; Genomic Systems, Inc.; St. Louis, MO). Since herpes simplex virus thymidine kinase (HSV-TK) interferes with spermatogenesis (Al-Shawi et al., 1991), pFLOXΔRI was generated by deleting the HSV-TK sequences from pFLOX (Orban et al., 1992) by *Eco*RI digestion and religation. Three contiguous *Pitx2* genomic fragments were inserted sequentially into pFLOXΔRI: a 631-bp fragment spanning the homeodomain-encoding exon 4 was placed between loxP2 and loxP3, a 1.7 kb fragment was placed upstream of loxP1 and the phosphoglycerate kinase-*neo*^{R} cassette, and a 3.2 kb fragment was placed downstream of loxP3 (Fig. 1).

The targeting vector was linearized with *Hin*dIII and electroporated into R1 ES cells (Nagy et al., 1993). G418-resistant colonies were screened by PCR to identify homologous recombinants. The 29 positive clones (3.3%) were rescreened by Southern blot to confirm homologous recombination 5′ and 3′ of the *neo*^{R} cassette, and by PCR to demonstrate the incorporation of loxP3. Two cell lines heterozygous for *Pitx2*^{neo} (6A.C10 and 8A.A9) were injected into C57BL/6J blastocysts to generate chimeric mice, which were mated to C57BL/6J females to transmit the neo allele.

The two neo/+ cell lines were also transiently transfected with a cre expression vector (pMC-Cre, (Gu et al., 1993)) to derive the null (–) and floxed alleles by cre-mediated site-specific recombination. The +/– and +/flox cell lines were identified by PCR (Fig 1). Two +/– cell lines were injected into C57BL/6J blastocysts; resulting chimeric founder males were bred to C57BL/6J females to transmit the null allele. Heterozygotes of the N1-N3 generations were mated to generate timed pregnancies. The morning after mating was designated e0.5.

Experiments with mice and recombinant DNA were approved by university committees.

RT-PCR

Total RNA was isolated from embryos or cell line pellets using TRIzol reagent (Gibco; Gaithersburg, MD) (Gage and Camper, 1997). PCR was performed on first-strand synthesis product using *Pitx2*-specific primers located in exons 1C and 4.

Histology, immunohistochemistry and in situ hybridization

Embryos were flash frozen or fixed overnight in 4% phosphate-buffered saline (pH 7.2). Fixed embryos were embedded in ParaPlast (Oxford Labware; St Louis, MO). Sections were stained in Hematoxylin (Fisher; Fair Lawn, NJ) and Eosin (Sigma; St. Louis, MO). In situ histochemistry was previously described (Schaeren-

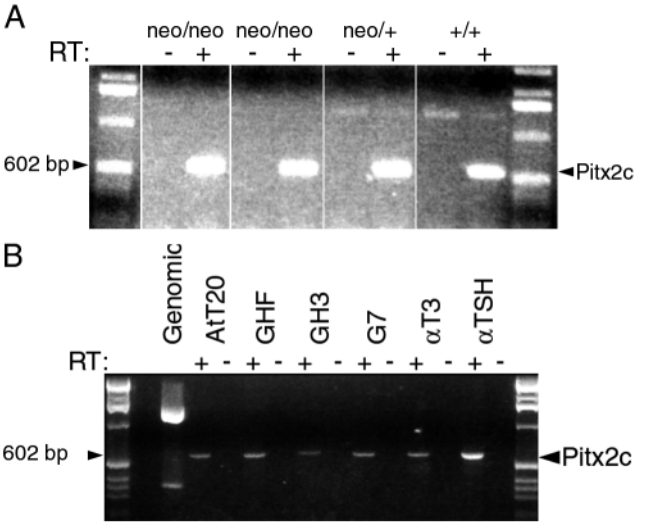

Fig. 2. RT-PCR detection of *Pitx2* mRNA. (A) Detection of *Pitx2c* in total RNA from *neo/neo*, *neo/+* and +/+ embryos at e12.5. Each reaction was carried out in the presence (+) and absence (–) of reverse transcriptase. (B) Detection of *Pitx2c* in total RNA representing all 5 anterior pituitary cell lineages. Cell lines: AtT20, corticotrope; GHFT-1, *Pit1*-dependent lineages; GH3, somatomammotrope; G7, AtT20 subclone; aT3, gonadotrope; aTSH, thyrotrope.

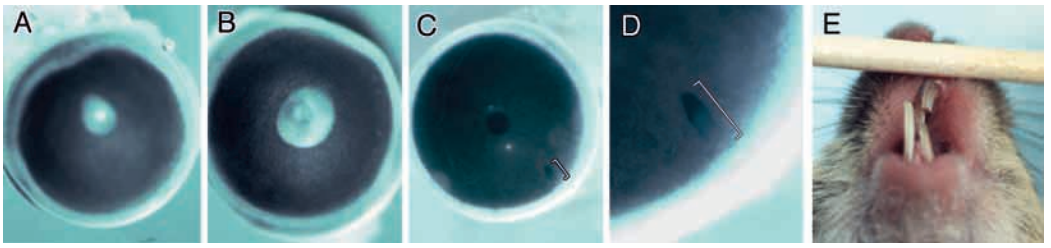

Fig. 3. Eyes and teeth of *Pitx2* heterozygotes. (A,B) *Pitx2*$^{neo/+}$ eye pair showing corectopia (A) and anisocoria (B). (C) neo/+ eye with multiple iris openings (bracket). (D) Magnification of eye in C. (E) Mal-occluded teeth in –/+ mouse.

Weimers and Gerfin-Moser, 1993). Plasmid templates for *Lhx3* (from H. Westphal) and *Rpx* (officially designated as *Hesx1*, from K. Mahon) were used as described (Hermesz et al., 1996; Zhadanov et al., 1995).

RESULTS

Construction of *Pitx2* allelic series

Analysis of *Pitx2* mRNA transcripts reveals the potential for three different protein isoforms of wild-type Pitx2 that arise through the use of alternative promoters and splicing (Fig. 1A) (Gage et al., 1999). *Pitx2a* and *Pitx2b* use an upstream promoter and differ from each other by alternative splicing of exon 3. *Pitx2c* utilizes a downstream promoter located in intron 3. Each Pitx2 isoform would contain the homeodomain, encoded primarily in exon 4, and have identical C termini but different N termini (Gage et al., 1999).

We used gene targeting in ES cells and cre-lox technology (Gu et al., 1994) to construct two *Pitx2* alleles that differ in severity. Gene targeting was performed with a vector engineered to contain loxP sites flanking the critical exon 4 and a neomycin resistance selectable marker cassette (*neo*^{R}) flanked by loxP sites and inserted into intron 4 (Fig. 1A). The *Pitx2*^{neo} allele was generated by homologous recombination after electroporation of the targeting vector into ES cells. Transfection of these clones with a cre expression vector produced the *Pitx2*^{null} and *Pitx2*^{flox} alleles. Clones heterozygous for *Pix2*^{null} (+/–) and *Pitx2*^{flox} (flox/+) were identified by PCR (Fig. 1C). Deletion of both the *neo*^{R} cassette and exon 4 via recombination at sites P1 and P3 produced a presumptive null allele due to loss of the essential homeodomain and a frameshift leading to a series of missense codons and a premature stop codon. Deletion of only the *neo*^{R} cassette, by recombination between lox P1 and P2, produced *Pitx2*^{flox}, an allele that should be fully functional and conditionally converted to *Pitx2*^{null} in the presence of tissue-specific or temporally regulated cre transgenes.

Two neo/+ clones and two +/– clones were injected into C57BL/6J blastocysts to generate chimeric mice. Chimeric males were bred to C57BL/6J females to transmit the modified *Pitx2* alleles, and independent mouse colonies derived from each injected cell line were established. Progeny were genotyped by PCR (Fig 1C). Heterozygotes for both alleles exhibited normal fertility. The phenotypes associated with each allele were replicated in two independent lines.

Pitx2^{neo} is hypomorphic

Insertion of a *neo*^{R} cassette into an intron by gene targeting can alter or disrupt gene expression by virtue of the cryptic splice sites and polyadenylation signals in the cassette, leading to hypomorphic or null alleles (Meyers et al., 1998). neo/+ mice were intercrossed to test for viable progeny. No neo/neo mice were identified in 334 two-week old progeny (Table 1), demonstrating that the neo allele is not functioning normally. Genetic analysis of timed pregnant litters from e9.5 to p1 revealed that individual homozygotes die throughout gestation beginning at e10.5. The loss of viable embryos is significant at e18.5 when only 2.4% (1/41) of the fetuses were neo homozygotes instead of the expected 25% (P<0.01). Death this late in gestation is usually attributable to structural cardiovascular defects or hematopoietic problems (Copp, 1995).

Normal *Pitx2* transcripts, produced by splicing from exon 1A to exon 4, were detected in mRNA from neo/neo embryos by RT-PCR (Fig. 2A). This observation and the more severe effects of the null

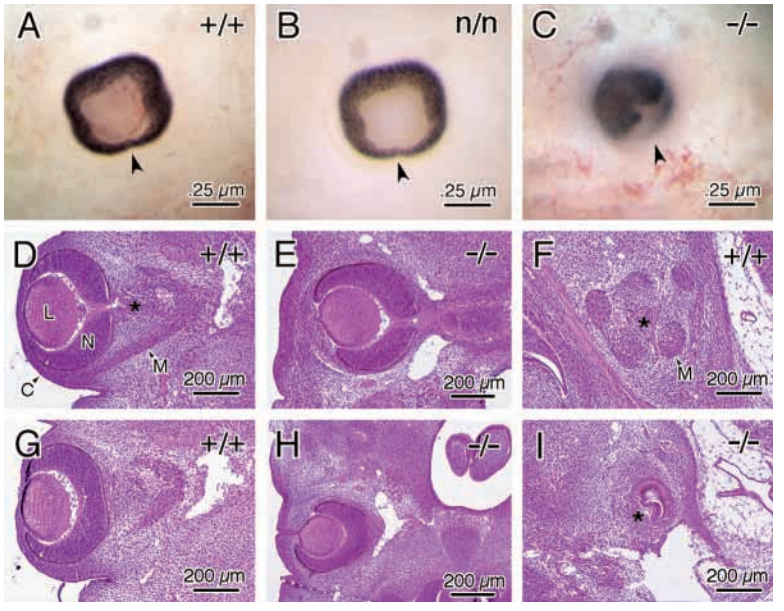

Fig. 4. Lack of *Pitx2* effects eye development. (A-C) Embryo eyes at e12.5 with choroidal fissure oriented toward lower right (arrowhead). (D,E,G,H) Transverse sections at e12.5 illustrate the lens (L), neural retina (N), cornea (C), extrinsic ocular muscles (M), and optic nerve (*) in +/+ (D,G) and –/– eyes (E,H). Mutant eyes (E,H) are inappropriately positioned, corneal epithelia and mesenchyme are hypercellular, closure of the optic fissure and stalk are defective or severely delayed, extrinsic ocular muscles are absent and a vestigial lens pit is present. (F,I) Sagittal sections behind +/+ (F) and –/– (I) eyes. Note the unclosed optic stalk and absent ocular muscles in the mutant eyes.

Table 1. Transmission of *Pitx2^{neo}*

Age	+/+	neo/+	neo/neo	Total
Prenatal				
e9.5	4	6	5	15
e10.5	7	16/18*	5/7	32
e11.5	4	11/12	3	19
e12.5	5	20/21	7/10	36
e13.5	6	15	7	28
e16.5	8	14	10/13	35
e18.5	14	24	1/3	41
Postnatal				
P1	1	15/16	2	19
P14	120 (36%)	214 (64%)		334

*live/total

allele (see below) demonstrate that the neo allele is a hypomorph, rather than a null (Table 2).

Pitx2 is required for both anterior and posterior eye development

The RGS phenotype can be highly variable, even within families. However, all patients exhibit congenital defects of the anterior segment of the eye. Abnormalities consistent with RGS1 were evident. These included corectopia (asymmetrically placed pupils), anisocoria (uneven pupil size), multiple pupillary openings and clouded lenses (Fig. 3). Like RGS in humans, these features were not present in all heterozygotes for the neo or null (–) allele (~10%). The majority of the +/– mice (n>30) had grossly normal teeth and body size, however two +/– males were approximately 1/3 normal body size, had mal-occluded incisors and obviously small eyes.

Examination of –/– embryos revealed a broad requirement for *Pitx2* during eye development (Fig. 4A,B,D,G). *Pitx2* is expressed in the perioptic mesenchyme by e9.5, but it is not expressed in the optic stalk or eminence. At e12.5, +/+ and neo/neo eyes are located immediately beneath the surface with the rim of the optic cup in contact with the developing cornea. In contrast, –/– eyes are smaller than normal and lie below the surface epithelium (Fig. 4C,E,H). They are rotated toward the nose so that the lens does not directly face the surface ectoderm. The developing corneal epithelium is thicker in –/– mice, and the optic cup is separated from the surface ectoderm by an abnormally thick mesenchymal layer (Fig. 4E,H). We confirmed the increased thickness of corneal epithelium by staining with a Pax6 antibody (data not shown). Vestigial lens pits are present in some –/– eyes at e12.5 (Fig. 4H), while lens pit closure is complete in +/+ eyes by e11.

Mesenchymal condensations leading to extrinsic eye muscle formation are clearly evident in wild-type eyes but absent in –/– embryos at e12.5 (Fig. 4D-I). Muscles form in neo/neo eyes but they are reduced in size (data not shown). Closure of the optic fissure is also defective or significantly delayed in the –/– embryos. No other gross defects were noted in the development of the neo/neo eyes or in the neural retina and retinal pigmented epithelium of –/– eyes.

Pitx2 regulates pituitary gland development

The anterior and intermediate lobes of the pituitary gland derive from Rathke's pouch, an invagination of oral ectoderm within the roof of the mouth that begins at e8.5. Sequentially,

Table 2. *Pitx2* allelic series

Genotype	Features
+/neo	variable, mild Rieger syndrome corectopia, anisocoria, multiple pupillary openings
+/–	variable, severe Rieger syndrome small body size, eye and tooth defects
neo/neo	embryonic lethal (e18.5) Heart: initiates rightward looping, positioned on right, septal and valve defects Lung: right isomerized Stomach: positioned normally on left
–/–	embryonic lethal Incomplete turning of embryo Ventral body wall open: gastroschisis, ectopia cordis Heart: initiates rightward looping, positioned on left but malrotated, single atrium, valve defects Lung: right isomerized Stomach: positioned on left Eye: malpositioned, optic nerve coloboma, vestigial lens pit, lack ocular muscles Pituitary: primoridium arrested at committed Rathke pouch stage

the early steps are formation of the pouch rudiment, development of the committed pouch and pouch expansion (Sheng et al., 1996). *Pitx2* is broadly expressed early in Rathke's pouch, including in the rudiment (Gage and Camper, 1997; Muccielli et al., 1996). The *Pitx2a* and *Pitx2b* mRNA isoforms are expressed in the anterior pituitary cells of the thyrotrope, somatotrope, lactotrope and gonadotrope lineages, but not corticotropes (Gage and Camper, 1997). The *Pitx2c* isoform is expressed in all five anterior pituitary cell lineages, including corticotropes (Fig. 2B). Examination of –/– embryos revealed a committed pouch at e10.5, indicating that *Pitx2* is not required for the initial stages of pituitary development (Fig. 5A,B). However, the mesenchymal layer surrounding the pouch was clearly hypercellular at this time and comparison of serial sagittal sections spanning the midline from +/+ and –/– littermates at e10.5 demonstrated that the pouch was smaller. Arrest of growth and differentiation is clearly evident in –/– pouches by e12.5. The wild-type pouch wall is thicker and cell proliferation on the ventral side of the pouch has formed the nascent anterior lobe. In contrast, the ventral and dorsal aspects of the –/– pouch wall remain relatively thin (Fig. 5D). This indicates that *Pitx2* is required for pituitary gland development shortly after formation of the committed pouch. No changes were noted in the nascent posterior pituitary gland. Pituitary glands from neo/neo embryos were morphologically normal at e16.5 and e18.5.

Hesx1 (*Rpx*) and *Lhx3* are homeobox genes that are required early during early pituitary development (Hermesz et al., 1996; Sheng et al., 1996). *Pitx2* is expressed before *Hesx1* and *Lhx3* during pouch development, making them candidates for downstream targets of *Pitx2*. *Hesx1* expression was detectable throughout the pouch in +/+ embryos at e12.5, but no transcripts were evident in –/– littermates (Fig. 5G,H). This indicated that *Pitx2* is a regulator of *Hesx1* expression during pituitary development. In contrast, similar levels of *Lhx3* expression were detected in both +/+ and –/– pouches, demonstrating that *Lhx3* expression is not dependent on *Pitx2* (Fig. 5I,J). Essential inductive interactions occur between Rathke's pouch and the base of the diencephalon early during pituitary development, including induction of pro-

opiomelanocortin (*Pomc*) in the diencephalon (Diakoku et al., 1982; Sheng et al., 1996). We demonstrated that *Pomc* expression in the diencephalon of +/+ versus –/– embryos is indistinguishable, indicating that some inductive interactions are intact in –/– mice (Fig. 5K,L).

Null homozygotes have gross morphological defects

Neo/neo embryos are indistinguishable externally from wild type (Fig. 6A,B). The –/– embryos are moderately undersized relative to their heterozygous or wild-type littermates, although there were no signs of edema (Fig. 6C,D). All the mutant embryos bend severely rightward rostral to the developing hindlimb. In some embryos (~5%), the crown of the head is open over the midbrain. Other brain defects were also varied but included excess mesenchyme and abnormalities of the expansion of the pontine and midbrain flexures. Rarely, embryos lacked the left forelimb (~5%, Fig. 6J), mandibular arch, tongue and/or aspects of the caudal region.

Individuals with RGS1 have phenotypes consistent with variable defects in development of the ventral body wall. Failure of umbilical cord involution ranges in severity from a protruding navel to omphalocele (umbilical hernia) requiring surgical repair (Jorgenson et al., 1978). In wild-type mice, the thoracic and abdominal organs are normally internalized by e12.5 (Fig. 6A). Remarkably, the heart, liver and other abdominal organs are almost universally external and displaced profoundly leftward and outward in –/– embryos at e12.5 due to a large fissure in the ventral body wall (Fig. 6C,D). This fissure results from incomplete ventral closure and is generally displaced towards the left of the midline. The open ventral walls thicken as they extend outwardly and dorsally (Fig. 6L,P). They remain contiguous with the amnion, similar to their relationship during and shortly after turning of the embryo. The heart and liver are usually within thin membranes, presumably the remnants of the pericardial sac and liver capsule, respectively (Fig. 6K). Thus, the phenotype of –/– mice appears to be a more severe expression of the omphalocele characteristic of some RGS patients.

Hearts of –/– embryos are dramatically enlarged by e12.5 and continue to increase in size (Fig. 6 G,H). A septal ridge is clearly visible extending across the ventricle, indicating that septation has at least initiated. However, even as late as e12.5-13.5 only a single, hugely distended atrium can be identified. Contraction of this single atrium injects blood into both ventricles. Blood flows from the atrium into the ventricles between contractions and atrial contraction can inject blood backward into the vena cava.

Requirement of Pitx2 for asymmetry of lung

Several organs of the body exhibit left-right asymmetry, including the lungs, heart and stomach. The heart begins as a symmetrical, straight tube and initates asymmetry by looping to the right in an ‘S-shaped’ structure. The atria and inflow tracts at the caudal end of the tube move dorsally and anteriorly behind the ventricular regions. Right looping is initated properly in both neo/neo and –/– embryos and the asymmetry appears grossly normal at later stages (Fig. 7A-H). In rodents, the left lung is a single lobe but the right lung consists of four lobes. Both neo/neo and –/– embryos exhibit right-isomerization of the lungs (Fig. 7I-L). Both the right and left lungs were mirror images with multiple lobes normally characteristic of the right lung. The stomach was positioned on the left, as expected, in both neo/neo and –/– mutants (data not shown). Positioning of the tail and umbilicus over the right flank indicated that mutant embryos turn in the normal direction (data not shown). However, the orientation of the hindlimbs is sometimes in the opposite direction to the forelimbs, suggesting that the process of turning is not always complete.

Partial and complete loss of *Pitx2* differentially affect heart development

The extremely abnormal heart morphology indicated that –/– embryos probably died of heart defects. A massive proliferation of mesoderm cells has displaced the mutant heart ventrally and leftward at e12.5 which, combined with the deficit in ventral body wall closure, results in its grossly external position (Fig. 8A,D). There is a single, distended atrium located to the left of the ventricles (Fig. 8E). Ventricular septation in –/– hearts is dysmorphic and there is little evidence of atrial septation. In contrast, +/+ hearts have distinct left and right atria developing on either side of the ventricles and ventricular septation is complete (Fig. 8B). Both the leaflets of the vena cava and atrial-ventricular (A-V) valve formation are defective in –/– embryos (Fig. 8E,F). In the case of the A-V valve, this appears to result from a reduction in the membranous component of the valve. Although the hearts of neo/neo animals were located within the thoracic cavity (Fig. 8I), they exhibited ventricular septation defects possibly resulting from a reduction in the membranous component (Fig. 8J). The structural defects of the heart are likely to account for the death of both the neo/neo and –/– embyros.

Malpositioning of the heart is evident in homozygotes for both alleles. The position of the hearts in neo/neo embryos was biased toward the right and the ventricular septum appeared reversed relative to wild-type hearts (compare Fig. 8I,J with 8G,H). The isomerization of the lungs results in a larger than normal left lung. This may contribute to the apparent shift in position of the heart, as heart looping initiated properly. While the heart was placed on the left in –/– embryos, its orientation was off by approximately 90°. The size of the left lung and the mass of mesoderm cells on the left could contribute to this rotation.

DISCUSSION

An allelic series ranging from partial to complete loss of function can be especially valuable in assessing gene function (Greco et al., 1996). The embryonic lethality frequently resulting from complete loss-of-function mutations in homeobox genes makes the availability of milder alleles highly desirable and relevant as models for human birth defects. We have generated an allelic series for the murine *Pitx2* gene (Table 2). Two alleles differ in severity and the floxed allele has the potential for tissue-specific and temporally induced disruption of Pitx2 function. The constellation of phenotypes produced in heterozygotes indicate that these mice are a model for Rieger syndrome, type I. *Pitx2* is essential for many aspects of normal development, with striking roles in eye, pituitary

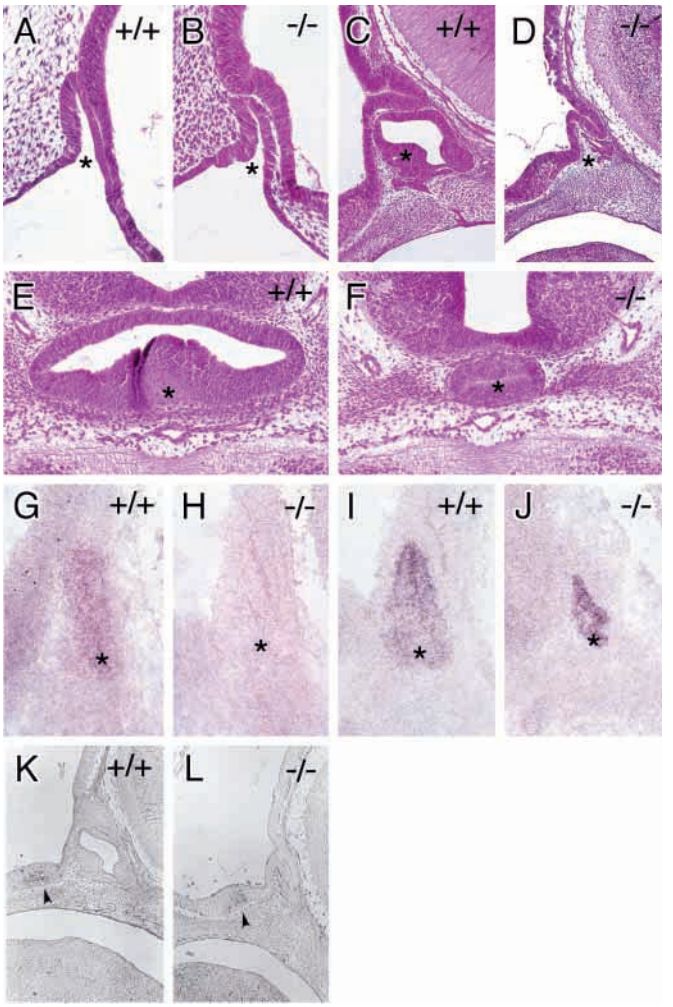

Fig. 5. Lack of *Pitx2* affects pituitary gland development. (A,B) Midline sagittal comparison of +/+ and –/– Rathke's pouch (*) at e10.5. Note the hypercellularity of the mesenchymal cells surrounding the mutant pouch. (C-F) Midline sagittal (C,D) and transverse (E,F) comparison of +/+ and –/– Rathke's pouch at e12.5. In the mutant, dorsal and ventral walls are threadlike and there is little evidence of cell proliferation at the ventral side. Lack of lateral development is clear in transverse sections (F). (G-L) In situ hybridization of midline sagittal sections with probes for *Hesx1* (G,H), *Lhx3* (I,J), and immunohistochemical staining with *Pomc* antibodies (K,L) in normal (G,I,K) and –/– pituitaries (H,J,L).

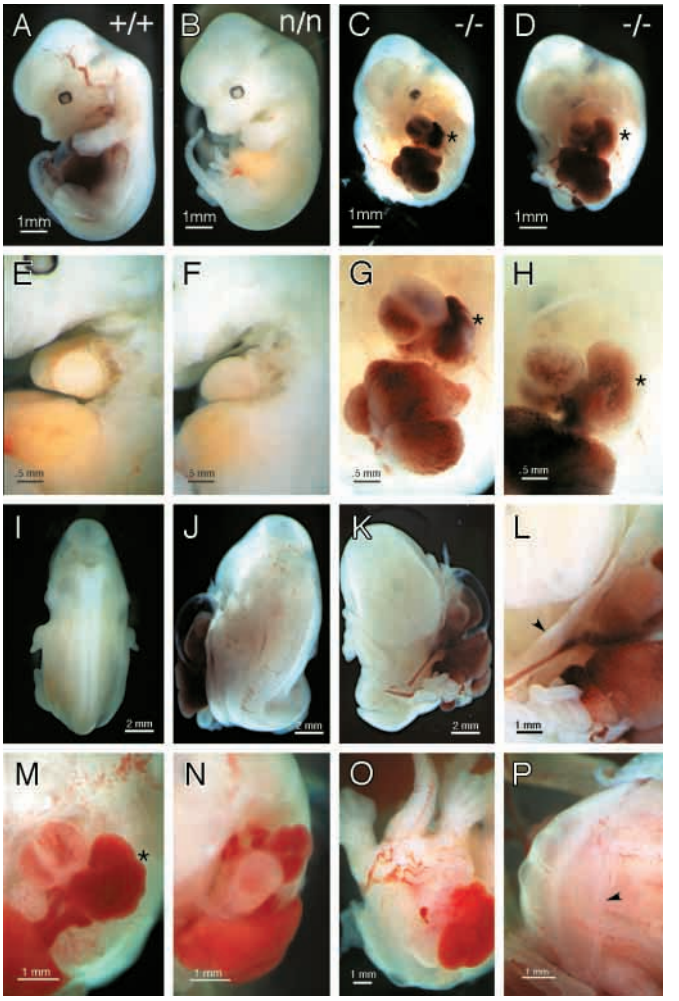

Fig. 6. Gross phenotypes of *Pitx2*^{neo} and *Pitx2*^{null} homozygotes. (A-D) Left lateral aspects of +/+ (A), neo/neo (B) and –/– (C,D) embryos at e12.5. Note reduced body size and externalization of ventral organs in –/– embryos. (E-H) Magnified left lateral flanks from embryos in A-D. Heart of *Pitx2*$^{null/null}$ embryos are external (ectopia cordis), as are the liver and other developing gut organs (gastroschisis). Note the distended, single atrium in both mutants (asterisk). Chest wall, and left forelimb and shoulder of +/+ and *neo/neo* embryos were dissected away for visualization. (I,J) Dorsal aspects of embryos in A and D. A-P axis of –/– mice bend severely right rostral to the hindlimbs. Note the extreme lateral placement of the heart and liver, and the absence of a left forelimb. (K,L,P) Low and high magnification ventral views. (L,P) Edge of ventral wall (arrowhead) was contiguous with the amnion prior to resection in this view of the right flank from the dorsal side. (M,N) Heart and liver of e13.5 –/– embryo with intact amnion. (O) Caudal view of e13.5 embryo.

gland, heart and lung ontogeny, including developing functional heart valves and establishing the asymmetry of the lungs.

The congenital eye defects of RGS patients indicate an essential role for *PITX2* in development of the anterior segment of the eye. In –/– embryos, the anterior segment is abnormally thick, due to an increase in both the epithelial and mesenchymal components of the cornea. It is striking that both layers are affected since *Pitx2* is not expressed in the corneal epithelium (Semina et al., 1996). RGS patients exhibit corneal opacity. Therefore, the changes observed in –/– mice are consistent with the defects seen in RGS patients. The two posterior eye defects, lack of ocular muscles and optic nerve coloboma, were unexpected because RGS involves anterior segment abnormalities. However, the majority of RGS patients develop early onset glaucoma, a progressive loss of retinal ganglion cells and degeneration of the optic nerve head (Chang et al., 1999). The optic fissure defects in –/– embryos suggest that subtle optic nerve defects in heterozygotes might result in glaucoma as the animals age, providing another parallel with RGS patients.

Only a small fraction of adult +/neo or +/– mice exhibited anterior chamber defects of the eye, consistent with the

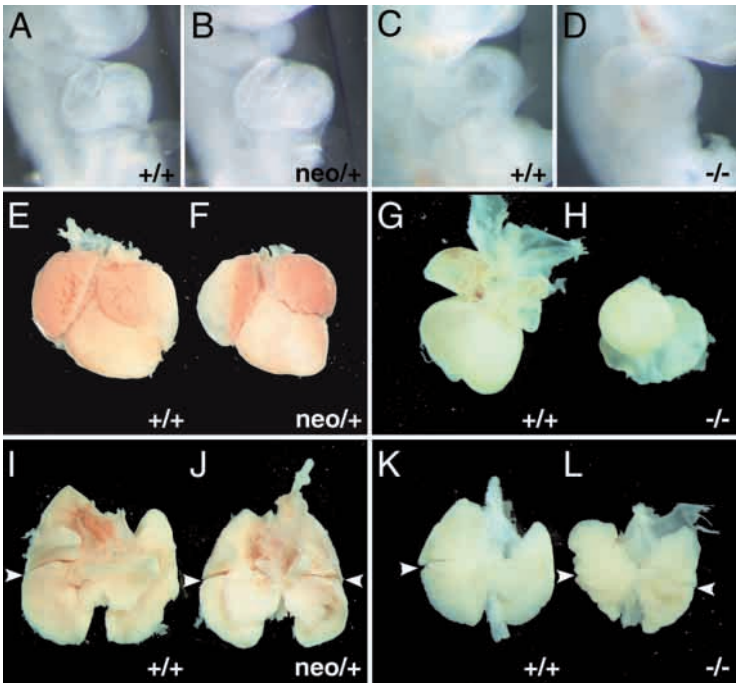

Fig. 7. Pitx2 deficiency causes right isomerization of the lungs.(A-D) Heart tubes loop rightward in neo/neo (B) and –/– (D) embryos at e9.5, similar to normal littermates (A, C). (E,F) Hearts dissected from neo/neo embryos at e16.5 (F) have asymmetric morphology similar to normal littermates (E). (G,H) Hearts from –/– embryos at e14.5 (H) are severely dysmorphic . (I-L) The lungs of neo/neo and –/– embryos are right-isomerized. Note the separation between the median and posteror lobes of the lungs (arrowhead). Lungs of normal e16.5 (I) and e14.5 (K) embryos and neo/neo (J) and –/– embryos (L) at the same time points.

remarkable variability in RGS and septo-optic dysplasia (*Hesx1* MIM #182230) (Dattani et al., 1998). In contrast to the variability in heterozygotes, profound disturbances in eye development were observed consistently in the absence of *Pitx2*. The dosage sensitivity of Pitx2 may contribute to the variability of the phenotype. Inbred strains of mice and strains with sensitizing mutations in *Pax6*, *Chx10* and *Trp1* have been used to demonstrate the effects of genetic background on eye development and glaucoma (Burmeister et al., 1996; Chang et al., 1999; Glaser et al., 1999). Modifiers of PITX2 may be responsible for unlinked cases of RGS in addition to other eye abnormalities and diseases such as cataracts and glaucoma. Thus, the *Pitx2* mutants we have generated could be valuable for identifying other genes important for eye development and disease.

A genetic hierarchy of homeobox genes, including *Hesx1*, *Lhx3*, *Prop1* and *Pit1*, is essential for pituitary gland development. (Dattani et al., 1998; Gage et al., 1996; Sheng et al., 1996; Sornson et al., 1996). Loss of *Hesx1*, *Lhx3* and *Prop1* results in dysmorphology of the pituitary primordium. *Pit1* is required later during pituitary development for specification of three anterior pituitary cell types (Camper et al., 1990; Li et al., 1990). Molecular genetic analyses suggest that some of the genes act sequentially in pituitary development: *Hesx1*→*Prop1*→*Pit1* (Anderson et al., 1995; Gage et al., 1996; Sornson et al., 1996). Our demonstration of altered *Hesx1* expression in –/– mice places Pitx2 early in this pathway. *Lhx3* and *Pitx2* affect pouch development

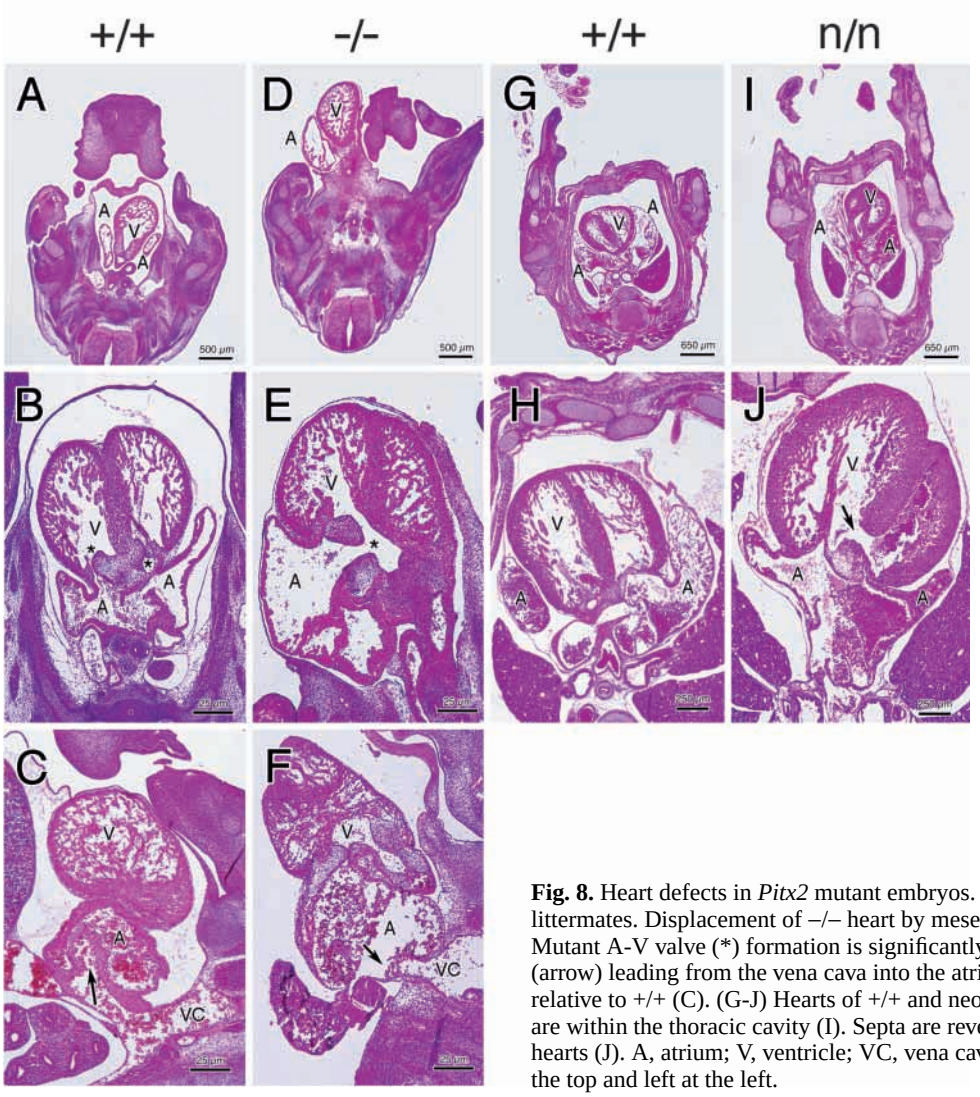

Fig. 8. Heart defects in *Pitx2* mutant embryos. (A-F) Hearts of e12.5 +/+ and –/– littermates. Displacement of –/– heart by mesenchymal proliferation is evident (A,D). Mutant A-V valve (*) formation is significantly reduced relative to +/+ (B,E). Leaflets (arrow) leading from the vena cava into the atrium are reduced in –/– embryos (F) relative to +/+ (C). (G-J) Hearts of +/+ and neo/neo littermates at e16.5. Mutant hearts are within the thoracic cavity (I). Septa are reversed and incomplete (arrow) in neo/neo hearts (J). A, atrium; V, ventricle; VC, vena cava. All panels are oriented with ventral at the top and left at the left.

similarly and both genes are required for *Hesx1* expression. *Lhx3* expression does not require *Pitx2*. Thus, *Pitx2* is an early regulator of pituitary ontogeny that acts with *Hesx1* and *Lhx3*, prior to the action of *Prop1* and *Pit1* to advance development of the committed Rathke pouch.

The expression patterns of *Pitx1* and *Pitx2* overlap in the developing pituitary and they have nearly identical homeodomains, suggesting functional redundancy (Lamonerie et al., 1996; Lanctot et al., 1997; Muccielli et al., 1996; Szeto et al., 1996; Tremblay et al., 1998). *Pitx1*$^{-/-}$ fetuses have normal pituitary morphology and only modest quantitative changes in three of five pituitary cell types (Szeto et al., 1999). In contrast, development is arrested at the committed pouch stage in *Pitx2*$^{-/-}$ fetuses. The relative roles of *Pitx1* and *Pitx2* may be similar to the relative roles of *Lhx4* and *Lhx3*. Loss of *Lhx4* alone results in a relatively mild pituitary phenotype, similar to *Pitx1* null mice (Sheng et al., 1997), but loss of *Lhx3* has an early and dramatic effect on pouch development, like *Pitx2*. Loss of both *Lhx3* and *Lhx4* affected pouch development more profoundly, indicating a partial ability of these two genes to compensate for each other (Sheng et al., 1997). Analysis of *Pitx1*, *Pitx2* double mutants will be necessary to reveal any functional overlap between these two genes.

Prior reports of asymmetric expression of *Pitx2* in early mouse development, before the onset of visceral organogenesis, suggested it might have a role in development of left-right asymmetry (Logan et al., 1998; Piedra et al., 1998; Ryan et al., 1998; Yoshioka et al., 1998). Its expression is perturbed in mouse mutants with reversals of the heart and visceral organs (Meno et al., 1998; Piedra et al., 1998; Ryan et al., 1998). Manipulation of *Pitx2* expression was sufficient to reprogram the left/right axis in chick and frog, providing further circumstantial evidence for an important role. In these experiments, *Pitx2* was expressed ectopically on the right side and an early player in the pathway was blocked (sonic hedgehog, *Shh*), preventing activation of *Pitx2* on the left. Isomerized or reversed development of the heart and gastrointestinal tract resulted. The defects in the ventral body wall that caused externalization of the viscera in –/– embryos revealed the importance of Pitx2 at the time the embryo turns. However, the right-ward looping of the heart initiated properly and the stomach was located on the left in both neo/neo and –/– fetuses, indicating that Pitx2 is not required to initiate the left-right asymmetry of the heart or the gastrointestinal tract. However, the lungs were right-isomerized, suggesting that Pitx2 is required for establishing the lobular pattern characteristic of the left lung. Thus, there is a dramatic difference between ectopic expression of Pitx2, and absent or low levels of Pitx2 in establishing the left-right asymmetry of the body axis. Ectopic expression of Pitx2 is sufficient to reprogram the left-right body axis, but it is only required for developing the asymmetry of the lungs. This suggests that there are other genes that partially compensate for the lack of Pitx2.

The defects that we observed in homozygous mutant mice clarify some questions about the Rieger syndrome phenotype. Retrospective studies have demonstrated that defects of the heart and umbilicus are more common than previously appreciated (Jorgenson et al., 1978; Mammi et al., 1998). However, the possibility existed that these features resulted from a contiguous gene syndrome. This is unlikely because reduced Pitx2 levels cause heart defects similar to those found in Rieger patients and failure of the ventral body wall to close that is consistent with omphalocele requiring surgical repair.

The concentration of Pitx2 is important for normal development. This is evident by comparing the mild eye phenotypes of adult +/– and +/neo mice with the severe eye defects in –/– embryos and by comparing the heart development of neo/neo and –/– fetuses. The sensitivity of each organ to Pitx2 levels is variable. The eye and heart appear to be more sensitive than the pituitary, for example. Pitx1 is expressed in the pituitary but not the eye or heart, thus functional redundancy may account for some of the differential sensitivity of each organ. In conjunction with tissue-specific, developmentally regulated, and/or inducible cre transgenes, the *Pitx2*^{flox} allele will allow spatial and temporal requirements of *Pitx2* function to be dissected.

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REFERENCES

Al-Shawi, R., Burke, J., Wallace, H., Jones, C., Harrison, S., Buxton, D., Maley, S., Chandley, A. and Bishop, J. O. (1991). The herpes simplex virus type 1 thymidine kinase is expressed in the testes of transgenic mice under the control of a cryptic promoter. *Mol. Cell Biol.* **11**, 4207-4216.

Amendt, B., Sutherland, L., Semina, E. and Russo, A. (1998). The molecular basis of Rieger syndrome: Analysis of Pitx2 homeodomain protein activities. *J. Biol. Chem.* **273**, 20066-20072.

Anderson, B., Pearse II, R. V., Jenne, K., Sornson, M., Lin, S.-C., Bartke, A. and Rosenfeld, M. G. (1995). The Ames dwarf gene is required for Pit-1 gene activation. *Dev. Biol.* **172**, 495-503.

Arakawa, H., Nakamura, T., Zhadanov, A. B., Fidanza, V., Yano, T., Bullrich, F., Shimizu, M., Blechman, J., Mazo, A., Canaani, E. et al. (1998). Identification and characterization of the ARP1 gene, a target for the human acute leukemia ALL1 gene. *Proc. Natl Acad. Sci. USA* **95**, 4573-4578.

Burmeister, M., Novak, J., Liang, M., Basu, S., Ploder, L., Hawes, N., Vidgen, D., Hoover, F., Goldman, D., Kalnins, V. et al. (1996). Ocular retardation mouse caused Chx10 homeobox null allele: inpaired retinal progenitor proliferation and bipolar cell differentiation. *Nat. Genet.* **12**, 376-384.

Camper, S. A., Saunders, T. L., Katz, R. W. and Reeves, R. H. (1990). The *Pit-1* transcription factor gene is a candidate for the Snell dwarf mutation. *Genomics* **8**, 586-590.

Chang, B., Smith, R., Hawes, N., Anderson, M., Zabaleta, A., Savinova, O., Roderick, T., Heckenlively, J., Davisson, M. and John, S. (1999). Interacting loci cause severe iris atrophy and glaucoma in DBA/2J mice. *Nat. Genet.* **21**, 405-409.

Copp, A. (1995). Death before birth: clues from gene knockouts and mutations. *Trends Genet.* **11**, 87-93.

Dattani, M. T., Martinez-Barbera, J.-P., Thomas, P. Q., Brickman, J. M., Gupta, R., Mårtensson, I.-L., Toresson, H., Fox, M., Wales, J. K. H., Hindmarsh, P. C. et al. (1998). Mutations in the homeobox gene *HESX1/Hesx1* associated with septo-optic dysplasia in human and mouse. *Nat. Genet.* **19**, 125-133.

Diakoku, S., Chikamori, M., Adachi, T. and Maki, Y. (1982). Effect of basal diencephalon on the development of Rathke's pouch in rats: a study in combined organ cultures. *Dev. Biol.* **90**, 198-202.

Gage, P. J., Brinkmeier, M. L., Scarlett, L. M., Knapp, L. T., Camper, S. A. and Mahon, K. A. (1996). The Ames dwarf gene, *df*, is required early in pituitary ontogeny for the extinction of *Rpx* transcription and initiation of lineage specific cell proliferation. *Mol. Endocrinol.* **10**, 1570-1581.

Gage, P. J. and Camper, S. A. (1997). Pituitary homeobox 2, a novel member of the *bicoid*-related family of homeobox genes, is a potential regulator of anterior structure formation. *Hum. Mol. Genet.* **6**, 457-464.

Gage, P. J., Suh, H. and Camper, S. A. (1999). Review: The *bicoid*-related Pitx gene family in development. *Mammalian Genome* **10**, 197-200.

Glaser, T., Tucker, P., Munson, A., Kanekar, S., Vetter, M. and Laemle, L. (1999). Analysis of an eyeless strain of mice: Identification of a Rx/rax homeobox gene mutation. *Invest. Ophthalmol. Vis. Sci.* **40**, S205.

Greco, T. L., Takada, S., Newhouse, M. M., McMahon, J. A., McMahon, A. P. and Camper, S. A. (1996). Analysis of the vestigial tail mutation demonstrates that *Wnt-3a* gene dosage regulates mouse axial development. *Genes Dev.* **10**, 313-324.

Gu, H., Marth, J. D., Orban, P. C., Mossmann, H. and Rajewsky, K. (1994). Deletion of a DNA polymerase β gene segment in T cells using cell type-specific gene targeting. *Science* **265**, 103-106.

Gu, H., Zou, Y. R. and Rajevsky, K. (1993). Independent control of immunoglobulin switch recombination at individual switch regions evidenced through Cre-loxP-mediate gene targeting. *Cell* **73**, 1155-1164.

Hermesz, E., Mackem, S. and Mahon, K. A. (1996). *Rpx:* A novel anterior-restricted homeobox gene progressively activated in the prechordal plate, anterior neural plate, and Rathke's pouch of the mouse embryo. *Development* **122**, 41-52.

Jorgenson, R., Levin, L., Cross, H., Yoder, F. and Kelly, T. (1978). The Rieger Syndrome. *Am. J. Med. Genet.* **2**, 307-318.

Kitamura, K., Miura, H., Yanazawa, M., Miyashita, T. and Kato, K. (1997). Expression patterns of Brx1 (Rieg gene), Sonic hedgehog, Nkx2.2, Dlx1 and Arx during zona limitans intrathalamica and embryonic ventral lateral geniculate nuclear formation. *Mech. Dev.* **67**, 83-96.

Lamonerie, T., Tremblay, J. J., Lanctot, C., Therrien, M., Gauthier, Y. and Drouin, J. (1996). Ptx1, a bicoid-related homeo box transcription factor involved in transcription of the pro-opiomelanocortin gene. *Genes Dev.* **10**, 1284-1295.

Lanctot, C., Lamolet, B. and Drouin, J. (1997). The bicoid-related homeoprotein Ptx1 defines the most anterior domain of the embryo and differentiates posterior from anterior lateral mesoderm. *Development* **124**, 2807-2817.

Lanctot, C., Moreau, A., Chamberland, M., Tremblay, M. L. and Drouin, J. (1999). Hindlimb patterning and mandible development require the Ptx1 gene. *Development* **126**, 1805-1810.

Li, S., Crenshaw 3rd, E. B., Rawson, E. J., Simmons, D. M., Swanson, L. W. and Rosenfeld, M. G. (1990). Dwarf locus mutants lacking three pituitary cell types result from mutations in the POU-domain gene *Pit-1*. *Nature* **347**, 528-533.

Logan, M., Pagan-Westphal, S. M., Smith, D. M., Paganessi, L. and Tabin, C. J. (1998). The transcription factor Pitx2 mediates situs-specific morphogenesis in response to left-right asymmetric signals. *Cell* **94**, 307-317.

Mammi, I., De Giorgio, P., Clementi, M. and Renconi, R. (1998). Cardiovascular anomaly in Rieger Syndrome: Heterogeneity or contiguity? *Acta Ophthalmologica Scandinavica* **76**, 509-512.

Meno, C., Shimono, A., Saijoh, Y., Yashiro, K., Mochida, K., Ohishi, S., Noji, S., Kondoh, H. and Hamada, H. (1998). *lefty-1* is required for left-right determination as a regulator of *lefty-2* and *nodal*. *Cell* **94**, 287-297.

Meyers, E., Lewandoski, M. and Martin, G. (1998). An Fgf8 mutant allelic series generated by Cre- and FLP-mediated recombination. *Nat. Genet.* **18**, 136-141.

Muccielli, M. L., Martinez, S., Pattyn, A., Goridis, C. and Brunet, J. F. (1996). Otlx2, an Otx-related homeobox gene expressed in the pituitary gland and in a restricted pattern in the forebrain. *Molec. Cell. Neurosci.* **8**, 258-271.

Nagy, A., Rossant, J., Nagy, R., Abramow-Newerly, W. and Roder, J. C. (1993). Derivation of completely cell culture-derived mice from early-passage embryonic stem cells. *Proc. Natl. Acad. Sci. USA* **90**, 8424-8428.

Orban, P. C., Chui, D. and Marth, J. D. (1992). Tissue- and site-specific DNA recombination in transgenic mice. *Proc. Natl. Acad. Sci. USA* **89**, 6861-6865.

Piedra, M. E., Icardo, J. M., Albajar, M., Rodriquez-Rey, J. C. and Ros, M. A. (1998). Pitx2 participates in the late phase of the pathway conrolling left-right asymmetry. *Cell* **94**, 319-324.

Ryan, A. K., Blumberg, B., Rodriguez-Estaban, C., Yonei-Tamura, S., Tamura, K., Tsukui, T., de al Peña, J., Sabbagh, W., Greenwald, J., Choe, S. et al. (1998). Pitx2 determines left-right asymmetry of internal organs in vertebrates. *Nature* **394**, 1998.

Sadeghi-Nedjad, A. and Senior, B. (1974). Autosomal dominant transmission of isolated growth hormone deficiency in iris-dental dysplasia (Rieger's Syndrome). *J. Pediatr.* **85**, 644-648.

Schaeren-Weimers, N. and Gerfin-Moser, A. (1993). A single protocol to detect transcripts of various types and expression levels in neural and cultured cells: in situ hybridization using digoxigenin-labeled cRNA probes. *Histochemistry* **100**, 431-440.

Semina, E. V., Ferrell, R. E., Mintz-Hittner, H. A., Bitoun, P., Alward, W. L., Reiter, R. S., Funkhauser, C., Daack-Hirsch, S. and Murray, J. C. (1998). A novel homeobox gene PITX3 is mutated in families with autosomal- dominant cataracts and ASMD. *Nat. Genet.* **19**, 167-170.

Semina, E. V., Reiter, R., Leysens, N. J., Alward, W. L., Small, K. W., Datson, N. A., Siegel-Bartelt, J., Bierke-Nelson, D., Bitoun, P., Zabel, B. U. et al. (1996). Cloning and characterization of a novel bicoid-related homeobox transcription factor gene, RIEG, involved in Rieger syndrome. *Nat. Genet.* **14**, 392-399.

Sheng, H., Moriyama, K., Yamashita, T., Li, H., Potter, S., Mahon, K. and Westphal, H. (1997). Multistep control of pituitary organogenesis. *Science* **278**, 1809-1812.

Sheng, H. Z., Zhadanov, A. B., Mosinger, B. J., Fujii, T., Bertuzzi, S., Grinberg, A., Lee, E. J., Huang, S. P., Mahon, K. A. and Westphal, H. (1996). Specification of pituitary cell lineages by the LIM homeobox gene Lhx3. *Science* **272**, 1004-1007.

Sornson, M. W., Wu, W., Dasen, J. S., Flynn, S. E., Norman, D. J., O'Connell, S. M., Gukovsky, I., Carrière, C., Ryan, A. K., Miller, A. P. et al. (1996). Pituitary lineage determination by the *Prophet* of *Pit-1* homeodomain factor defective in Ames dwarfism. *Nature* **384**, 327-333.

Sternberg, N. L. (1992). Cloning high molecular weight DNA fragments by the bacteriophage P1 system. *Trends Genet.* **8**, 11-16.

Szeto, D., Ryan, A., O'Connell, S. and Rosenfeld, M. (1996). P-OTX: A PIT-1-interacting homeodomain factor expressed during anterior pituitary gland development. *Proc. Natl. Acad. Sci. USA* **93**, 7706-7710.

Szeto, D. P., Rodriquez-Estaban, C., Ryan, A. K., O'Connell, S. M., Liu, F., C., K., Gleiberman, A. S., Izpisua-Belmonte, J. C. and Rosenfeld, M. G. (1999). Role of Bicoid-related homeodomain factor Pitx1 in specifying hindlimb morphogenesis and pituitary development. *Genes Dev.* **13**, 484-494.

Tremblay, J. J., Lanctôt, C. and Drouin, J. (1998). The pan-Pituitary Activator of Transcription, Ptx1 (Pituitary Homeobox 1), Acts in Synergy with SF-1 and Pit1 and Is an Upstream Regulator of the Lim-Homeodomain gene Lim3/Lhx3. *Mol. Endocrinol.* **12**, 428-441.

Vorbruggen, G., Constien, R., Zilian, O., Wimmer, E. A., Dowe, G., Taubert, H., Noll, M. and Jackle, H. (1997). Embryonic expression and characterization of a Ptx1 homolog in Drosophila. *Mech. Dev.* **68**, 139-147.

Yoshioka, H., Meno, C., Koshiba, K., Sugihara, M., Itoh, H., Ishimaru, Y., Inoue, T., Ohuchi, H., Semina, E. V., Murray, J. C. et al. (1998). *Pitx2*, a bicoid-type homeobox gene, is involved in a lefty signaling pathway in determination of left-right asymmetry. *Cell* **94**, 299-305.

Zhadanov, A., Bertuzzi, S., Taira, M., Dawid, I. and Westphal, H. (1995). Expression pattern of the murine LIM class homeobox gene Lhx3 in subsets of neural and neuroendocrine tissues. *Dev. Dyn.* **202**, 354-364.