Development 129, 329-337 (2002)
Printed in Great Britain © The Company of Biologists Limited 2002
DEV3558

Pitx2 is required at multiple stages of pituitary organogenesis: pituitary primordium formation and cell specification

Hoonkyo Suh[1], Philip J. Gage[2], Jacques Drouin[3] and Sally A. Camper[1,2,*]

[1]Neuroscience Program and [2]Department of Human Genetics, University of Michigan, Ann Arbor, MI 48109-0638, USA
[3]Institut de Recherches Cliniques de Montreal, Montréal Québec, Canada
*Author for correspondence (e-mail: scamper@umich.edu)

Accepted 23 October 2001

SUMMARY

Analysis of an allelic series in mice revealed that the *Pitx2* homeobox gene is required at multiple stages of pituitary development. It is necessary for initiating expansion of Rathke's pouch and maintaining expression of the fetal-specific transcription factors *Hesx1* and *Prop1*. At later stages *Pitx2* is necessary for specification and expansion of the gonadotropes and *Pit1* lineage within the ventral and caudomedial anterior pituitary. Mechanistically, this is due to the dependence of several critical lineage-specific transcription factors, *Pit1*, *Gata2*, *Egr1* and *Sf1*, on a threshold level of PITX2. The related *Pitx1* gene has a role in hormone gene transcription, and it is important late in ontogeny for the final expansion of the differentiated cell types. *Pitx1* and *Pitx2* have overlapping functions in the expansion of Rathke's pouch, revealing the sensitivity of pituitary organogenesis to the dosage of the PITX family. The model developed for PITX gene function in pituitary development provides a better understanding of the etiology of Rieger syndrome and may extend to other PITX-sensitive developmental processes.

Key words: *Pitx2*, *Pitx1*, *Gata2*, Allelic series, Gene dosage, Cell specification, Mouse

INTRODUCTION

The mammalian *Pitx* gene family consists of three bicoid-related homeobox genes, each with an important role in the development of multiple organs (Gage et al., 1999b). Mice deficient in *Pitx1* display severe defects in hindlimb development and cleft palate formation with additional mild pituitary phenotypes (Lanctot et al., 1999b; Szeto et al., 1999). *Pitx3* deficiency results in microphathalmia, and agenesis of the lens and anterior segment structures in aphakia (*ak*) mice (Semina et al., 2000). Mutations in *PITX2* are one cause of Rieger syndrome (RGS) in humans, a phenotypically and genetically heterogeneous, dominant disorder. RGS is characterized by eye, tooth and umbilical abnormalities, and occasionally heart defects (Semina et al., 1996). Both dominant and loss of function mutations have been found in *PITX2*, providing evidence that haploinsufficiency is one of the underlying mechanisms for RGS (Amendt et al., 1998; Flomen et al., 1997; Kozlowski and Walter, 2000; Priston et al., 2001).

The phenotype of mice with reduced *Pitx2* function mimics Rieger syndrome. Mice heterozygous for a null (*Pitx2*$^{-}$) allele have a low frequency of eye and tooth abnormalities, consistent with RGS. This is suggestive of semi-dominant inheritance with very low penetrance (Gage et al., 1999a). Null homozygotes exhibit severe defects in the same organs that are mildly affected in Rieger patients (Gage et al., 1999a; Kitamura et al., 1999; Lin et al., 1999; Lu et al., 1999). Failure of ventral body wall closure in mutant mice correlates with the umbilical hernia observed in humans (Jorgenson et al., 1978). Development of the heart, eyes and teeth is also profoundly disrupted in the mutant mice, and they die by embryonic day (E) 14.5. These features correspond to the heart defects, abnormalities in the anterior chamber of the eye, and missing or misplaced teeth in Rieger patients. Analysis of *Pitx2* null mice established the critical role of PITX2 in the development of craniofacial structures, eyes, teeth and multiple organs including the pituitary, heart and lungs (Gage et al., 1999a; Kitamura et al., 1999; Lin et al., 1999; Lu et al., 1999).

Both *Pitx1* and *Pitx2* are expressed at E8.5 in the stomodeal ectoderm or oral plate, which gives rise to Rathke's pouch and, ultimately, the glandular part of the pituitary gland. Neither gene is expressed in the adjacent ventral diencephalon, which forms the infundibular process and neural portion of the pituitary. *Pitx1* and *Pitx2* are expressed uniformly and constitutively in the prospective anterior and intermediate lobes of the developing pituitary during the two major waves of cell proliferation and throughout the time that hormone-specific expression ensues. In addition, *Pitx1* and *Pitx2* transcripts are apparently present in all five adult anterior pituitary cell types (Gage and Camper, 1997; Tremblay et al., 1998). Persistent expression of *Pitx1* and *Pitx2* contrasts with the fetal specificity of the critical transcription factors *Hesx1* (also known as *Rpx*), *Prop1* and *Lhx4* (Hermesz et al., 1996; Li et al., 1996; Sornson et al., 1996). The uniform distribution of *Pitx* transcripts in the pituitary primordium contrasts with the stratified patterns of expression that characterize transcription factors implicated in

lineage determination such as *Gata2*, steroidogenic factor 1 (*Sf1* or *Nr5a1*), *Egr1* and *Pit1* (Dasen et al., 1999; Ingraham et al., 1994; Simmons et al., 1990; Topilko et al., 1998). These features of *Pitx* expression and the reduced growth hormone levels of some Rieger patients (Sadeghi-Nedjad and Senior, 1974) led to the hypothesis that PITX2 has a role in specification of multiple cell types and function in the pituitary gland.

Rathke's pouch formation occurs normally in *Pitx1*$^{-/-}$ mice, suggesting that *Pitx1* is not essential for the initial steps in pituitary development, or that the loss of *Pitx1* function is compensated by *Pitx2*. *Pitx2*$^{-/-}$ mutant embryos have a small Rathke's pouch in early development that fails to undergo further expansion (Gage et al., 1999a). This suggests that PITX2 is required early in the cascade of transcription factors that influence pituitary development. The role of PITX2 in cell fate decisions or specialized cell function could not be determined because of the absolute requirement for PITX2 in earlier stages of pituitary development.

We produced a hypomorphic (reduced function, *Pitx2*^{neo}) allele of *Pitx2* (Gage et al., 1999a). Mice homozygotes for this allele live until postnatal day 1 (P1), much longer than null homozygotes. This makes it feasible to analyze the effect of reduced *Pitx2* on all aspects of pituitary development and cell specification in homozygotes for the reduced function allele, *Pitx2*^{neo}. The results provide evidence that the dosage of PITX2 is critical for expansion of the pituitary primordium and the somatotrope and thyrotrope lineages, as well as for activation of transcription factors required for gonadotropes, the cells that make luteinizing hormone (LH) and follicle stimulating hormone (FSH). In addition, we reveal that pituitary development relies not only *Pitx2* gene dosage but also requires the combined gene dosage of *Pitx1* and *Pitx2*. Mechanisms similar to the one we present for *Pitx2* haploinsufficiency in pituitary development are likely to underlie the dosage requirement of other organs for *Pitx2* and could be pertinent to understanding the mechanism of other haploinsufficiency disorders.

MATERIALS AND METHODS

Animal husbandry

Mice carrying the *Pitx2*^{neo}, *Pitx2*$^{-}$ and *Pitx1*$^{-}$ alleles were generated by gene targeting (Gage et al., 1999a; Lanctot et al., 1999b), and bred at the University of Michigan. All animals were maintained according to the NIH guidelines for animal care. Genotypes were determined by polymerase chain reaction (PCR) amplification of genomic DNA from tail biopsies or yolk sacs (Gage et al., 1999a).

Histology, in situ hybridization and immunohistochemistry

Timed pregnancies were produced using sexually mature females. The morning after mating was designated as E0.5. Collected embryos were frozen and embedded in OCT (Sakura) and sectioned for in situ hybridization, or fixed for 2-4 hours in 4% paraformaldehyde in phosphate-buffered saline (PBS) at room temperature, embedded in paraffin and sectioned for morphology and immunohistochemistry as described (Cushman et al., 2001). In situ hybridization and immunohistochemistry for hormone genes and transcription factors were carried out according to standard methods (Cushman et al., 2001; Gage et al., 1996b).

A plasmid containing mouse *Gata2* (*mGata2*) genomic sequences in pGEM13 was provided by Dr David Gordon (University of Colorado, Health Science Center, Denver, CO, USA). This plasmid was linearized by digestion with *Bam*HI to generate an antisense probe spanning 365 base pairs (bp) of C-terminal coding sequences and 1.5 kb of 3′ untranslated region (3′UTR). 700 bp mouse *Egr1* and 923 bp *Pomc* cDNAs provided by Dr Vikas Sukhatme (Harvard Medical Center, Boston, MA, USA) and Dr Michael Uhler (University of Michigan, Ann Arbor, MI, USA), respectively, were subcloned into pBLUESCRIPT SK (+) (pSK+, Stratagene), and digested with *Hin*dIII to make antisense probes. *Pit1* cDNA in pKS (–) was linearized with *Hin*dIII digestion and used for 672 bp antisense probe generation. All riboprobes used in these experiments were generated and labeled with digoxigenin (Roche Molecular Biochemicals), and some slides were counterstained with nuclear Methyl Green following the manufacturer's instructions (Vectastain).

Antisera against SF1 and PROP1 were kindly provided by Drs Ken-Ichirou Morohashi (National Institute for Basic Biology, Okazaki, Japan), and Aimee Ryan (Montreal Children's Hospital Research Institute, Montreal, Canada), respectively. SF1 immunostaining was performed on frozen sections with 1:1500 dilution as described previously (Cushman et al., 2001). The LHX3 monoclonal antibody developed by Thomas Jessell (Columbia University, New York, NY, USA) was obtained from the Developmental Studies Hybridoma bank developed under the auspices of the NICHD and maintained by The University of Iowa. PITX2 antibody was provided by Dr Tord Hjalt (University of Iowa, Iowa City, IA, USA). Epitopes were retrieved by boiling paraffin-embedded sections in 10 mM citrate for 10 minutes. Sections were incubated in biotinylated secondary antibodies (Jackson Immuno Research), and signals were amplified by using TSA tetramethylrhodamine kit (NEN) or MOM kit (Vector Laboratories)

Transient cell transfection assays

All expression constructs were made in pCGN2 plasmid provided by Dr David Gordon. Open reading frames (ORFs) of *Pitx2a*, *Pitx2b* and *Pitx2c* were amplified with *PWO* polymerase (Roche Molecular Chemicals) and ligated into *Hin*dIII and *Bam*HI sites of pCGN2. *Pitx1* and *Egr1* cDNAs were generated by reverse transcriptase (RT)-PCR from AtT-20 pituitary corticotrope-derived cells (G7 subclone of AtT-20 cells provided by Dr Audrey Seasholtz, Ann Arbor, MI, USA), and cloned into pCGN2 expression vector. All clones were confirmed by DNA sequencing. Genomic DNA containing mouse *Gata2* regulatory elements was a gift from Dr Masayuki Yamamoto (University of Tsukuba, Tsukuba, Japan) and Dr Stuart Orkin (Harvard Medical School, Boston, MA, USA). *Not*I-*Nco*I fragments containing approximately –7 kb to +216 bp upstream of *Gata2* were ligated into luciferase reporter plasmid, pGL3 (Promega) (Minegishi et al., 1998). Transient cell transfection assays were performed in cell lines representing pregonadotropes, αT3-1 (kindly provided by Dr Pamela Mellon, University of California at San Diego, La Jolla, CA, USA) (Alarid et al., 1996), and heterologous cells, CV-1. Briefly, 3×10^5 cells were plated in 60 mm plates 1 day before transfection. 48 hours after Fugene 6-mediated transfection (Roche Molecular Chemicals), cells were harvested for the measurement of luciferase reporter gene activity (Promega). *CMV-βgal* construct (Clontech) was cotransfected to normalize the transfection efficiency. 1 μg each of *Gata2* luciferase construct, PITX2 expression vector and *CMV-βgal* construct were transfected.

RESULTS

Pitx2 affects pituitary expansion in a dosage sensitive manner

We utilized three *Pitx2* mutant genotypes to assess the effect of varied *Pitx2* gene dosage on pituitary gland development: *Pitx2* null (*Pitx2*$^{-/-}$), *Pitx2* compound heterozygote (*Pitx2*$^{neo/-}$)

and *Pitx2* hypomorph (*Pitx2*$^{neo/neo}$) (Fig. 1A-C). The *Pitx2*^{neo} allele was generated by the standard method of inserting a neomycin resistance cassette (neo) with splicing and polyadenylation signals into an intron (Meyers et al., 1998; Meyers and Martin, 1999; Nagy et al., 1998). This allelic series of *Pitx2* mutants is a valuable resource for assessing gene function because each genotype represents a different level of gene expression (Gage et al., 1999a).

We examined the effect of reduced *Pitx2* dosage on pituitary gland morphology at E12.5, a stage at which all genotypes are viable (Fig. 2A). Midsagittal sections of embryos were stained with Hematoxylin and Eosin (Fig. 2E-H). Both PITX1 and PITX2 are expressed in the anterior lobe and intermediate lobe, but not in the posterior lobe at this time point (Lanctot et al., 1999a) (Fig. 2B-D). Although little difference is observed in the pituitary gland morphology of the prospective anterior lobe in wild-type mice and *Pitx2*$^{neo/neo}$ mutants (Fig. 2E,F), *Pitx2*$^{-/-}$ embryos exhibit profound anterior pituitary hypoplasia (Fig. 2H). An intermediate reduction in the size of Rathke's pouch was observed in the compound heterozygotes (*Pitx2*$^{neo/-}$; Fig. 2G). The reduction in the size of Rathke's pouch parallels the decrease in *Pitx2* expression, demonstrating the dosage sensitivity of the pituitary gland to PITX2. In contrast, the infundibulum, or prospective posterior lobe, appears to develop normally in all *Pitx2* genotypes. This is consistent with the lack of PITX2 expression in this neural ectoderm-derived structure (Fig. 2C).

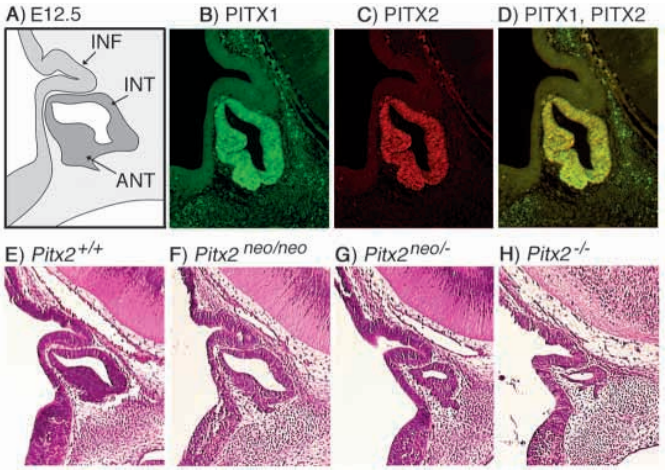

Fig. 2. Pituitary organ size diminishes with reduced PITX2 dosage. (A) Diagram of pituitary morphology. INF, infundibulum, the prospective posterior lobe; INT, intermediate lobe; ANT, anterior lobe. Immunohistochemical examination shows that the expression of PITX1 (B) and PITX2 (C) overlaps in the anterior lobe and intermediate lobe, but not in the posterior lobe (D). Histological examination of E12.5 embryo sagittal sections of *Pitx2* wild type (E), *Pitx2*$^{neo/neo}$ (F), *Pitx2*$^{neo/-}$ (G) and *Pitx2*$^{-/-}$ (H) shows that the size of Rathke's pouch decreases as the *Pitx2* level is reduced. Note that the *Pitx2*$^{neo/-}$ embryo has an intermediate pouch size.

Transcription of hormone genes is altered in *Pitx2* hypomorphs

The five major cell types in the anterior pituitary gland are defined by the hormones they produce. We investigated the role of *Pitx2* in the specification of each cell type by assessing the expression of their respective hormone genes by in situ hybridization. P1 embryos from *Pitx2*$^{neo/neo}$ mice were examined because the five pituitary cell types are fully differentiated by this time point. The most profound effect of reduced *Pitx2* dosage was observed in the ventral-most cell type, the gonadotropes. Both *Lhb* and *Fshb* transcripts are nearly absent in *Pitx2*^{neo} homozygotes (Fig. 3A-D). Gonadotropin releasing hormone receptor (*Gnrhr*) is another gonadotrope marker. This receptor is required for the full expansion of this cell type and is normally detected in the ventral pituitary where gonadotropes form (Fig. 3E). However, *Gnrhr* expression is nearly abolished in the *Pitx2*^{neo} homozygotes (Fig. 3F). The deficiency of these three differentiated gonadotrope markers suggests that gonadotrope development is especially sensitive to reduction in PITX2.

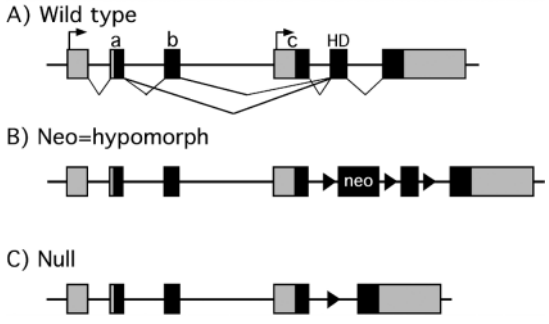

Fig. 1. Three *Pitx2* alleles. (A) Genomic organization of the *Pitx2* locus. Among three expressed *Pitx2* cDNA isoforms, *Pitx2a* and *Pitx2b* arise by alternative splicing and *Pitx2c* utilizes an alternative promoter. Arrows above exons indicate transcriptional initiation sites. (B) *Pitx2*^{neo} is a hypomorphic allele containing a neo resistance gene within the intronic site of *Pitx2*. (C) *Pitx2* null allele results from the excision of exon 4, which encodes most homeodomain sequences. Black boxes, coding sequences; gray boxes, non-coding sequences; a, *Pitx2a*-specific exon; b, *Pitx2b*-specific exon; c, transcriptional start site of *Pitx2c* isoform; HD, homeodomain.

Both the somatotropes (GH producing cells) and thyrotropes (TSH producing cells) appear in the caudomedial aspect of the anterior lobe and are dependent upon the transcription factor PIT1 (Simmons et al., 1990). The pattern and intensity of *Gh* and thyroid stimulating hormone beta-subunit, *Tshb*, hybridization is moderately reduced in *Pitx2*^{neo} homozygotes compared to normal mice, suggesting that there are fewer fully differentiated somatotropes and thyrotropes (Fig. 3G,H,K,L). Growth hormone releasing hormone receptor, *Ghrhr*, is required for the GH secretion and expansion of the somatotrope population (Jansson et al., 1986). Its expression is also reduced in *Pitx2*$^{neo/neo}$ mutants, consistent with the apparent reduction in the number of somatotropes (Fig. 3I,J). This observation is intriguing in light of the short stature and reduced growth hormone secretion in some RGS patients.

The first complete hormone to be produced in anterior pituitary is adrenocorticotropin (ACTH), which is cleaved from the pro-opiomelanocortin (POMC) precursor protein in corticotropes. *Pomc* transcripts appear in both the anterior pituitary and the intermediate lobe. Neither the pattern nor the intensity of *Pomc* hybridization is altered substantially at E18-P1 in *Pitx2*^{neo} homozygotes relative to wild type (Fig. 3M,N). This, together with the reduction in thyrotropes and somatotropes and the absence of gonadotropes, indicates that

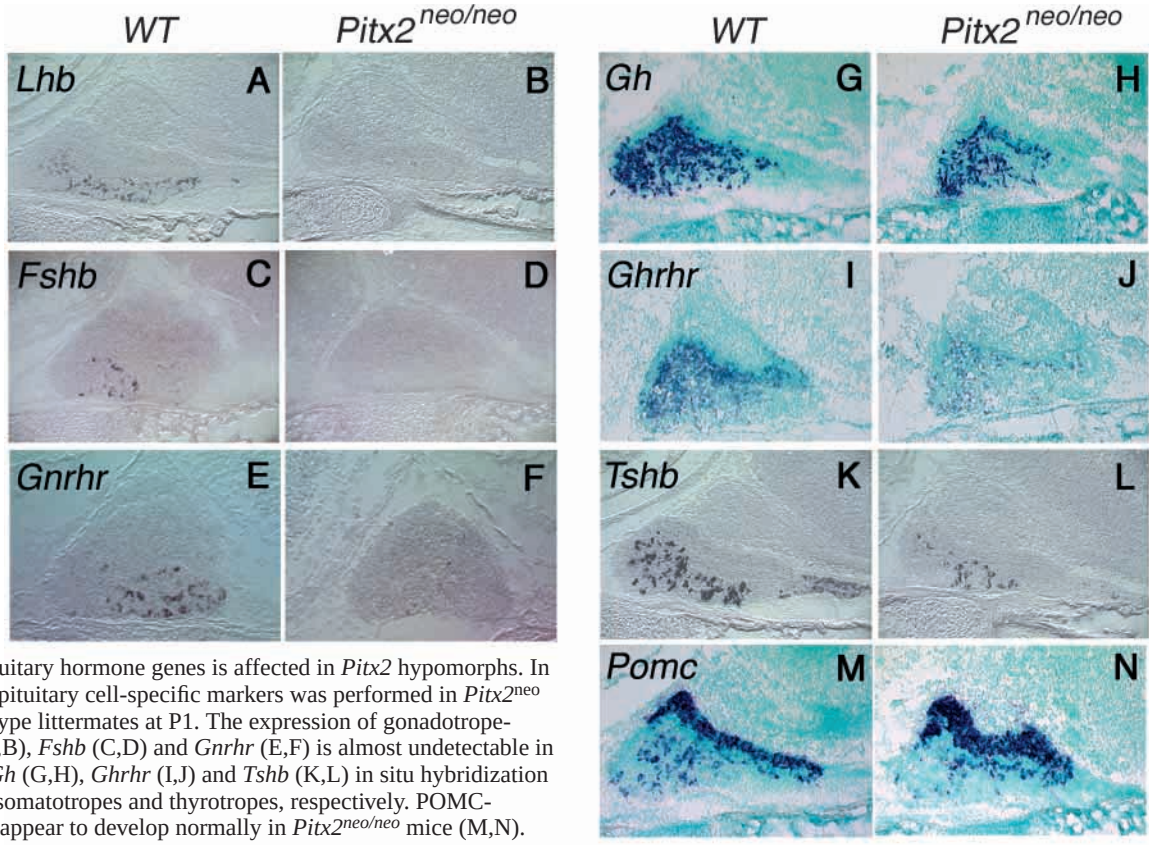

Fig. 3. Expression of pituitary hormone genes is affected in *Pitx2* hypomorphs. In situ hybridization using pituitary cell-specific markers was performed in *Pitx2*^{neo} homozygotes and wild-type littermates at P1. The expression of gonadotrope-specific markers *Lhb* (A,B), *Fshb* (C,D) and *Gnrhr* (E,F) is almost undetectable in *Pitx2*$^{neo/neo}$ pituitaries. *Gh* (G,H), *Ghrhr* (I,J) and *Tshb* (K,L) in situ hybridization reveals the reduction in somatotropes and thyrotropes, respectively. POMC-producing corticotropes appear to develop normally in *Pitx2*$^{neo/neo}$ mice (M,N).

the number of fully differentiated hormone producing cells is decreased differentially along the dorsoventral axis in response to a reduction in *Pitx2*.

Altered transcription factor expression in *Pitx2* hypomorphs

Gata2, *Egr1*, *Sf1* and *Pitx1* are thought to be important for differentiation and/or function of the gonadotropes (Dasen et al., 1999; Ingraham et al., 1994; Szeto et al., 1999; Topilko et al., 1998). We examined the expression of these transcription factors in *Pitx2*$^{neo/neo}$ mice to determine whether *Pitx2* affected *Lhb* and *Fshb* transcription by influencing expression of these transcription factors. *Gata2* expression normally initiates at E12.5 in the rostral tip of the pituitary primordium (Fig. 4A,B), but it is absent in the ventral and caudomedial aspect of mutant pituitaries at P1 (Fig. 4C,D). *Egr1* transcript and immunoreactive SF1 are nearly absent compared to wild type at P1 (Fig. 4E-H). The reduction in *Egr1* and *Sf1* expression, and the failure of *Gata2* transcripts to expand through the most ventral portion of the anterior lobe, is consistent with the hypothesis that *Pitx2* is essential for activation of *Gata2*, *Sf1* and *Egr1*. Immunohistochemstry did not reveal any difference in PITX1 immunoreactivity during embryogenesis (Fig. 4I-L). Therefore, reduction in PITX2 dosage specifically affects a subset of critical transcription factors.

Mutations in pituitary-specific 'paired'-like homeobox genes, *Prop1* and *Pit1*, interfere with the development of three pituitary cell types: somatotropes, thyrotropes, and lactotropes (the prolactin (PRL) producing cells) (Camper et al., 1990; Simmons et al., 1990; Sornson et al., 1996). PROP1 is required for the activation of *Pit1* and for normal gonadotrope function (Cushman et al., 2001; Gage et al., 1996a; Tang et al., 1993; Wu et al., 1998). To test whether alterations in the expression of these transcription factors account for the reduced thyrotrope and somatotrope number in *Pitx2*$^{neo/neo}$ embryos, immunohistochemistry and in situ hybridization were performed for PROP1 and *Pit1*, respectively. PROP1 expression is not changed dramatically in *Pitx2*^{neo} homozygotes, but *Pit1* expression is clearly reduced (Fig. 4M-P). This suggests that *Pitx2* affects the activation of *Pit1*, and the decrease in *Pit1* may be responsible for the reduction in thyrotropes and somatotropes.

Pitx2 enhances *Gata2* expression

The earliest molecular effect of reduced *Pitx2* expression is the failure of *Gata2* to be activated in the ventral aspect of the developing gland. We established a transient transfection assay system to test whether *Gata2* expression is dependent on PITX2. Two isoforms of *Gata2* cDNA arise by tissue-specific promoter usage (Minegishi et al., 1998). The isoform containing *Gata2* exon 1G is expressed in the pituitary gland (data not shown). We used the 5′ upstream sequences (–7kb to +216bp) of this isoform to generate a reporter construct for *Gata2* transcription. DNA sequence analysis confirmed the presence of a consensus bicoid binding site at –1242 to –1236 (GenBank accession no. AF448814) (Amendt et al., 1998; Tremblay et al., 1998). PITX2 expression vectors were assembled for the three known isoforms of PITX2 that are generated by alternative splicing and alternative promoter usage (Gage et al., 1999b). These three PITX2 proteins have

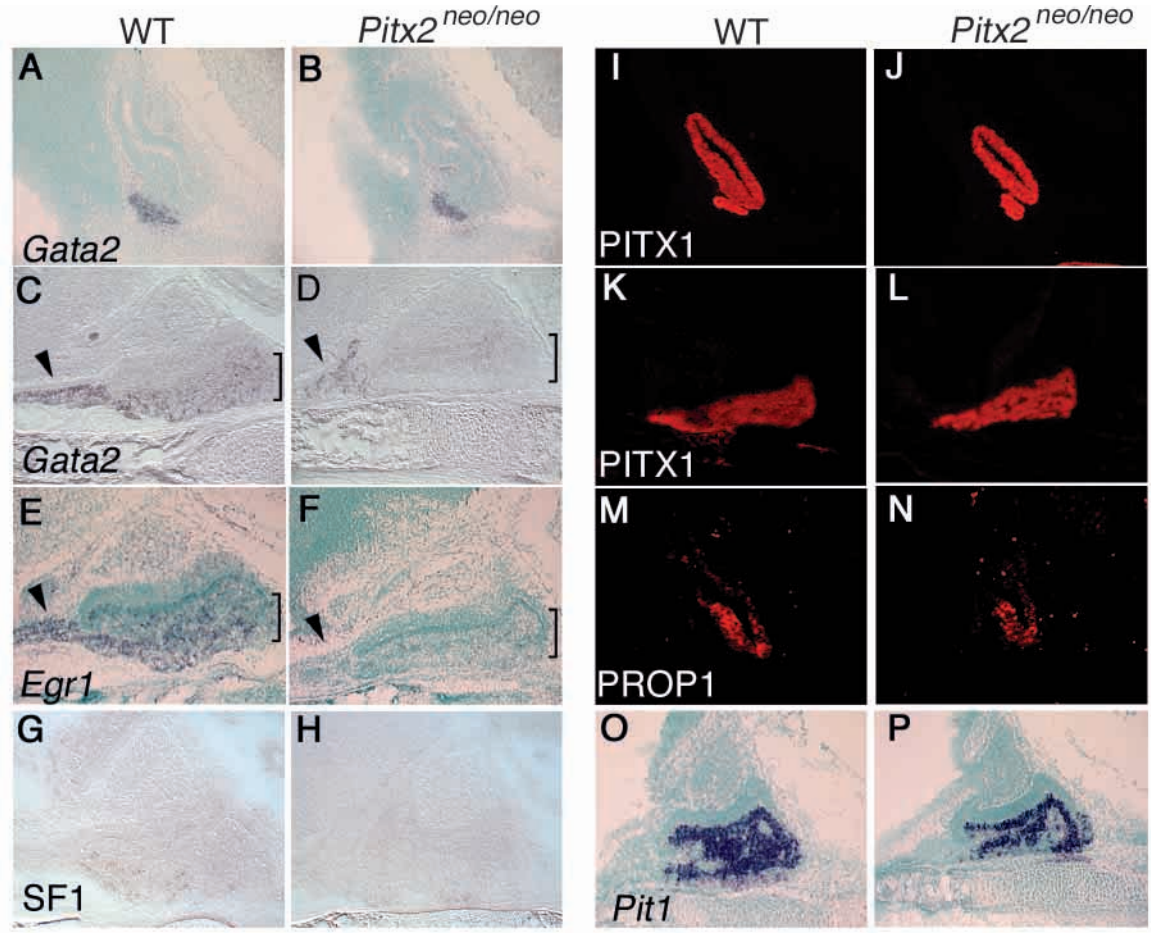

Fig. 4. Reduced *Pitx2* causes decreased expression of lineage-specific transcription factors. In situ hybridization or immunohistochemistry was performed to examine the change of transcription factor expression in *Pitx2*$^{neo/neo}$ mice and wild-type littermates at E12.5 and P1. *Gata2* expression initiates normally at early stages (A,B), but fails to be maintained (C,D). The expression of *Egr1* (E,F) and SF1 (G,H) is nearly undetectable in *Pitx2*$^{neo/neo}$ animals. PITX1 (I,J and K,L) and PROP1 (M,N) expression was unchanged in homozygotes for *Pitx2*^{neo}. *Pit1* in situ hybridization shows a reduction in the number of *Pit1*-producing cells (O,P). Note *Gata2* (A,B), PITX1 (I,J) and PROP1 (M,N) expression at E12.5. Arrowhead, rostral tip of pituitary gland; the brackets in C-F indicate the ventral and caudomedial aspect of the pituitary gland.

233 common amino acids including the homeodomain and putative C-terminal transactivation domains, but differ in the N termini. PITX2A, PITX2B and PITX2C have 38, 84 and 91 N-terminal amino acids, respectively, and PITX2B and PITX2C N-terminal sequences share only 28% and 35% amino acid sequence similarities compared to PITX2A. Two cell lines were used for transfection: a pituitary-derived αT3-1 cell line that expresses all three isoforms of PITX2 and represents pre-gonadotropes (Gage and Camper, 1997; Gage et al., 1999a), and a heterologous CV-1 cell line derived from monkey kidney fibroblasts.

PITX2 expression vectors were cotransfected with *Gata2* reporter constructs. PITX2B induces *Gata2* reporter gene expression in a statistically significant manner in αT3-1 cell lines (P<0.05, Fig. 5A). PITX2B enhanced *Gata2*-directed luciferase activity better than other isoforms in αT3-1 cells, suggesting that different N termini of PITX2 can influence function. All three isoforms of PITX2 showed a statistically significant transactivation of the *Gata2* reporter gene in CV-1 cells (P<0.05, Fig. 5B). These data clearly demonstrate that *Pitx2* is an upstream regulator of *Gata2*.

Pitx1 and *Pitx2* act synergistically in early pituitary development

Pitx1 and *Pitx2* have 97% similarity in the homeodomain, 67% identity in the C-terminal putative transactivational domain, and overlapping expression patterns in the pituitary gland (Fig. 2D). The pituitary phenotypes of *Pitx1*$^{-/-}$ mice bear some similarities to the changes that result from reduced *Pitx2* dosage. *Pitx1*$^{-/-}$ mutants have more corticotropes with fewer gonadotropes and thyrotropes (Szeto et al., 1999), although the reduction in gonadotropes is very subtle compared to near ablation observed in *Pitx2*$^{neo/neo}$ mice. The similarities between the *Pitx1*$^{-/-}$ and *Pitx2*$^{neo/neo}$ phenotypes led us to hypothesize that there is functional redundancy between *Pitx1* and *Pitx2* during early pituitary development. We also suspected that these two transcription factors might act synergistically in specifying certain cell lineages.

We tested for overlapping functions by generating double mutants carrying *Pitx1*$^{-}$ and *Pitx2*^{neo} alleles. The progeny of a double heterozygote intercross represented all possible genotypes in the expected Mendelian ratio at E12.5. The oral ectoderm invaginated normally in all genotypes, making a

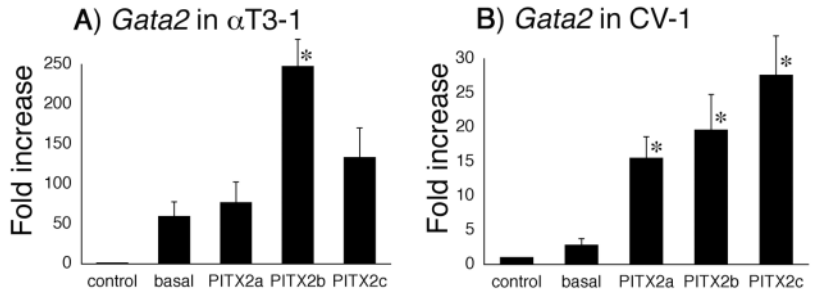

Fig. 5. PITX2 enhances *Gata2* transcription. The fold increase (*y*-axis) of luciferase gene activity compared to control (transfection of empty vectors only) is shown. Three PITX2 isoform expression vectors were individually cotransfected with *Gata2* luciferase construct into αT3-1 (A) or CV-1 cells (B). PITX2 showed a significant induction of *Gata2* luciferase expression in both cell lines (A,B; $P<0.05$). Different PITX2 isoforms may have different transactivation properties on *Gata2* promoter (A). Asterisk indicates statistically significant induction compared to basal level (transfection of reporter construct only without PITX2 expression vector, $P<0.05$). Results from more than 4 independent experiments were averaged.

direct contact with the ventral diencephalon at E10.5 (data not shown). However, Rathke's pouch failed to expand in *Pitx1*$^{-/-}$;*Pitx2*$^{neo/neo}$ mice at E12.5 (Fig. 6D), while normal pituitary size and morphology was observed in mice homozygous for only *Pitx1*$^{-}$ or *Pitx2*^{neo} alleles (Fig. 6B,C). An intermediate reduction in pouch size was observed in *Pitx1*$^{-/-}$;*Pitx2*$^{neo/+}$ and *Pitx1*$^{+/-}$;*Pitx2*$^{neo/neo}$ mutants (data not shown). Thus, these data reveal that pituitary development is sensitive to the total dosage of the *Pitx* gene family, and that *Pitx1* and *Pitx2* have overlapping functions during the early stages of pituitary development. However, pituitary development in the double mutants does not progress far enough to assess the effects on individual cell types.

In order to ascertain the molecular basis for this synergistic effect, we considered additional potential target genes whose expression might be affected in the double mutants. The LIM homeobox gene, *Lhx3*, is expressed early during pituitary development, and mice deficient in *Lhx3* exhibit arrested pituitary development that is similar to *Pitx1*$^{-/-}$;*Pitx2*$^{neo/neo}$ mutants (Sheng et al., 1996). This suggests that *Pitx1* and *Pitx2* might have overlapping functions that are required to activate *Lhx3* transcription. However, immunoreactive LHX3 was indistinguishable in *Pitx1*$^{-/-}$;*Pitx2*$^{neo/neo}$ and wild-type mice (Fig. 6E-H). This indicates that low amounts of PITX2 are sufficient for LHX3 expression in the absence of PITX1.

DISCUSSION

Using a *Pitx2* allelic series we demonstrate that *Pitx2* is required at multiple stages of pituitary organogenesis and that proper *Pitx2* gene dosage is essential for these processes. *Pitx2* is required for Rathke's pouch expansion in early development, and probably influences this process through the regulation of *Hesx1* (*Rpx*) and *Prop1* expression. Mutations in *Rpx* and *Prop1* cause pituitary hypoplasia, and transcription of both genes is dependent upon *Pitx2* (Dattani and Robinson, 2000; Gage et al., 1999a; Lin et al., 1999). Pituitary gland development progresses further in *Pitx2*$^{neo/neo}$ hypomorphs than in mice completely deficient in *Pitx2*. Analysis of the hypomorphs revealed that normal levels of PITX2 are necessary for cell-lineage specification of ventral and caudomedial cell types at later stages of organogenesis. These observations led to a new model for pituitary development (Fig. 7).

The *Pitx2*^{neo} hypomorphic allele has little effect on pituitary morphology. Low levels of PITX2 are sufficient for *Prop1* expression and the waves of cell proliferation that control expansion of the pouch. Several differentiated cell types are reduced or absent in *Pitx2* hypomorphs, however, revealing the important role of *Pitx2* in cell specification. Gonadotropes are the most sensitive cell type in the pituitary to reductions in *Pitx2*. The gonadotrope markers *Lhb*, *Fshb* and *Gnrhr* are expressed at a low level or completely absent, as are three critical factors for gonadotrope function, *Gata2*, *Sf1* and *Egr1*. The dependence of these factors on *Pitx2* is consistent with the hypothesis that *Pitx2* acts early in the pathway, upstream of *Gata2*, *Sf1* and *Egr1* in the specification of gonadotropes (Fig. 7). Cell transfection studies demonstrated that PITX2 is a

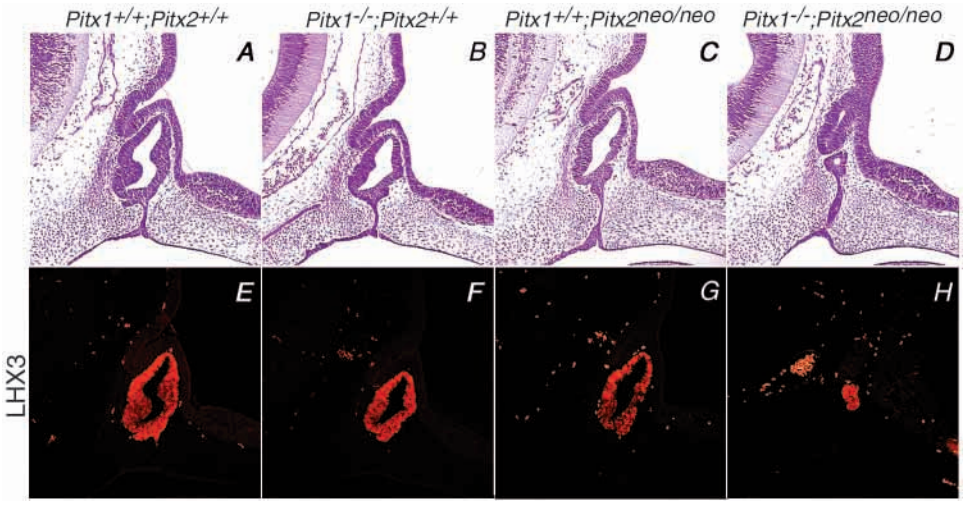

Fig. 6. *Pitx1* and *Pix2* have functional overlaps in pituitary ontogeny. Histological examinations of double mutants carrying *Pitx1*$^{-}$ and *Pitx2*^{neo} alleles show the normal formation of Rathke's pouch in all genotypes (A-C), but not in *Pitx1*$^{-/-}$;*Pitx2*$^{neo/neo}$ mice at E12.5 (D). Note that Rathke's pouch in *Pitx1*$^{-/-}$;*Pitx2*$^{neo/neo}$ mice does not expand (D). Immunoreactive LHX3 is present all genotypes (E-H).

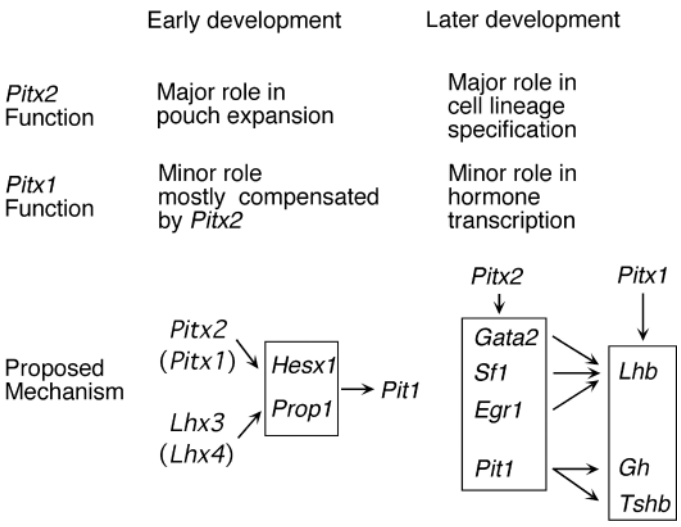

Fig. 7. *Pitx2* functions at multiple developmental stages. Analysis of *Pitx2*$^{-/-}$ mice revealed that *Pitx2* is required for Rathke's pouch expansion at early stages of embryogenesis, and studies from *Pitx2*$^{neo/neo}$ homozygotes showed that *Pitx2* is also required for cell lineage specification at later stages of organogenesis, influencing expression of downstream transcription factors. In addition to *Pitx1* function in pituitary hormone transcription, *Pitx1* acts synergistically with *Pitx2* at an early stage of pituitary development.

direct transcriptional activator of *Gata2*. This suggests that normal levels of PITX2 are required for activation of *Gata2* in the ventral and caudomedial aspect of the pituitary gland and that GATA2 may be vital for gonadotrope specification. A role for GATA2 in gonadotrope specification was suggested based on the results of dominant negative and ectopic transgene expression experiments (Dasen et al., 1999). The model proposed that a higher concentration of GATA2 in the ventral pituitary is important in gonadotrope specification and that a ventrodorsal gradient of GATA2 prevents *Pit1* expression from expanding from the caudomedial area into the gonadotrope field. However, we demonstrate that the expression of both *Gata2* and *Pit1* is decreased when *Pitx2* is reduced, and the specification of gonadotropes and thyrotropes was subsequently affected. These data indicate that the temporal and spatial expression of *Gata2* and *Pit1* are regulated by more than the concentration gradient and that *Pitx2* is critically involved in this cell-specification mechanism.

We considered the possibility that reduced expression of pituitary hormones in *Pitx2*$^{neo/neo}$ mice is due to developmental delay. This seems unlikely for several reasons. *Pitx2*^{neo} homozygotes activate expression of *Gata2* and *Prop1* normally at E10.5-12.5, and the pituitary volume is nearly normal at birth. *Gata2* expression normally spreads ventrally and caudomedially by E14.5, but there was no detectable expression of *Gata2* in the ventral and caudomedial regions of mutants even 5 days later at P1. The pattern of *Egr1* expression normally changes dynamically along the anterior-posterior axis of the gland. *Egr1* expression is initially highest in the posterior pituitary at E14.5, peaks in the intermediate lobe at the later stages in development, and is predominately expressed in the anterior lobe in adults (Topilko et al., 1998). However, *Egr1* was not expressed in any part of the pituitaries of *Pitx2*$^{neo/neo}$ mice at P1. Thus, the best explanation for the phenotype is that the PITX2 level in *Pitx2*$^{neo/neo}$ mutants is not sufficient to initiate or maintain *Gata2* expression in the ventral and caudomedial regions, which disrupts specification of gonadotropes and the *Pit1* lineage. These observations of dosage-sensitive effects suggest the possibility that a specific threshold of PITX2 must be reached for normal development and that the requirements of each cell type are different.

Pitx1 and *Pitx2* are highly homologous members of the *Pitx* gene family. Although they have similar temporal and spatial expression patterns in the pituitary and hindlimb, they also have unique expression regions (Gage et al., 1999b). *Pitx1*$^{-/-}$ mice have cleft palate and severe defects in the hindlimb, whereas *Pitx2*-deficient mice have severe heart and lung defects. In spite of these differences, loss of *Pitx1* function causes a constellation of changes in pituitary hormone transcription (Szeto et al., 1999), which share some similarities with the pituitary phenotype that we observe in mice with reduced levels of *Pitx2*. *Pitx1*-deficient mice have slightly elevated levels of *Pomc* transcripts (Szeto et al., 1999). The expression of *Tshb*, *Fshb* and *Lhb* was reduced, and no change was noted in *Gh* expression. Reduced *Pitx2* expression also influenced gonadotropes and thyrotropes, but the effect on gonadotropes was much more profound than that observed in *Pitx1* mutants. Another difference is that corticotropes were not affected by reduced *Pitx2* dosage. A role for *Pitx1* and *Pitx2* in gonadotropes was suggested by their ability to induce *Lhb* gene transcription in cell transfection experiments and transgenic studies (Quirk et al., 2001; Tremblay et al., 2000). SF1 and EGR1 act synergistically with PITX1 to transactivate *Lhb* reporter expression (Tremblay and Drouin, 1999; Tremblay et al., 1999). Here, we report the dependence of the gonadotrope transcription factors SF1 and EGR1 on *Pitx2*, and a strong transcriptional effect of PITX2 on the activation of *Gata2* expression (Fig. 5). This indicates that the role of *Pitx2* in gonadotropes goes well beyond a potential role in transactivation of *Lhb*, and is important for the activation of several transcription factors critical in gonadotrope cell lineage specification.

Mutations in *Prop1* are responsible for multiple pituitary hormone deficiencies in mice and humans (Cushman and Camper, 2001). Both *Prop1* deficiency and persistent expression of *Prop1* interfere with gonadotrope differentiation (Cushman et al., 2001; Tang et al., 1993; Wu et al., 1998). Thus, appropriately regulated *Prop1* is critical for gonadotrope development and function. However, no dramatic changes in PROP1 expression were noted in *Pitx2*$^{neo/neo}$ mutants, suggesting that the gonadotrope defects in *Pitx2*$^{neo/neo}$ mice probably involve other downstream targets of *Pitx2*.

Reduced *Pitx2* dosage caused decreased expression of *Pit1*, which may be responsible for the reduced number of thyrotropes and somatotropes. *Pitx2* might also have a role in transcription of some target genes in these cells, such as *Tshb*, *Gh* and *Ghrhr* (Tremblay et al., 2000). Some Rieger patients have short stature that is attributable to an inability to secrete growth hormone (GH) in response to insulin (Feingold et al., 1969; Polomeno et al., 1980; Sadeghi-Nedjad and Senior, 1974). The occasional growth defects in human patients could be caused by lesions in other genes associated with Rieger syndrome (Mears et al., 1998; Phillips et al., 1996). While this issue may be resolved as mutation analysis in Rieger patients

becomes more complete, the phenotype of *Pitx2*$^{neo/neo}$ embryos clearly revealed a crucial role for PITX2 in somatotrope development and expression of *Gh* and *Ghrhr*. Thus, isolated GH insufficiency in RGS patients could result from haploinsufficiency for *PITX2*.

Lhx3 and *Lhx4* have overlapping functions in the formation of the definitive pouch and expansion of the pituitary primordium (Sheng et al., 1997; Sheng et al., 1996). One functional allele of *Lhx3* is sufficient for lineage specification, but absence of both LHX3 and LHX4 causes arrested development of the small pouch rudiment. Reduced dosage of these *Lhx* genes affects pouch expansion and lineage specification. The examination of *Pitx1*$^{-/-}$;*Pitx2*$^{neo/neo}$ revealed a similar functional overlap between *Pitx1* and *Pitx2* at early stages of pituitary development. While Rathke's pouch appears to have normal morphology in homozygotes for either allele, pouch formation was barely detectable in *Pitx1*$^{-/-}$;*Pitx2*$^{neo/neo}$ mice. Thus, these *Pitx* genes act as early upstream regulators in the transcriptional cascade, important for the patterning and cell specification processes during pituitary development.

Mutations in several homeobox transcription factors required for organ development exhibit semi-dominant inheritance in humans and mice due to haploinsufficiency (Glaser et al., 1994; Sheng et al., 1997; Smith et al., 2000). Heterozygotes display variable, but less severe, phenotypes than null homozygotes because the presence of one functional allele cannot fully compensate for the loss of function of the other allele. This indicates that the function of these transcription factors is sensitive to gene dosage. Determining the underlying mechanisms of haploinsufficiency is an important goal for understanding organogenesis. Evidence for several different mechanisms has been collected, including monoallelic expression and threshold effects on cell proliferation and programmed cell death (Nutt et al., 1999; Ostrom et al., 2000; van Raamsdonk and Tilghman, 2000). Here we propose the threshold model to explain the underlying mechanism for *Pitx2* haploinsufficiency. Lower levels of PITX2 are not sufficient to activate the cascade of transcription factors, starting with GATA2, that are critical for cell lineage specification and proliferation. Similar dosage effects may apply in the eyes, teeth, mandible and other structures dependent upon PITX2.

We thank Drs Audrey Seasholtz, Michael Uhler, Tom Glaser and Greg Dressler for sharing reagents, instruments, and for discussion. We also acknowledge Dr Thom Saunders, Patrick Gillespie and Sandra Lindert for ES cell work and mouse colony maintenance. This work was supported by funding from the Rackham Graduate School (H.S.), the Glaucoma Research Foundation (P.J.G.), and the NICHD 34283 (S.A.C.).

REFERENCES

Alarid, E. T., Windle, J. J., Whyte, D. B. and Mellon, P. L. (1996). Immortalization of pituitary cells at discrete stages of development by directed oncogenesis in transgenic mice. *Development* **122**, 3319-3329.

Amendt, B., Sutherland, L., Murray, J. and Russo, A. (1998). The molecular basis of Rieger Syndrome: analysis of PITX2 homeobox activities. *J. Biol. Chem.* **111**, 20066-20072.

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.

Cushman, L. J. and Camper, S. A. (2001). Molecular basis of pituitary dysfunction in mouse and human. *Mamm. Genome* **12**, 485-494.

Cushman, L. J., Watkins-Chow, D. E., Brinkmeier, M. L., Raetzman, L. T., Radak, A. L., Lloyd, R. V. and Camper, S. A. (2001). Persistent *Prop1* expression delays gonadotrope differentiation and enhances pituitary tumor susceptibility. *Hum. Mol. Genet.* **10**, 1141-1153.

Dasen, J. S., O'Connell, S. M., Flynn, S. E., Treier, M., Gleiberman, A. S., Szeto, D. P., Hooshmand, F., Aggrawal, A. K. and Rosenfeld, M. G. (1999). Reciprocal Interactions of Pit1 and GATA2 Mediate Signaling Gradient-Induced Determination of Pituitary Cell Types. *Cell* **97**, 587-598.

Dattani, M. T. and Robinson, I. C. (2000). The molecular basis for developmental disorders of the pituitary gland in man. *Clin. Genet.* **57**, 337-346.

Feingold, M., Shiere, F., Fogels, H. R. and Donaldson, D. (1969). Rieger's syndrome. *Pediatrics* **44**, 564-569.

Flomen, R. H., Gorman, P. A., Vatcheva, R., Groet, J., Barisic, I., Ligutic, I., Sheer, D. and Nizetic, D. (1997). Rieger syndrome locus: a new reciprocal translocation t(4;12)(q25;q15) and a deletion del(4)(q25q27) both break between markers D4S2945 and D4S193. *J. Med. Genet.* **34**, 191-195.

Gage, P. J., Brinkmeier, M. L., Scarlett, L. M., Knapp, L. T., Camper, S. A. and Mahon, K. A. (1996a). 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., Roller, M. L., Saunders, T. L., Scarlett, L. M. and Camper, S. A. (1996b). Anterior pituitary cells defective in the cell autonomous factor, *df*, undergo cell lineage specification but not expansion. *Development* **122**, 151-160.

Gage, P. J., Suh, H. and Camper, S. A. (1999a). Dosage requirement of *Pitx2* for development of multiple organs. *Development* **126**, 4643-4651.

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

Glaser, T., Jepeal, L., Edwards, J. G., Young, S. R., Favor, J. and Maas, R. L. (1994). PAX6 gene dosage effect in a family with congenital cataracts, aniridia, anophthalmia and central nervous system defects [published erratum appears in Nat Genet 1994 Oct;8(2):203]. *Nat. Genet.* **7**, 463-471.

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.

Ingraham, H. A., Lala, D. S., Ikeda, Y., Luo, X., Shen, W.-H., Nachtigal, M. W., Abbud, R., Nilson, J. H. and Parker, K. L. (1994). The nuclear receptor steroidogenic factor 1 acts at multiple levels of the reproductive axis. *Genes Dev.* **8**, 2302-2312.

Jansson, J. O., Downs, T. R., Beamer, W. G. and Frohman, L. A. (1986). Receptor-associated resistance to growth hormone-releasing factor in dwarf 'Little' mice. *Science* **232**, 511-512.

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, K., Miyagawa-Tomita, S., Yanazawa, M., Katoh-Fukui, Y., Suzuki, R., Ohuchi, H., Suehiro, A., Motegi, Y., Nakahara, Y. et al. (1999). Mouse Pitx2 deficiency leads to anomalies of the ventral body wall, heart, extra- and periocular mesoderm and right pulmonary isomerism. *Development* **126**, 5749-5758.

Kozlowski, K. and Walter, M. A. (2000). Variation in residual PITX2 activity underlies the phenotypic spectrum of anterior segment developmental disorders. *Hum. Mol. Genet.* **9**, 2131-2139.

Lanctot, C., Gauthier, Y. and Drouin, J. (1999a). Pituitary homeobox 1 (Ptx1) is differentially expressed during pituitary development. *Endocrinol.* **140**, 1416-1422.

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

Li, H., Zeitler, P. S., Valerius, M. T., Small, K. and Potter, S. S. (1996). Gsh-1, an orphan Hox gene, is required for normal pituitary development. *EMBO J.* **15**, 714-724.

Lin, C. R., Kioussi, C., O'Connell, S., Briata, P., Szeto, D., Liu, F., Izpisua-Belmonte, J. C. and Rosenfeld, M. G. (1999). Pitx2 regulates lung asymmetry, cardiac positioning and pituitary and tooth morphogenesis. *Nature* **401**, 279-282.

Lu, M.-F., Pressman, C., Dyer, R., Johnson, R. L. and Martin, J. F. (1999).

Function of Rieger syndrome gene in left-right asymmetry and craniofacial development. *Nature* **401**, 276-278.

Mears, A. J., Jordan, T., Mirzayans, F., Dubois, S., Kume, T., Parlee, M., Ritch, R., Koop, B., Kuo, W. L., Collins, C. et al. (1998). Mutations of the forkhead/winged-helix gene, FKHL7, in patients with Axenfeld-Rieger anomaly. *Am. J. Hum. Gen.* **63**, 1316-1328.

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.

Meyers, E. N. and Martin, G. R. (1999). Differences in left-right axis pathways in mouse and chick: functions of FGF8 and SHH. *Science* **285**, 403-406.

Minegishi, N., Ohta, J., Suwabe, N., Nakauchi, H., Ishihara, H., Hayashi, N. and Yamamoto, M. (1998). Alternative Promoters Regulate Transcription of the Mouse GATA-2 Gene. *J. Biol. Chem.* **273**, 3625-3634.

Nagy, A., Moens, C., Ivanyi, E., Pawling, J., Gertsenstein, M., Hadjantonakis, A. K., Pirity, M. and Rossant, J. (1998). Dissecting the role of N-myc in development using a single targeting vector to generate a series of alleles. *Curr. Biol.* **8**, 661-664.

Nutt, S. L., Vambrie, S., Steinlein, P., Kozmik, Z., Rolink, A., Weith, A. and Busslinger, M. (1999). Independent regulation of the two Pax5 alleles during B-cell development. *Nat. Genet.* **21**, 390-395.

Ostrom, L., Tang, M., Gruss, P. and Dressler, G. (2000). Reduced *Pax2* Gene Dosage Increases Apoptosis and Slows the Progression of Renal Cystic Disease. *Dev. Biol.* **219**, 250-258.

Phillips, J. C., del Bono, E. A., Haines, J. L., Pralea, A. M., Cohen, J. S., Greff, L. J. and Wiggs, J. L. (1996). A second locus for Rieger syndrome maps to chromosome 13q14. *Am. J. Hum. Genet.* **59**, 613-619.

Polomeno, R. C., Staudenmaier, C., Guyda, H. J. and Little, J. M. (1980). Ocular defects and short stature. *Can. J. Ophthalmol.* **15**, 125-130.

Priston, M., Kozlowski, K., Gill, D., Letwin, K., Buys, Y., Levin, A., Walter, M. and Heon, E. (2001). Functional analysis of two newly identified PITX2 mutants reveal a novel molecular mechanism for Axenfel-Rieger syndrome. *Hum. Mol. Genet.* **10**, 1631-1638.

Quirk, C. C., Lozada, K. L., Keri, R. A. and Nilson, J. H. (2001). A Single Pitx1 Binding Site Is Essential for Activity of the LH{beta} Promoter in Transgenic Mice. *Mol. Endocrinol.* **15**, 734-746.

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.

Semina, E. V., Murray, J. C., Reiter, R., Hrstka, R. F. and Graw, J. (2000). Deletion in the promoter region and altered expression of Pitx3 homeobox gene in aphakia mice. *Hum. Mol. Genet.* **9**, 1575-1585.

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.

Simmons, D. M., Voss, J. W., Ingraham, H. A., Holloway, J. M., Broide, R. S., Rosenfeld, M. G. and Swanson, L. W. (1990). Pituitary cell phenotypes involve cell-specific *Pit-1* mRNA translation and synergistic interactions with other classes of transcription factors. *Genes Dev.* **4**, 695-711.

Smith, R. S., Zabaleta, A., Kume, T., Savinova, O. V., Kidson, S. H., Martin, J. E., Nishimura, D. Y., Alward, W. L., Hogan, B. L. and John, S. W. (2000). Haploinsufficiency of the transcription factors FOXC1 and FOXC2 results in aberrant ocular development. *Hum. Mol. Genet.* **9**, 1021-1032.

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.

Szeto, D. P., Rodriquez-Estaban, C., Ryan, A. K., O'Connell, S. M., Liu, F., Kioussi, C., 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.

Tang, K., Bartke, A., Gardiner, C. S., Wagner, T. E. and Yun, J. S. (1993). Gonadotropin secretion, synthesis, and gene expression in human growth hormone transgenic mice and in Ames dwarf mice. *Endocrinology* **132**, 2518-2524.

Topilko, P., Schneider-Maunoury, S., Levi, G., Trembleau, A., Gourdji, D., Driancourt, M.-A., Rao, C. V. and Charnay, P. (1998). Multiple Pituitary and Ovarian Defects in *Krox-24* (*NGF1-A, Egr-1*)-Targeted Mice. *Mol. Endocrinol.* **12**, 107-122.

Tremblay, J. J. and Drouin, J. (1999). Egr-1 is a downstream effector of GnRH and synergizes by direct interaction with Ptx1 and SF-1 to enhance luteinizing hormone beta gene transcription. *Mol. Cell. Biol.* **19**, 2567-2576.

Tremblay, J. J., Goodyer, C. G. and Drouin, J. (2000). Transcriptional properties of Ptx1 and Ptx2 isoforms. *Neuroendocrinol.* **71**, 277-286.

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.

Tremblay, J. J., Marcil, A., Gauthier, Y. and Drouin, J. (1999). Ptx1 regulates Sf-1 activity by an interaction that mimics the role of the ligand-binding domain. *EMBO J.* **18**, 3431-3441.

van Raamsdonk, C. D. and Tilghman, S. M. (2000). Dosage requirement and allelic expression of PAX6 during lens placode formation. *Development* **127**, 5439-5448.

Wu, W., Cogan, J. D., Pfaffle, R. W., Dasen, J. S., Frisch, H., O'Connell, S. M., Flynn, S. E., Brown, M. R., Mullis, P. E., Parks, J. S. et al. (1998). Mutations in PROP1 cause familial combined pituitary hormone deficiency. *Nat. Genet.* **18**, 147-149.