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

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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 Sfi1, on a threshold level of PITX2. The related Pitx1 gene has a role in hormone gene transcription, and it is important late in development 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 microphthalmia, and agenesis of the lens and anterior segment structures in aphakia (ak) mice (Semia 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 (Semia 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., 1999; 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 Hlx4 (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
requirement of other organs for pituitary development are likely to underlie the dosage similar to the one we present for specification in homozygotes for the reduced function allele, reduced homozygotes. This makes it feasible to analyze the effect of allele live until postnatal day 1 (P1), much longer than null

MATERIALS AND METHODS

Animal husbandry
Mice carrying the Pitx2neo, 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 BamHI 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 Pome 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 HindIII to make antisense probes. Pitr1 cDNA in pKS (–) was linearized with HindIII 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-Ichi Hori Morohashi (National Institute for Basic Biology, Okazaki, Japan), and Aimée 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 LH3 monoclonal antibody developed by Drs 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 HindIII and BamHI sites of pCGN2. Pitx1 and Egr1 cDNAs were generated by reverse transcriptase (RT)-PCR from AT-20 pituitary corticotrope-derived cells (G7 subclone of AT-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). NotI-NcoI 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, 3×10⁵ 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 (Pitx2neo–)}
and Pitx2 hypomorph (Pitx2<sup>neo/neo</sup>) (Fig. 1A-C). The Pitx2<sup>neo</sup> 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<sup>neo/neo</sup> mutants (Fig. 2E,F), Pitx2<sup>−/−</sup> 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<sup>2neo/2H</sup>), 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).

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<sup>neo/neo</sup> 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<sup>neo</sup> 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<sup>neo</sup> homozygotes (Fig. 3F). The deficiency of these three differentiated gonadotrope markers suggests that gonadotrope development is especially sensitive to reduction in PITX2.

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<sup>neo</sup> homoyzogotes 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<sup>neo/neo</sup> 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 adrenocorticotropic (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<sup>neo</sup> homozygotes relative to wild type (Fig. 3M,N). This, together with the reduction in thyrotropes and somatotropes and the absence of gonadotropes, indicates that
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 Pitx2neo/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. Immunohistochemistry 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 Pitx2neo/neo embryos, immunohistochemistry and in situ hybridization were performed for PROP1 and Pit1, respectively. PROP1 expression is not changed dramatically in Pitx2neo 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
Pituitary dependence on Pitx2 dosage

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 Pitx2neo/neo mice. The similarities between the Pitx1–/– and Pitx2neo/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 Pitx2neo 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...
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.

![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.](image)

![Fig. 6. Pitx1 and Pitx2 have functional overlaps in pituitary ontogeny. Histological examinations of double mutants carrying Pitx1+/−;Pitx2neo/+ alleles show the normal formation of Rathke’s pouch in all genotypes (A-C), but not in Pitx1+/−;Pitx2neo/neo mice at E12.5 (D). Note that Rathke’s pouch in Pitx1+/−;Pitx2neo/neo mice does not expand (D). Immunoreactive LHX3 is present all genotypes (E-H).](image)

direct contact with the ventral diencephalon at E10.5 (data not shown). However, Rathke’s pouch failed to expand in Pitx1+/−;Pitx2neo/neo mice at E12.5 (Fig. 6D), while normal pituitary size and morphology was observed in mice homozygous for only Pitx1−/− or Pitx2neo/neo alleles (Fig. 6B, C). An intermediate reduction in pouch size was observed in Pitx1+/−;Pitx2neo/+ and Pitx1+/−;Pitx2neo/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 duringpituitary development, and mice deficient in Lhx3 exhibit arrested pituitary development that is similar to Pitx1+/−;Pitx2neo/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+/−;Pitx2neo/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 Hex1 (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 Pitx2neo/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 Pitx2neo 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...
anterior lobe in adults (Topilko et al., 1998). However, stages in development, and is predominately expressed in the pituitary at E14.5, peaks in the intermediate lobe at the later of the gland. expression is initially highest in the posterior mutants even 5 days later at P1. The pattern of Ptx2 neo/neo was not expressed in any part of the pituitaries of Gata2 mutants. 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 Pome 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). SFI 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 SFI 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<sup>neo/neo</sup> 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<sup>+/−</sup>;Pitx2<sup>neo/neo</sup> 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<sup>+/−</sup>;Pitx2<sup>neo/neo</sup> 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.

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