Candidate genes for panhypopituitarism identified by gene expression profiling

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STUDIES OF PITUITARY DEVELOPMENT have focused on identification of signaling pathways that are necessary for induction of the pituitary primordium known as Rathke’s pouch and transcription factors that activate hormone gene expression (reviewed in Refs. 27, 47). There are many other critical processes in pituitary organogenesis, including cell migration, coordinating the transition from proliferation to differentiation, establishing connections between cells to form functional multicellular networks, development of vasculature including the hypophyseal portal system, and establishing connections with the hypothalamic neurons that regulate hormone secretion (5, 11, 63). Identification of genes involved in these processes not only would be a significant step forward for understanding the fundamentals of pituitary organogenesis but could also uncover candidate genes for cases of pituitary hypoplasia or dysfunction that currently remain elusive. Pituitary stalk disruption is an important cause of hypopituitarism, and while some congenital cases could be attributable to birth trauma or traumatic brain injury, others are likely to be genetic, and the molecular mechanisms remain to be defined (9, 61).

Mouse models of hypopituitarism have been invaluable for enhancing the understanding of basic pituitary gland development and uncovering the basis for human pituitary disease, yet they have not been heavily exploited for discovering differences in gene expression. A comparison of gene expression in Prop1 and Pit1 mutants would be ideal for identifying new candidate genes. The Prop1/Prop1 mutant mice, also known as Ames dwarfs, carry an inactivating S83P missense mutation, and the Pit1/Pit1 mutants, known as Snell dwarfs, have a W261C missense mutation leading to loss of function (13, 56, 64, 80). These nonallelic mutants have many similar features. Both mutants have multiple pituitary hormone deficiency (MPHD) with little or no GH, PRL, or TSH, a reduction in circulating LH and FSH, and adult pituitary hypoplasia (8, 16, 84). Because of the similar hormone deficiencies, both mutants are the same size as their littermates at birth and begin to display obvious signs of growth insufficiency at −2 wk of age. By adulthood, the mutant mice are approximately one-third to one-fourth the size of their littermates. Comparative studies have uncovered several important differences in pituitary gland development between the two mutants (37, 89, 90). Differences are expected because Prop1 expression is detectable before Pit1, and Prop1 would likely have many downstream target genes besides Pit1 (4, 36). Thus, differences in gene expression between Prop1 and Pit1 mutants could reveal specific actions of each gene.

The developmental onset of anterior pituitary hypoplasia, organ morphology, and vascularization are different in Prop1 and Pit1 mutants (36, 89, 90). Hypoplasia of the anterior lobe and pituitary gland dysmorphology are apparent in the Prop1-deficient mice at embryonic day 14.5 (e14.5), although the overall volume of the organ is similar to normal mice until postnatal day 1 (P1). This phenotype is attributed to the failure of precursor cells to migrate away from the proliferative zone and colonize the anterior lobe, causing the striking dysmorphology, decreased cell proliferation, and increased cell death. In contrast, the pituitaries of Pit1-deficient mice are morphologically indistinguishable from normal mice from e14 through birth. Prop1 mutant pituitaries are poorly vascularized, while those of Pit1 mutant mice appear normal. These differences suggest several biological processes are likely to be regulated by Prop1 but not Pit1.

To identify the Prop1- and Pit1-specific target genes that guide pituitary development, we used gene expression microarrays to compare the transcripts in normal, newborn mouse pituitaries with those of Prop1/Prop1 and Pit1/Pit1 mutants. We discovered that the homeobox transcription factor gene Otx2 has uniquely altered expression in Prop1 mutants. The develop-
opmental regulation of Otx2 expression in normal mice suggests that it could have roles in development of the hypothalamus and pituitary stalk, as well as Rathke’s pouch derivatives, explaining the requirement for OTX2 for normal pituitary function in mouse and human (59). The differentially expressed genes reported here constitute a resource of candidate genes for roles in pituitary development and cases of MPH of unknown etiology.

MATERIALS AND METHODS

Mice. Ames dwarf mice (Prop1<sup>−/−</sup>), originally obtained from Dr. A. Bartke (Southern Illinois University, Carbondale, IL) from a noninbred stock (DF/B), and Snell dwarf mice (Pit1<sup>−/−</sup>) or originally obtained from The Jackson Laboratory from an inbred stock (DWJ) (Bar Harbor, ME), have been maintained as colonies at the University of Michigan through heterozygous matings. The morning after conception is designated e0.5 and the day of birth is designated as P1. All mice were housed in a 12-h light, 12-h dark cycle in ventilated cages with unlimited access to tap water and Purina 5020 chow. All procedures using mice were approved by the University of Michigan Committee on Use and Care of Animals, and all experiments were conducted in accordance with the principles and procedures outlined in the National Institutes of Health Guidelines of the Care and Use of Experimental Animals.

PCR genotyping. The genotypes of Prop1<sup>−/−</sup> and Pit1<sup>−/−</sup> mice were determined as described (31, 36).

Isolation of total RNA from newborn pituitaries. Pituitaries were collected, stored, homogenized, and RNA isolated as described (88). RNA was isolated from individual pituitaries from Ames wild-type, Ames Prop1<sup>−/−</sup>, Snell wild-type, and Snell Pit1<sup>−/−</sup> P1 pituitaries for validation by RT quantification PCR (RT-qPCR). To assess RNA quality for the microarray, total RNA was analyzed by capillary electrophoresis on an Agilent Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA) in the UMCCC Affymetrix (Santa Clara, CA) and cDNA Microarray Core to visualize and quantitate 18S and 28S ribosomal bands. UV spectrophotometry was used to assess the quality and concentration of total RNA for RT-qPCR.

Microarray analysis. Pituitary gene expression was determined with Mouse Genome 430 2.0 GeneChip oligonucleotide arrays (Affymetrix). Total RNA (1 μg) from three pooled pituitary tissue were used. Amplification of mRNA samples was performed using the Ovation RNA Amplification System (NuGENE, San Carlos, CA) according to the manufacturer’s recommendations. Synthesis of cRNA, hybridization to chips, and washes were performed in the University of Michigan Affymetrix and cDNA Microarray Core according to the manufacturer’s protocol and as described previously (38). RNA samples were processed together, pooling three P1 pituitaries into each RNA sample and then collecting five RNA pooled samples for each of the four experimental groups (Ames background wild type, Ames background Prop1<sup>−/−</sup>, Snell background wild type, and Snell background Pit1<sup>−/−</sup>). After hybridization, GeneChips were scanned at 1.5-μm density with GeneChip Scanner 3000 (Affymetrix).

Data analysis. Data analysis was performed in the University of Michigan Affymetrix and cDNA Microarray Core as previously described (44, 88). Probe-sets were remapped with Molecular and Behavioral Neuroscience Institute probe-sets (21). Differentially expressed genes were detected by fitting a gene-wise linear model to the data and computing contrasts of interest. Significance for each comparison was calculated using a nested F-test, which is simply a way to determine which comparisons are significant when there may be more than one significant comparison (79). Testing for overrepresented gene ontology terms was performed using a conditional hypergeometric model according to Alexa et al., 2006 (3). The unadjusted P value of 0.05 was adjusted for multiplicity using false discovery rate (10).

We used RT-qPCR to validate gene expression changes for genes that exhibited a similar fold change in Prop1 mutants vs. wild type and in the Prop1 mutants vs. Pit1 mutants.

Heat maps were created with differentially expressed genes between each of the groups, which were determined by fitting linear models using the LIMMA package from BioConductor. The genes that showed at least a twofold difference and a P value of 0.05 were included. A distance matrix was created using Euclidean distances or the square distance between two samples and then clustered using hierarchical clustering from the hclust function in the “base R” “stats” package.

RT-qPCR. Synthesis of cDNA and real-time PCR was carried out as described (88). Expression of the housekeeping gene GAPDH served as an internal control for each RNA sample. The fold change values and standard deviations were calculated as described (57, 88).

Immunohistochemistry and in situ hybridization. Timed pregnancies were produced using natural matings of sexually mature females and males. Collected embryos and P1 heads were fixed for 2–4 h in 4% paraformaldehyde in PBS (pH 7.2) at room temperature, dehydrated in a graded series of ethanol, and embedded in paraffin. Six-micrometer-thick sections were prepared for immunohistochemistry and in situ hybridization. Mengshuang Qiu (previously of Dr. John Rubenstein laboratory, University of California at San Francisco, San Francisco, CA) provided a plasmid-containing mouse Otx2 genomic sequence in pBluescriptSK-. The Otx2 containing plasmid was linearized with BamHI and labeled with T7 polymerase. The probe was diluted 1:100 and hybridized at 50°C. All riboprobes were generated and labeled with digoxigenin and precipitated with nitro-blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate (Roche Molecular Biochemicals, Indianapolis, IN) following previously described methods (20, 82).

Pituitary cell populations were examined by immunohistochemistry with antibodies against pituitary hormone markers. Immunostaining for the pituitary hormones was performed with polyclonal antisera against LHβ (1:1,500) and ACTH (1:100), which reacts with POMC as well as its cleavage product ACTH (National Hormone and Peptide Program, UCLA Medical Center, Torrance, CA). After antigen retrieval with a 10 min 0.01M citrate boil, immunostaining of pituitary gland transcription factor proteins was performed with antisera against NfixA1 at a 1:1,500 dilution (provided by Dr. Ken-Ichiro Morohashi, National Institute for Basic Biology, Okazaki, Japan), TPT used at a dilution of 1:1,000 (Provided by Dr. Jacques Drouin, Institut de Recherches Cliniques de Montreal, Montreal, Quebec, Canada), and OTX2 used at a dilution of 1:1,000 (Abcam, Cambridge, MA). All sections were incubated in biotinylated secondary antibodies for rabbit and guinea pig (Vector Laboratories, Burlingame, CA) and detected with either the tyramide signal amplification (TSA) fluoroscein isothiocyanate (FITC) kit (according to the manufacturer’s protocol, Perkin-Elmer, Boston, MA), or a streptavidin-conjugated Cy2 fluorophor (1:200, Jackson ImmunoResearch).

Gene pathway building. Genomatix Bibliosphere Software was used to generate gene networks from microarray data (http://www.genomatix.de). Bibliosphere search was based set on the lowest level Co-citation Filter with the most recent publications on April 2, 2008.

RESULTS

Experimental design for microarray analysis of gene expression. We selected newborn (P1) pituitaries as a source of tissue for microarray analysis of gene expression instead of embryonic stages at which Prop1 and Pit1 transcription are first initiated for several reasons. Many effects of Prop1 and Pit1 on gene expression can be detected at P1 (89, 90). The size of a P1 pituitary gland is sufficient to provide enough RNA for microarray analyses with limited pooling, and animal use was...
minimized because mother mice could be bred multiple times. P1 pituitaries were dissected from newborns produced from heterozygote matings, and individuals were genotyped. We pooled pituitaries from three newborn mice of the following genotypes for RNA preparation: DF/B-Prop1<sup>df/df</sup>, DF/B-Prop1<sup>+/+</sup>, DW/J-Pit1<sup>dw/dw</sup>, and DW/J-Pit1<sup>+/+</sup>. Five independent samples of total RNA for each experimental group were collected and analyzed using Affymetrix microarray technology.

Global analysis of gene expression changes. The raw microarray expression values were fit to a principal components analysis (PCA) and plotted (data not shown). The results indicate that the greatest amount of difference in gene expression exists between the two strains (DF/B and DW/J). Among the four samples: DW/J-<i>Pit1</i><sup>dw/dw</sup>, DW/J-<i>Pit1</i><sup>+/+</sup>, DF/B-Prop1<sup>+/+</sup>, and DF/B-Prop1<sup>df/df</sup>, the mutant and wild-type samples from within a strain were more similar to each other than the two wild types were comparing across strains. To understand the nature of the strain-specific differences in pituitary gene expression, we identified biological processes that differed between the two wild-type strains using overrepresented gene ontology (GO) terms. A total of 43 GO terms were identified with unadjusted <i>P</i> values of < 0.01 between the background strains DF/B and DW/J (Table 1). It is intriguing that the vascular endothelial growth factor receptor signaling pathway, blood vessel development, and grooming behavior are among the strain-specific biological processes terms.

Individual gene expression differences can be visualized by plotting the genes with at least a twofold difference in expression between the two mutants and the two wild-type strains (Fig. 1A). A total of 524 genes are differentially expressed by at least twofold between the two mutants. A similar degree of differential expression is detected by comparing the two wild types to each other: 418 genes. Fewer differentially expressed genes are detected in comparing the mutants to their normal littermates. There are 118 total genes that are differentially expressed in <i>Prop1</i> mutants compared with their wild-type littermates, and a total of 85 that are differentially expressed in <i>Pit1</i> dwarfs compared with wild type. The two mutants share a known as SF1, a nuclear hormone receptor expressed in pituitary gonadotropes that is important for transcription of <i>Lhb</i>, <i>Fshb</i>, and the gonadotropin releasing hormone receptor, <i>Ghrhr</i> (30, 40, 48).

Pituitary transcription factor expression in <i>Prop1</i> and <i>Pit1</i> mutant mice. Both mutants have reduced circulating gonadotropins, yet <i>Nr5a1</i> transcripts are elevated in the pituitary gland (8, 73, 84). The increase in <i>Nr5a1</i> expression was expected because of absent antagonizing effects of <i>Pit1</i> in the mutants (23). If <i>Pit1</i> were the only factor leading to increased <i>Nr5a1</i> expression, however, the degree of elevation would be similar in both mutants, and it is not (Table 2). The T-box transcription factor TPIT is necessary for suppression of ectopic <i>Nr5a1</i> expression and ectopic gonadotrope differentiation (70). TPIT activates the transcription of Pomc in corticotropes and melanotropes and hence the production of ACTH and MSH (55). To determine whether alterations in Tpit expression contributed to the elevation in <i>Nr5a1</i> transcription we carried out immunostaining for Tpit and Pomc, and we found similar expression in the <i>Prop1</i><sup>df/df</sup> and <i>Pit1</i><sup>dw/dw</sup> dwarf mutants and in their normal littermates at P1 (Fig. 2, A–H). NR5A1 immunostaining is expanded in the anterior pituitary glands of both the <i>Prop1</i><sup>df/df</sup> and <i>Pit1</i><sup>dw/dw</sup> dwarf mutants (Fig. 2, N and P), relative to their normal littermates (Fig. 2, M and O), and the increase is more profound in <i>Pit1</i> mutants. Thus, altered <i>Tpit</i> expression does not appear to contribute to the elevation in <i>Nr5a1</i> expression in either mutant. Furthermore, unknown factors that are lacking in <i>Prop1</i> mutants must contribute to the profound elevation of <i>Nr5a1</i> and LHB (Fig. 2L) in <i>Pit1</i> mutants.

Biological processes represented by differentially expressed genes. To identify possible <i>Prop1</i>-specific biological processes we searched for significantly overrepresented GO terms among the genes that were differentially expressed between <i>Prop1</i> and <i>Pit1</i> mutants (Table 3). There are 17 GO terms that had unadjusted <i>P</i> values of < 0.05. Among these are five biological processes that were anticipated to exhibit differential expression based on prior knowledge of <i>Prop1</i> action: tissue development and remodeling, frizzled signaling pathway (14, 49, 69), organ morphogenesis (36, 89), and anterior/posterior patterning (73). Some of the novel processes identified by overrepresentation of GO terms may be mechanistically relevant to <i>Prop1</i> molecular actions including vesicle-mediated transport and gland development.

<i>Prop1</i>-specific changes in gene expression. We used RT-qPCR to verify differential expression of nine novel genes that appeared to be altered specifically in <i>Prop1</i> mutants (Table 4). Each RT-qPCR fold change was based on a sample size of four

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### Table 1. Biological processes affected by genetic background

<table>
<thead>
<tr>
<th>GO Term</th>
<th>Description</th>
<th>P Value</th>
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<tr>
<td>GO:00006814</td>
<td>sodium ion transport</td>
<td>0.001</td>
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<tr>
<td>GO:00009887</td>
<td>organ morphogenesis</td>
<td>0.001</td>
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<td>GO:00048010</td>
<td>vascular endothelial growth factor receptor signaling pathway</td>
<td>0.001</td>
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<td>GO:0035295</td>
<td>tube development</td>
<td>0.01</td>
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<td>GO:00068986</td>
<td>receptor-mediated endocytosis</td>
<td>0.001</td>
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<tr>
<td>GO:0001568</td>
<td>blood vessel development</td>
<td>0.001</td>
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<tr>
<td>GO:00032501</td>
<td>multacellular organism process</td>
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<td>GO:0006811</td>
<td>ion transport</td>
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<td>GO:0007166</td>
<td>cell surface receptor linked signal transduction</td>
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</tr>
<tr>
<td>GO:0007625</td>
<td>grooming behavior</td>
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</tr>
<tr>
<td>GO:00088576</td>
<td>chemical homeostasis</td>
<td>0.001</td>
</tr>
<tr>
<td>GO:0001654</td>
<td>eye development</td>
<td>0.001</td>
</tr>
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</table>

Biological processes differentially expressed in <i>Prop1</i><sup>+/+</sup> (DF/B) relative to <i>Pit1</i><sup>+/+</sup> (DW/J). GO, Gene Ontology.

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The internal control of GAPDH expression remained relatively constant between the four experimental groups. In each case, a greater change in expression was evident by qPCR than by the expression microarray (93). It is not uncommon for microarrays to underestimate differences in gene expression due to the limited dynamic range of the chip.

The expression of three genes is reduced in the Prop1 mutant relative to wild-type littermates, suggesting that Prop1 normally activates them, as it does for Pit1 (66, 80). The reduction in expression of these genes in Prop1 mutants compared with wild-type ranges from >21-fold for Cart (cocaine and amphetamine regulated transcript) to three- to fourfold for Rgs2 (Regulator of G protein signaling 2), and Lbxcor1 or Corl1 (Ladybird homeobox 1 homolog co-repressor 1 or Co-repressor for Lbx1) (Table 4).

The majority of these putative Prop1-specific targets (6/9) exhibit elevated expression in the mutant, ranging from >200-fold to twofold (Table 4). Thus, Prop1 normally represses these genes, similar to the role of Prop1 in silencing Hesx1 expression (36, 66). Most of these putative Prop1 target genes have never been studied in the pituitary gland. However, pituitary expression of Hey1 (Hairy/enhancer-of-split related with YRPW motif 1) and Otx2 (orthodenticle homolog 2) has been reported (59, 74). Hey1 has not been studied extensively in the pituitary, but Prop1 is essential for Notch signaling, and Hey1 is a potential downstream effector of this pathway, suggesting that Hey1 merits further analysis (72).

Spatial and temporal regulation of Otx2 expression in developing pituitary gland. Little is known about the spatial and temporal regulation of Otx2 expression, other than its detection...
at e12.5 in the mouse pituitary gland (78). If Prop1 were a direct repressor of Otx2, then we would expect Otx2 expression to be present in the dorsal aspect of Rathke’s pouch and to wane in normal mice as Prop1 expression peaks at e12.5, and we would expect Otx2 expression to persist in Rathke’s pouch through e14.5 in Prop1 mutants, similar to the effects of Prop1 deficiency on Hesx1 expression (36). To explore this idea we analyzed the expression of Otx2 throughout pituitary gland development in normal and Prop1 mutant mice using both in situ hybridization and immunohistochemical staining.

Table 2. Expression changes in both dwarf mutants

<table>
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<tr>
<th>Gene Symbol</th>
<th>Gene Name</th>
<th>Prop1&lt;sup&gt;df/df&lt;/sup&gt;</th>
<th>Prop1&lt;sup&gt;+/+&lt;/sup&gt;</th>
<th>Pit1&lt;sup&gt;df/df&lt;/sup&gt;</th>
<th>Pit1&lt;sup&gt;+/+&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>Pappa2</td>
<td>pappalysin 2</td>
<td>−4.66</td>
<td>−12.21</td>
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<tr>
<td>Rtnx</td>
<td>retinoid X receptor gamma</td>
<td>−3.84</td>
<td>−5.50</td>
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<tr>
<td>Gmnt</td>
<td>glycine-N-methyltransferase</td>
<td>−2.77</td>
<td>−4.06</td>
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<tr>
<td>Igf1</td>
<td>immunoglobulin superfamily, memember 1</td>
<td>−2.60</td>
<td>−3.25</td>
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<td></td>
</tr>
<tr>
<td>Plac8</td>
<td>placenta-specific 8</td>
<td>−2.36</td>
<td>−2.73</td>
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<td>C77370</td>
<td>expressed sequence C77370</td>
<td>−2.35</td>
<td>−2.64</td>
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<td>Pad2</td>
<td>peptidyl arginine deiminase, type II</td>
<td>−2.31</td>
<td>−2.91</td>
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<td>Cyp27a1</td>
<td>cytochrome P450, family 27, subfamily a, polypeptide 1</td>
<td>−2.08</td>
<td>−3.03</td>
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<td>Huisf1</td>
<td>HIV TAT specific factor 1</td>
<td>−1.91</td>
<td>−2.79</td>
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<tr>
<td>Galnt11</td>
<td>UDP-N-acetyl-alpha-D-galactosamine:polypeptide-N-acetylgalactosaminyltransferase-like 1</td>
<td>−2.23</td>
<td>−2.38</td>
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<td>Epas3</td>
<td>ectonucleoside triphosphate diphosphohydrolase 3</td>
<td>−2.14</td>
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<td>Ango6</td>
<td>angiotensin-like 6</td>
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<td>Nqo1</td>
<td>NAD(P)H dehydrogenase, quinone 1</td>
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<td>Bruno6</td>
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Novel Genes

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<tr>
<th>Gene Symbol</th>
<th>Gene Name</th>
<th>Fold Change</th>
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<tr>
<td>Ghrhr</td>
<td>growth hormone releasing hormone receptor</td>
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</tr>
<tr>
<td>Tshb</td>
<td>thyroid stimulating hormone, beta subunit</td>
<td>4.38</td>
</tr>
<tr>
<td>Trhr</td>
<td>thyrotropin releasing hormone receptor</td>
<td>2.08</td>
</tr>
<tr>
<td>Nr5a1</td>
<td>nuclear receptor subfamily 5, group A, member 1</td>
<td>2.03</td>
</tr>
</tbody>
</table>

Expected Genes

Fold change in expression between genotype 1 (top) and genotype 2 (bottom), i.e., negative number means expression is reduced in genotype 1 relative to genotype 2.

Otx2 transcripts and protein are normally detectable at e10.5 in both the ventral diencephalon, from which the posterior pituitary lobe develops, and Rathke’s pouch (Fig. 3, A and B), indicating both intrinsic and extrinsic effects of Otx2 on pituitary development are possible. No transcripts are detected with the Otx2 sense control probe (Fig. 3 Ab), and no OTX2 immunoreactivity is detected in the absence of OTX2 antibody (data not shown). By e12.5 Otx2 transcripts are undetectable in Rathke’s pouch, but transcription persists in the region of the ventral diencephalon that acts as a signaling center for pouch development.
growth by expressing fibroblast growth factors 8 and 10 (FGF8, FGF10) and bone morphogenic protein 4 (BMP4) (Fig. 3ac) (14, 26, 32, 86). This pattern of Otx2 transcripts, present in the ventral diencephalon but absent in the pouch, continues at e14.5 (Fig. 3ae). No Otx2 transcripts are detected in the normal ventral diencephalon or any of the lobes of the pituitary gland at e16.5 or P1 (Fig. 3a, g, i). OTX2 protein exhibits perdurance: immunoreactivity is still detectable in the intermediate lobe at e12.5 and e14.5 even though the transcripts have faded (Fig. 3b, c and e). By e16.5 OTX2 immunoreactivity is reduced in both the posterior and intermediate lobes of normal mice (Fig. 3bg). The spatial and temporal pattern of Otx2 expression reveals the potentially important expression of Otx2 in the ventral diencephalon and posterior pituitary lobe in addition to transient, early expression in Rathke’s pouch.

The temporal and spatial patterns of Otx2 transcripts and protein are similar in Prop1 mutants and wild-type mice at e10.5, e12.5, and e14.5 (compare Fig. 3b, a and b; Fig. 3a, c and d; Fig. 3b, c and d; Fig. 3a, e and f). OTX2 immunoreactivity is slightly expanded in the Prop1 mutant at e14.5, when the dysmorphology of the mutant becomes evident (Fig. 3b, e and f) (36). By e16.5, both Otx2 transcription and protein accumulation are elevated in Prop1 mutant intermediate lobes relative to wild type (compare Fig. 3a, g and h; Fig. 3b, g and h). At P1 ectopic Otx2 expression is evident in patches of both the intermediate and anterior lobes (black arrows) (compare Fig. 3a, i and j). No ectopic expression is detectable in the posterior lobe of Prop1 mutants (white arrow) at e16.5 (Fig. 3ah). Thus, Prop1 deficiency causes abnormalities in Otx2 expression in Rathke’s pouch derivatives after Prop1 expression has waned in wild-type mice. It does not cause abnormalities in Otx2 expression in the ventral diencephalon or posterior lobe. Although we cannot rule out direct effects, Prop1 deficiency appears to affect Otx2 expression indirectly.

**Gene networks potentially regulated by Pit1 and Prop1.** We identified a number of putative gene networks amongst the genes differentially expressed in mutants relative to wild type using Bibliosphere software by Genomatix (http://www.genomatix.de), which uses scientific literature to build networks of potentially interacting genes. We selected two compelling networks that are anchored by Otx2 and Nr5a1, respectively (Fig. 4, A and B), for presentation of significant differentially expressed genes. Both gene networks include Nr5a1. We present an updated representation of the likely upstream and downstream factors for Prop1 in the developing mouse pituitary gland (Fig. 4c).

**Otx2 expression is increased specifically in Prop1 mutants** (Table 4), and it is the anchor for a novel pituitary gene network (Fig. 4a). The increase in Otx2 expression is associated with elevated Hesx1 expression in the microarray and in Prop1 mutant mice (36). The increase in Hesx1 transcripts is associated with decreases in Cga and Tshb expression. It is intriguing that expression of the stem cell markers Sox2 (SRY-box containing gene 2) and Nrl2/1 (nuclear receptor subfamily 2, group E, member 1)(33, 77) are increased, given that Prop1 mutants exhibit difficulty transitioning from the proliferative zone to differentiation zone (89).

**Nr5a1 expression is elevated in both Prop1 and Pit1 mutants** (Fig. 2) (23, 73), and it anchors a gene network that contains Gnrh, a known target of Nr5a1 (Fig. 4b) (43). Another network member is thyrotropin releasing hormone receptor (Trhr), which is important for the production of TSH and PRL in the pituitary gland (95). Increased Nr5a1 expression leads to increased transcription of Tpit and Nr1ob1 (nuclear receptor subfamily 0, group B, member 1), also known as Dax1, which can have either antagonistic (51, 94) or cooperative relationships with Nr5a1 depending on the context (67).

### Table 3. Multiple processes altered specifically in Prop1<sup>df/df</sup> pituitary glands

<table>
<thead>
<tr>
<th>GO Term</th>
<th>Description</th>
<th>P Value</th>
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<tr>
<td>GO:00042445</td>
<td>hormone metabolism</td>
<td>0.001</td>
</tr>
<tr>
<td>GO:0001501</td>
<td>skeletal development</td>
<td>0.002</td>
</tr>
<tr>
<td>GO:0004851</td>
<td>rhythmic process</td>
<td>0.004</td>
</tr>
<tr>
<td>GO:0016192</td>
<td>vesicle-mediated transport</td>
<td>0.009</td>
</tr>
<tr>
<td>GO:0048732</td>
<td>gland development</td>
<td>0.011</td>
</tr>
<tr>
<td>GO:0009888*</td>
<td>tissue development</td>
<td>0.023</td>
</tr>
<tr>
<td>GO:0051216</td>
<td>cartilage development</td>
<td>0.024</td>
</tr>
<tr>
<td>GO:0007222*</td>
<td>frizzled signaling pathway</td>
<td>0.026</td>
</tr>
<tr>
<td>GO:0017157</td>
<td>regulation of exocytosis</td>
<td>0.031</td>
</tr>
<tr>
<td>GO:0046849</td>
<td>bone remodeling</td>
<td>0.031</td>
</tr>
<tr>
<td>GO:0048771*</td>
<td>tissue remodeling</td>
<td>0.036</td>
</tr>
<tr>
<td>GO:00000160</td>
<td>two-component signal transduction system</td>
<td>0.041</td>
</tr>
<tr>
<td>GO:0001658</td>
<td>ureteric bud branching</td>
<td>0.041</td>
</tr>
<tr>
<td>GO:0001501</td>
<td>proteoglycan metabolism</td>
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</tr>
<tr>
<td>GO:0009887*</td>
<td>organ morphogenesis</td>
<td>0.042</td>
</tr>
<tr>
<td>GO:0009966</td>
<td>regulation of signal transduction</td>
<td>0.043</td>
</tr>
<tr>
<td>GO:0009952*</td>
<td>anterior/posterior pattern formation</td>
<td>0.044</td>
</tr>
</tbody>
</table>

*Biological processes expected to be changed based on previous data.

### Table 4. Validation of Prop1-specific changes in gene expression

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Name</th>
<th>Microarray</th>
<th>qPCR</th>
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<tbody>
<tr>
<td>Sult1e1</td>
<td>sulfotransferase family 1E, member 1</td>
<td>16.7</td>
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<tr>
<td>Chrb2</td>
<td>carbonyl reductase 2</td>
<td>19.2</td>
<td>19.2</td>
</tr>
<tr>
<td>Adam32e1</td>
<td>ADAM-like, decysin 1</td>
<td>14.9</td>
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<tr>
<td>Otx2</td>
<td>orthodenticle homolog 2</td>
<td>2.9</td>
<td>2.9</td>
</tr>
<tr>
<td>Hesl</td>
<td>hairy/enhancer-of-split related with YRPW motif 1</td>
<td>2.4</td>
<td>2.4</td>
</tr>
<tr>
<td>Cdh10</td>
<td>claudin 10</td>
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<td>2.2</td>
</tr>
<tr>
<td>Lbxcor1</td>
<td>ladybird homeobox 1 homolog corepressor 1</td>
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<td>−2.3</td>
</tr>
<tr>
<td>Rga2</td>
<td>regulator of G-protein signaling 2</td>
<td>−4.0</td>
<td>−4.0</td>
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<tr>
<td>Cart</td>
<td>cocaine and amphetamine regulated transcript</td>
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</table>

*Genes with reported expression in e12.5–e14.5 mouse pituitary gland.
DISCUSSION

The phenotypic similarities and differences between pituitary development and function in Prop1 and Pit1 mutants create a unique opportunity to identify new genes and pathways that may be direct targets of Prop1 and Pit1 action by comparative gene expression profiling. We have focused on genes that are differentially expressed in the Prop1<sup>df/df</sup> mutant and wild type, along with genes differentially expressed between the Prop1<sup>df/df</sup> mutant and the Pit1<sup>dw/dw</sup> mutant, through gene expression microarray analysis. We present a manageable sized collection of genes affected specifically by Prop1, and an inventory of probable Prop1-specific biological processes. In addition, we uncovered a list of novel genes that are affected by both mutants, and serendipitously, a cadre of genes whose pituitary expression at birth is strongly influenced by genetic background.

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Strain-specific differences in pituitary gene expression. The greatest degree of difference in gene expression exists between the two wild-type strains (DF/B and DW/J). We identified numerous biological processes underlying this unexpected finding. The reason for these gene expression differences is not clear, but some could be attributable to differences in gestation time, and thereby maturity of the newborn pups. Gestation time in mice varies from 18 to 22 days, and the strain of mouse and size of the litter both influence the gestation time (39). The anterior lobe of the pituitary gland grows laterally, producing an elongated shape in the coronal plane, in late gestation and the neonatal period. The DF/B strain appears to have more an elongated shape in the coronal plane, compared to the DW/J strain. What- ever mechanism underlies the strain specific differences in normal pituitary gland gene expression, the sheer abundance of them at birth underlines the importance of using normal litter-mate controls.

We were most intrigued by the strain-specific effects on the biological processes of vascular endothelial growth factor receptor signaling, blood vessel development, and grooming behavior. We expected differences in vascularization between Prop1<sup>df/df</sup> and Pit1<sup>dw/dw</sup>, but we did not anticipate strain-specific differences in blood vessel development, as the two background strains appeared to have similar organization of blood vessels at P1 and P8 using PECAM immunohistochemistry to visualize the vessels (90). Vascularization of the pituitary gland is important for allowing hormones to be secreted and taken to target organs and to allow negative feedback signals to reach the pituitary gland (60). Despite the importance of vascular development for transport of hormones, there is still much to be learned about how this process is regulated in development (41, 76, 83). In partic- ular, we are interested in whether the development of the vasculature stimulates pituitary cell differentiation as it does in the pancreas (52, 53).

Different effects of Prop1 and Pit1 on gonadotropes. NR5A1 expression is elevated in Prop1<sup>df/df</sup> and Pit1<sup>dw/dw</sup> mutants, although the increase is much more profound in Pit1 mutants. The expression of TPIT and POMC are similar in both mutants and wild types. Thus, elevated NR5A1 expression is independent of TPIT expression status in these cases. Thus, different mechanisms must underlie the elevation of NR5A1 transcription in the Prop1 and Pit1 mutants relative to Tap1 mutants. It is intriguing that LHB expression is diminished in Prop1<sup>df/df</sup> and expanded in Pit1<sup>dw/dw</sup>, even though NR5A1 is expanded in both mutants. We hypothesize that Prop1 plays a distinct role in the differentiation and function of gonadotropes, independent of gonadotrope-specific transcription factors such as Nr5a1 and Gata2. Transgenic mice that over-express Prop1 exhibit delayed gonadotrope differentiation, but there is no decrease in the known gonadotrope transcription regulators, Nr0b1, Nr5a1, Gata2, or Egr1 (20, 88). Furthermore, human PROP1 patients exhibit decreased gonadotropin production, while PIT1 deficient patients do not have this feature (68). The precise role of Prop1 in gonadotrope develop- ment is not clear.
Novel genes and pathways uncovered by expression analysis. Our analysis of genes with similar expression changes in both strains of dwarf mutants relative to wild type revealed several novel genes not previously analyzed in either Prop1<sup>df/df</sup> or Pit1<sup>dw/dw</sup> mutants (Table 1). A few of these, such as Pappa2, Rxrg, and Gmnt, play known roles in transcription factor activity and endocrinology (18, 58, 81). Pappalysin 2 (Pappa2) exhibits an enzymatic activity by cleaving insulin-like growth factor binding protein (IGFBP-5) in addition to being involved in normal postnatal growth (18). Retinoid X receptor gamma (Rxrg) regulates transcription by acting as a homodimer or heterodimer with a number of nuclear and orphan receptors (58). Rxrg is expressed in the thyrotrope cells in the pituitary gland (81). Glycine N-methylase (Gmnt) is directly regulated by growth hormone (1). Although these genes are unlikely to be direct targets of the Pit1 and Prop1 genes, they may be relevant participants in the adult phenotypes of both dwarf mutants.

Several biological processes that were expected to be different between the Prop1 and Pit1 dwarf mice based on their phenotypic variance were, in fact, altered in the microarray analysis of expression (Table 2). Due to the dysmorphology characteristic of the newborn Prop1<sup>df/df</sup>, we anticipated finding genes involved in tissue remodeling, organ morphogenesis, and anterior/posterior pattern formation. Some more noteworthy biological process GO terms were hormone metabolism, gland development, tissue development, and frizzled signaling pathway. Hormone metabolism included Sulf1el (sulfotransferase family 1E, member 1). Posttranslational modification by sulfonation of asparagine-linked oligosaccharides is shown to influence the bioactivity of LH by regulating its half-life in circulation (7). Sulfonated LH is more rapidly removed from the plasma, and this is necessary for the characteristic episodic rise and fall in levels of plasma LH that is essential for maximal bioactivity (6). The Prop1<sup>df/df</sup> mutant has relatively normal LHβ immunoreactivity in the pituitary gland, but it has low levels of circulating LH. The overexpression of Sulf1el might cause excessive sulfonation of LHβ, leading to rapid breakdown in the bloodstream.

Nine genes are differentially expressed in the Prop1<sup>df/df</sup> vs. Pit1<sup>df/df</sup> as well as in Prop1<sup>df/df</sup> vs. wild type, and were confirmed by RT-qPCR. These genes constitute an explicit list of possible Prop1-specific genes. The elevated expression of Adamdec1 (ADAM-like, decysin 1) and Cldn10 (Claudin 10) are of particular interest because of their potential roles in cell adhesion, which appears to be excessive and/or abnormally persistent in Prop1 mutants. Adamdec1 is significantly increased in the Prop1<sup>df/df</sup> pituitary gland. It is likely to be secreted and is a member of the Adam family of transmembrane genes that have cell adhesion and protease activities (62). Claudin proteins are important components of tight junctions (87). Tight junctions act as a barrier so that molecules do not pass between two interacting cells (2). An inability of cells to receive molecular signals that cue migration and/or differentiation may account for the dysmorphic pituitary seen in the Prop1 mutants. It has recently been shown that the Prop1 mutant mouse pituitary has an expanded expression pattern of N-cadherin (42), a cell adhesion molecule that must be down regulated for cell motility. Moreover, different pituitary cell types exhibit unique cadherin expression profiles, which could affect cell adhesion and consequently cell sorting and networks in the pituitary gland (15). One can hypothesize that Claudins, also cell adhesion molecules, may play a similar role in the organogenesis of the developing pituitary gland.

Differential notch signaling in Prop1 mutants. Various members of the Notch signaling pathway have been implicated in pituitary gland development (50, 71, 72, 97). In particular, expression of Hey1, a transcriptional repressor and potential downstream target of Notch2, is reduced in transgenic mice with constitutively activated Notch signaling (74). Consistent with this, we observed elevated expression of Hey1 in Prop1 mutants, which fail to activate Notch2 expression. Hey1 is normally expressed in the developing pituitary gland from e11.5 to e14.5 and then becomes greatly reduced at e16.5 (74). Hey1 promotes endothelial cell proliferation, migration, and organization of vessel formation (34). HEY1 inhibits bHLH function, which can impact establishment of cell lineages (85).

In particular, bHLH protein upstream stimulatory factor 1 (USF1) reduces the activity of α-subunit promoters (Cga) in pituitary cells (45, 46). Furthermore, misexpression of Hey genes during development, and their ability to repress helix-loop-helix genes, affects cell fate in neurons by maintaining neural precursor cells that are meant to differentiate into late-born cell types (75). It is possible that the role of Hey1 in the pituitary gland is similar, namely transforming progenitor gonadotropes into hormone producing gonadotropes.

Loss of Prop1 causes persistent Otx2 expression. The expression pattern of the transcriptional repressor OTX2 during pituitary development has important implications for understanding the role of OTX2 deficiency on hypopituitarism. Otx2 expression in the neural ectoderm suggests that it could affect FGF- and/or BMP-mediated induction of Rathke's pouch (26). Otx2 expression in the pouch itself suggests the possibility of an intrinsic role in pouch development as well. Otx2 is able to activate Hexx1 expression (28), and Hexx1 is necessary for normal pituitary development and function (22, 25). The expression pattern of Otx2 is consistent with the proposed role of regulating Hexx1 expression. In contrast, the idea that Otx2 regulates Pitl expression (24) seems unlikely based on the lack of expression of Otx2 in the caudo-medial area of the gland of wild-type mice where Pitl is normally activated at e14.5 (4, 36). The relative contributions of normal Otx2 expression in the ventral diencephalon and Rathke's pouch for induction and maintenance of Hexx1 expression could be assessed with tissue-specific disruptions of the Otx2 expression (35).

The normal expression patterns of Prop1 and Otx2 are not consistent with Prop1 directly repressing Otx2 expression. Aberrant Otx2 transcripts are not detected in Prop1 mutants until e16.5, 4 days after Prop1 expression peaks and 2 days after obvious dysmorphology is evident in Prop1 mutants. Prop1 may be required for activating expression of a gene or genes that suppress Otx2 in the anterior and intermediate lobes, or Prop1 could suppress an activator of Otx2 that is expressed in pouch derivatives. The consequences of ectopic expression of Otx2 in the pituitary of Prop1 mutants are not clear, but it could contribute to the unique defects in Prop1 mutant pituitary glands.

Pathway analysis identifies genes associated with Otx2. A hypothetical pathway generated by Bibliosphere software indicates a number of potential downstream targets for Otx2 that could contribute to the Prop1 mutant phenotype. A decrease in Otx2 results in a decrease in Neurod1 expression, which is
required for early corticotrope differentiation (54). According to this gene pathway, decreased Neuredl in the pituitary gland also leads to a decrease in Neurod4 expression, also reported in mouse retina (17). Neurod4 functions in the Notch signaling pathway by maintaining Notch ligand expression (65). It remains unclear whether Neurod4 is involved in the Notch pathway within the development of the pituitary gland, but it appears to be necessary for the proper onset of somatotrope specification (97).

Both Sox2 and Ntr2el are markers for stem cells, and their expression is increased concomitant with an increase of Otx2 in the Prop1 mutants. The pituitary gland has a population of multipotent progenitor cells that are marked by Sox2 (33). Prop1 mutants might be enriched for this stage of progenitor cells, due to the defects in transitioning from proliferation to differentiation (90). Later stages of progenitors are lacking in Prop1 mutants. Although Ntr2el expression has not been previously reported in the pituitary gland, it may be important for maintaining the multipotent and proliferative state of stem cells there, as it does in adult brain (77). These findings suggest that Prop1 may play an important role in regulating progenitor or stem cell activity as suggested by the coexpression of Prop1 with the stem-cell marker Sox2 (92).

Conclusion

Here we report a collection of potential downstream factors and pathways regulated by the Prop1 and Pitl genes. Otx2 expression is elevated of Prop1 mutants, and it plays a significant role in the formation of the gland in mouse and man. Pathway analyses suggest that Otx2 could be involved in the Notch pathway and stem cell perseverance in the developing pituitary gland. The other differentially expressed genes we report could also have important roles in pituitary development and function. Thus, this study lays a foundation for functional studies to define the roles of these novel genes and pathways in pituitary gland molecular processes.

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