The Forkhead Transcription Factor, Foxd1, Is Necessary for Pituitary Luteinizing Hormone Expression in Mice

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Abstract

The pituitary gland regulates numerous physiological functions including growth, reproduction, temperature and metabolic homeostasis, lactation, and response to stress. Pituitary organogenesis is dependent on signaling factors that are produced in and around the developing pituitary. The studies described in this report reveal that the forkhead transcription factor, Foxd1, is not expressed in the developing mouse pituitary gland, but rather in the mesenchyme surrounding the pituitary gland, which is an essential source of signaling factors that regulate pituitary organogenesis. Loss of Foxd1 causes a morphological defect in which the anterior lobe of the pituitary gland protrudes through the cartilage plate that is developing ventral to the pituitary at embryonic days (e)14.5, e16.5, and e18.5. The number of proliferating pituitary cells is increased at e14.5 and e16.5. Loss of Foxd1 also results in significantly decreased levels of Lhb expression at e18.5. This decrease in Lhb expression does not appear to be due to a change in the number of gonadotrope cells in the pituitary gland. Previous studies have shown that loss of the LIM homeodomain factor, Lhx3, which is activated by the FGF signaling pathway, results in loss of LH production. Although there is a difference in Lhb expression in Foxd1 null mice, these studies suggest that Foxd1 is indirectly required for normal Lhb expression and cartilage formation.

Introduction

The pituitary gland is a highly specialized organ that is essential for normal endocrine function. This essential gland secretes hormones that regulate growth, metabolism, reproduction, lactation, and response to stress [1]. Pituitary organogenesis begins in mice on embryonic day (e)8.5. By e10.5 the oral ectoderm invaginates and will form Rathke’s pouch by e12.5. The early stages of pituitary development are characterized by rapid proliferation. This is evident at e14.5 by the significant expansion of the anterior lobe of the pituitary. The last day of mouse embryonic development is e18.5 (the day before birth) [2].

The anterior lobe of the pituitary gland contains five specialized hormone-secreting cell types. Somatotropes produce growth hormone (GH) that targets the liver and bone. Lactotropes secrete prolactin (PRL) that acts on the mammary glands. Gonadotropes produce follicle-stimulating hormone (FSH) and luteinizing hormone (LH) that regulate function of the gonads. Thrytrotropes secrete thyroid-stimulating hormone (TSH) that targets the thyroid. FSH, LH, and TSH are dimeric hormones consisting of a common α-subunit (CGA) and a unique β-subunit (FSHB, LHb, TSHB). Finally, corticotropes produce adrenocorticotropic hormone (ACTH) that acts on the adrenal gland. The posterior lobe of the pituitary gland is stimulated by direct innervation from the hypothalamus and secretes oxytocin and anti-diuretic hormone. The intermediate lobe produces melanocyte-stimulating hormone.

Input from the hypothalamus stimulates the pituitary to secrete hormones that act on a number of target organs throughout the body to regulate a diverse range of physiological functions [2].

Differentiation of the different cell types is dependent on dorsal-ventral morphogenetic gradients that result in overlapping dorsal-ventral patterns of transcription factor expression. Signaling molecules such as bone morphogenetic proteins (BMPs), fibroblast growth factors (FGFs), and sonic hedgehog (SHH) are involved in initiating pituitary development [3,4].

Several forkhead factors have roles in pituitary development and function. Foxl2 (P66h) is the first forkhead to be described in the pituitary gland [5]. FOXL2 protein is detected in the prospective anterior lobe of the developing pituitary gland starting at e11.5 and continuing into adulthood in gonadotrope and thyrotrope cells of the anterior pituitary [6]. FOXL2 plays a role in regulating several gonadotropin genes including those coding for gonadotropin-releasing hormone receptor, the glycoprotein hormone α-subunit (Gα), and Fshb [6,7,8,9,10]. In fact, expression of Fshb is severely impaired in the absence of Foxl2, suggesting that Foxl2 is required for normal Fshb expression [10]. The forkhead factor, FOXP3, has a well-established role in the development and function of helper T cells [11,12]. While Foxp3 is not expressed in or even near the pituitary gland, it is important for pituitary function [13]. Scufy mice have a mutation in Foxp3 and have drastically reduced levels of Lhb and Fshb expression resulting in infertility [13,14,15]. FOXL1 represses growth hormone expres-
Foxd1 was originally known as brain factor-2 (Bf2) and is important for proper pituitary formation [20,21]. Heterozygous null mice have no obvious phenotype and are fertile [20]. Foxd1 homozygous null mice have small kidneys, decreased ureteric branching and die within 24 hours after birth due to renal failure. This is due, in part, to ectopic bone morphogenic (BMP) signaling, which causes mis-patterning of the kidney [20]. Foxd1 is also expressed in the retina and is required for normal development of the retina and optic chiasm [22]. The following studies demonstrate that while Foxd1 is not expressed in the developing pituitary gland, it is expressed in the mesenchyme surrounding the pituitary gland, which produces signaling factors that are essential for normal pituitary development.

Materials and Methods

Mice

Foxd1 heterozygous null mice were generated by Hatini et al. [20] and provided to us by Cathy Mendelsohn of Columbia University. To create the null Foxd1 allele, a LoxP cassette was inserted in place of the Foxd1 coding sequence. A phosphoglycerokinase (PGK)-neor cassette was inserted in the same orientation [20]. As Foxd1 is a single exon gene, this procedure eliminated nearly all of the Foxd1 coding sequence. Mice were maintained in a 12-hour dark-light cycle. Embryos were obtained from an intercross of Foxd1LacZ+/− mice. Foxd1 mutant mice are on a mixed Swiss Webster and C57BL/6J background. The morning the copulatory plug is detected is determined to be e0.5. Littermates were unmasked by boiling in 10 mM citric acid for 10 min, tissue sections were blocked using the Mouse on Mouse (M.O.M.) kit (Vector Laboratories) according to the manufacturer’s directions. Sections were unmasked by boiling in 10 mM citric acid for 10 min, tissue sections were blocked using the Mouse on Mouse (M.O.M.) kit (Vector Laboratories) according to the manufacturer’s directions.

Histology and Immunohistochemistry

Embryos were dissected and fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) (pH 7.2) for 45 min to 24 h (depending on stage of development). All samples were washed in PBS, dehydrated in a graded series of ethanol, and embedded in paraffin. Sections (3 μm) were deparaffinated in xylene, rehydrated through a series of graded ethanol washes, and stained in hematoxylin (Fisher Scientific) and eosin (Sigma) or used for immunohistochemistry.

To detect cell proliferation in embryonic pituitaries, pregnant mice were given an intraperitoneal injection of bromodeoxyuridine (BrdU) at 100 μg/g body weight 2 h before the embryos were harvested [23]. To visualize BrdU and LHX3, tissue sections were deparaffinated in xylene, rehydrated in ethanol, and incubated in 3% hydrogen peroxide to remove endogenous peroxidases. After epitopes were unmasked by boiling in 10 mM citric acid for 10 min, tissue sections were blocked using the Mouse on Mouse (M.O.M.) kit (Vector Laboratories) according to the manufacturer’s directions. Tissue sections were incubated overnight at 4°C with antibodies for BrdU (Invitrogen, clone ZBU30, 1:100). Histology and Immunohistochemistry

β-galactosidase Staining

Foxd1LacZ/+ embryos (e12.5-e18.5) were frozen and cryosectioned (5 μm). Frozen sections were post-fixed in 4% formaldehyde for 5 min, rinsed in PBS and stained overnight in β-galactosidase staining solution in 1X PBS (5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 1 mg/mL X-gal). Foxd1LacZ/+ embryos (e10.5) and adult pituitaries were stained whole mount for β-galactosidase as follows. Embryos and adult pituitaries were fixed in 4% formaldehyde for 1 hour, rinsed in PBS and stained overnight in β-galactosidase staining solution. After a series of graded ethanol washes, samples were embedded in paraffin and sectioned (3 μm).

Histology and Immunohistochemistry

Embryos were dissected and fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) (pH 7.2) for 45 min to 24 h (depending on stage of development). All samples were washed in PBS, dehydrated in a graded series of ethanol, and embedded in paraffin. Sections (3 μm) were deparaffinated in xylene, rehydrated through a series of graded ethanol washes, and stained in hematoxylin (Fisher Scientific) and eosin (Sigma) or used for immunohistochemistry.
Tissue sections were incubated with anti-mouse secondary (MOM kit, Vector Laboratories) 30 min at room temperature. Next, sections were incubated sequentially with strept-avidin-horseradish peroxidase and fluorescein from the Tyramide Signal Amplification kit (PerkinElmer). Following a 5 min incubation with water, sections were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (167 nM, Molecular Probes). One mid-sagittal section from each individual was photographed at a magnification of 200X. The total number of BrdU-positive cells in the intermediate and anterior lobe of each section was counted manually in three individuals per group.

To visualize pituitary hormones, tissue sections were deparaffinized and rehydrated as described above. Tissue sections were incubated with antibodies against GH (1:10,000; National Hormone and Peptide Program (NHPP)), POMC (1:500; NHPP), TSHB (1:2000, NHPP), LH (1:500, NHPP), CGA (1:300), or FSHB (1:250) for 1 hour at room temperature and then the appropriate secondary antibodies: anti-rabbit-TRITC (1:100, Jackson ImmunoResearch), anti-guinea pig-FITC (1:100, Jackson ImmunoResearch), or biotinylated anti-rabbit followed by horseradish peroxidase and FITC as described above. One mid-coronal section was photographed at a magnification of 200X for each individual. One half of the section was pictured in each photograph. Because coronal pituitary sections are symmetrical, this gives a good representation of cell types of the anterior lobe. The total number of LHB-positive cells in each photograph was counted manually. Three individuals per group were analyzed and values were set relative to wild type controls.

Programmed cell death in the pituitaries was also detected by the TUNEL method using the in situ cell detection kit POD (Roche) according to manufacturer’s instructions.

Cartilage was stained by Gomori’s aldehyde fuchsin stain as follows. Tissue sections were deparaffinized and rehydrated before incubating 10–60 min in 0.5% iodine. Tissue sections were then decolorized with 0.5% sodium bisulfite, washed in water and transferred to 70% alcohol. Tissue sections were stained in aldehyde fuchsin solution (0.5% basic fuchsin, 70% ethanol, 1% paraldehyde, pH 1.0) for 1–2 hr and rinsed in 70% ethanol.

Digital images of pituitary sections were captured with a Leica DM 5000B fluorescent microscope and Retiga 2000R digital camera. FITC and DAPI pictures were merged using Adobe Photoshop CS3.

RT-PCR
Pituitaries were dissected from e18.5 embryos. Total RNA was isolated with the RNAqueous-Micro kit (Ambion, Inc.) according to manufacturer’s directions. RNA concentrations were determined by spectrophotometry. RNA was treated with DNase I and DNase inactivating reagent from the TURBO DNase-free kit (Ambion, Inc.) as per manufacturer’s instructions. ImPromII reagents and random primers (Promega) were used to synthesize cDNA.

Real time RT-PCR was performed on a CFX96 Real Time System (BioRad). Amplification was accomplished using Taqman Gene Expression Assays (Applied Biosystems) as per manufacturer’s instructions. Five ng of cDNA was used in a 15 μL reaction volume. Samples and controls were run in triplicate. No-template controls and no-reverse transcriptase controls were used to assure the absence of contamination and efficacy of the DNase treatment, respectively. Five to six individuals were included in each group.

Data were analyzed by the ΔΔCT method [24,25]. The values for ΔΔCT were calculated by subtracting the average ΔCT of wild type controls from the ΔCT for each sample. Ct values over 30 were considered unreliable and were not included in our analyses.

Statistical Analysis
All results are expressed as mean ± SEM. Data were analyzed by Student’s t-test using Microsoft Excel. P-values less than 0.05 were considered significant (*). P-values less than 0.01 were considered very significant (**).
Results

Normal Foxd1 Expression

Foxd1 was identified in an expression library developed from pituitary at e14.5, suggesting that it may be important for pituitary development [26]. Foxd1 expression was confirmed by real-time RT-PCR (data not shown). To visualize Foxd1 expression, mice in which the Foxd1 allele has been replaced with the coding sequence for LacZ were analyzed [20]. Sections from mouse embryos that were heterozygous for the engineered Foxd1 allele (Foxd1<sup>-/-</sup>LacZ) were stained for β-galactosidase. This study reveals that Foxd1 is not expressed in the pituitary gland during development, but rather in the mesenchyme surrounding the pituitary (Fig. 1A–C). Many signaling factors that are important for pituitary organo-

Figure 3. Loss of Foxd1 expression results in disruption of the developing cartilage plate. Mid-sagittal (A–F) and coronal (G–H) sections from Foxd1<sup>LacZ</sup> embryos and wild type littermates were stained with Gomori’s aldehyde fuchsin to visualize cartilage. (A–B) The prospective pituitary gland and surrounding tissue appears normal at e12.5. (C–H) A break in the cartilage plate ventral to the pituitary is apparent in Foxd1<sup>LacZ</sup> embryos by e14.5 (arrows). Pictures were taken at 100X and scale bars represent 100 μm.

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genesis are expressed in the mesenchyme surrounding the pituitary, thus FOXD1 could contribute to transcriptional regulation of these factors [27,28].

Pituitary Morphology

The expression of Foxd1 in the mesenchyme surrounding the developing pituitary led us to investigate the role of FOXD1 in pituitary development. Morphology of the pituitary gland was examined in embryos lacking Foxd1 expression. Hematoxylin and eosin stains of Foxd1 null and wild type embryos revealed that the pituitary gland extends through the cartilage plate ventral to the pituitary gland (Fig. 2A–L). To better visualize the cartilage plate, Gomori’s aldehyde fuchsin stains were performed. These experiments showed that the developing pituitary extends through the cartilage plate evident by e14.5 (Fig. 3A–H).

Pituitary Hormone Expression

The pituitary gland is essential for orchestrating numerous physiological processes. To determine if Foxd1 expression is important for pituitary function, we performed real time RT-PCR on pituitary from e18.5 Foxd1 wild type and null embryos. This age was chosen because most pituitary hormones are detectable at this age and Foxd1 null mice die within 24 hours after birth. Pth was excluded from this analysis because C1 values for Pth at e18.5 are consistently over 30 and we feel that this does not represent an accurate measurement of expression (data not

![Graph showing relative expression of various factors in Foxd1 null and wild type embryos](image-url)

**Figure 4. Expression of luteinizing hormone is reduced in Foxd1lacZ/lacZ embryos.** (A) Real time RT-PCR revealed that expression of Lhb is significantly reduced in embryos lacking Foxd1 expression compared to wild type littermates at e18.5 (P<0.05). Expression of other pituitary hormones was not significantly reduced. (B–K) No apparent differences in GH, TSHB, POMC, FSHB, or CGA were observed. (L–M) The intensity of LHB staining is reduced in mutant pituitary glands. (N) The number of LHB-positive cells was counted manually and set relative to wild type controls. No significant difference was detected in Foxd1lacZ/lacZ pituitaries as compared to wild type. Cell nuclei are stained with DAPI (blue). Pictures were taken at 200X (B–K) or 630X (L–M). Scale bars represent 100 μm.

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shown). This is consistent with findings by Brannick et al., demonstrating that neither prolactin protein nor prolactin mRNA is detectable until after postnatal day 1 [29]. Expression of growth hormone, thyroid-stimulating hormone-β, pro-opiomelanocortin, follicle-stimulating hormone-β, and the glycoprotein hormone α-subunit is not significantly different between wild type and null embryos (Fig. 4A). However, expression of luteinizing hormone is significantly decreased in Foxd1 null embryos as compared to wild type littermates (Fig. 4A). No apparent loss of somatotrope, thyrotrrope, or corticotrope cells was observed in Foxd1 null embryos (Fig. 4B–K). While the intensity of staining is decreased for LHB, the number of gonadotrope cells is not decreased in Foxd1 null embryos, suggesting that it is the level of Lhb expression that is decreased and not an inability of the gonadotropes to differentiate that causes the lower Lhb expression (Fig. 4L–N). This suggests that FOXD1 is required for normal pituitary expression of luteinizing hormone.

**Pituitary Cell Apoptosis and Proliferation**

Apoptosis and proliferation are essential processes for normal pituitary development. Apoptosis occurs at e10.5 to separate Rathke’s pouch from the oral ectoderm that will form the lining of the mouth [30]. After Rathke’s pouch is separated from the rest of the oral ectoderm, a cartilage plate forms to separate the pituitary from the oral cavity. If apoptosis fails to occur, the pituitary will remain attached to the rest of the oral ectoderm and the cartilage plate will not form completely [31]. To determine if failure of apoptosis caused the pituitary of Foxd1 null embryos to protrude through the cartilage plate, apoptotic cells were labeled by TUNEL analysis. No significant difference in the number of apoptotic pituitary cells was detected in Foxd1 null embryos as compared to wild type littermates (Fig. 5A–F). This suggests that the pituitary/cartilage dysmorphology in Foxd1 null embryos is not due to a failure in apoptosis.

Excessive pituitary cell proliferation can cause the pituitary gland to increase in size and protrude through the cartilage plate. Actively dividing cells were labeled in Foxd1 wild type and null embryos from e10.5 through e18.5 with the thymidine analog...
bromodeoxyuridine (BrdU) (Fig. 6A–J). The number of BrdU-positive pituitary cells was counted manually. The number of proliferating pituitary cells is significantly increased in Foxd1LacZ/LacZ embryos at e14.5 and e16.5 (Fig. 6K).

Figure 6. Pituitary cell proliferation is increased in Foxd1LacZ/LacZ embryos. (A–J) Actively dividing cells were labeled with bromodeoxyuridine (BrdU). Immunohistochemistry was performed to detect BrdU in mid-sagittal sections from Foxd1LacZ/LacZ embryos and wild type littermates. (K) The number of BrdU-positive cells was manually counted in pituitary sections from Foxd1LacZ/LacZ embryos and wild type littermates. The number of BrdU-positive cells is significantly higher in Foxd1LacZ/LacZ embryonic pituitaries at e14.5 and e16.5 as compared to wild type littermates (P<0.01).

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LHX3

Fibroblast growth factor (FGF) signaling is essential for normal pituitary development. Pituitary expression of the LIM homeodomain factor, Lhx3, is regulated by FGF and loss of Lhx3 results in a...
decrease in LH production [31,32,33,34]. To determine if the expression patterns for LHX3 are altered in the developing pituitary in the absence of Foxd1, immunohistochemistry for LHX3 was performed. These studies indicate that LHX3 immunoreactivity is not different in Foxd1 null embryos as compared to wild type littermates (Fig. 7A–L).

Discussion

Forkhead transcription factors are important for diverse physiological functions including embryonic development of almost every type of tissue [35,36,37,38]. In order to identify genes that contribute to pituitary development and function, we have turned to the forkhead family. Foxd1 was identified in an embryonic pituitary expression library [26]. However, when Foxd1 expression was analyzed by β-galactosidase staining of Foxd1+/LacZ embryos, it was discovered that Foxd1 is expressed in the mesenchyme surrounding the pituitary, but not in the pituitary itself, suggesting that when dissecting pituitaries from e14.5 mouse embryos, some mesenchyme was obtained as well. The mesenchyme surrounding the developing pituitary gland is a rich source of signaling factors that are essential for normal pituitary development. Bmp2 is expressed in the mesenchyme ventral and rostral to Rathke’s pouch at e12.5 and e14.5 and is believed to be important for inducing proliferation and differentiation of ventral cell types [5,39]. The BMP inhibitors chordin and noggin are expressed in the mesenchyme caudally and ventrally, respectively, to the pituitary by approximately e12.5 [5,27,39]. FOXD1 is present in the mesenchyme ventral and caudal to the putative pituitary gland from e12.5 and continuing through development. This expression pattern places FOXD1 in a region where it could affect several signaling factors that are important for pituitary development.

Expression of Lhb was significantly reduced in Foxd1 mutant embryos as compared to wild type littermates. These data demonstrate that Foxd1 is indirectly required for Lhb expression in gonadotrope cells. Because Foxd1 is not expressed in the developing pituitary gland, but is present in the mesenchyme surrounding the developing pituitary, the reduction in Lhb expression may be due to the loss of signaling factors from the mesenchyme surrounding the pituitary gland. Signals from outside of the pituitary are important for proper specification of pituitary cells [34]. FGFs from the infundibulum can increase immunoreactivity for ACTH and decrease immunoreactivity for the LIM homeodomain factor, ISL1, as well as for αGSU [34]. In contrast, BMPs from the ventral juxtapituitary mesenchyme increases immunoreactivity for αGSU and decreases immunoreactivity for ACTH [34]. In embryos null for noggin, a BMP2 and 4 antagonist, a secondary pituitary is sometimes induced, however in the primary pituitary all hormones are produced normally [27]. The BMP antagonist, chordin, is expressed in the caudal mesenchyme adjacent to Rathke’s pouch at e12.0 and may be important for counteracting BMP2 signals [5]. It may be that FOXD1 is important for production of one or more of these signaling factors and that loss of FOXD1, and ultimately of certain signals from the mesenchyme, disrupts Lhb expression.

Another possibility is that loss of Foxd1 in the mesenchyme surrounding the hypothalamus affects hypothalamic function or that GnRH neuronal migration is abnormal. In mouse embryos lacking Foxd1 retinal ganglion cell axons that form the optic chiasm aberrantly project contralaterally [22]. Thus, FOXD1 appears to be important for proper neuronal migration. Hpg mice, which have a mutation of the Gnrh gene resulting in a loss of GnRH secretion, exhibit reduced expression of Lhb and Fshb [40,41]. In Foxd1 mice expression of the GnRH-responsive genes Fshb and Cga is normal. Thus, it seems unlikely that GnRH stimulation of gonadotrope cells is altered in Foxd1 mutants.
Apoptosis is an important process in pituitary development, allowing the oral ectoderm that will form the pituitary gland to separate from the oral ectoderm that will form the lining of the mouth with a cartilage plate forming between. Foxd1 mutants exhibit normal apoptosis, however abnormal apoptosis has been observed with other mutations [27,31,42,43]. The pituitary fails to separate from the lining of the oral cavity in the absence of Lhx3 expression, however it has not been determined if a failure in apoptosis during early development is responsible [31]. Increased apoptosis later in development does contribute to the hypopituitarism observed in Lhx3 null embryos [42,43]. In the absence of the BMP inhibitor, noggin, apoptosis in Rathke’s pouch is decreased and the pituitary fails to separate from the oral ectoderm, disrupting formation of the cartilage plate [27].

Proliferation is an essential process in organogenesis. During pituitary development, proliferation occurs in Rathke’s pouch, but not in the rostral tip of the pituitary gland [6]. Loss of TCF4, which mediates WNT signaling, causes pituitary cell hyperplasia, leading to a disruption of the cartilage plate ventral to the pituitary gland [44,45]. The number of proliferating pituitary cells was increased at e14.5 and e16.6 in embryos lacking Foxd1, suggesting that Foxd1 is important for regulating proliferation in the developing pituitary gland. This increase in pituitary cell proliferation may contribute to the break in the cartilage plate ventral to the pituitary gland as seen in Tgf4 mutants.

There is much to be learned about how signaling factors from the juxtapituitary mesenchyme regulate pituitary hormone production. The data described in this manuscript demonstrate that Foxd1 indirectly contributes to normal expression of Lhb, but not the gonadotropin genes Fshb and Cga. In the absence of Foxd1, pituitary cell proliferation is increased and the cartilage plate ventral to the pituitary fails to fuse. These studies provide important clues as to the role of FOXD1 and the juxtapituitary mesenchyme during pituitary development and for pituitary function.

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Author Contributions

Conceived and designed the experiments: JHG EMP BEK DQJ BSE. Performed the experiments: JHG EMP SAO JS TH BSE. Analyzed the data: JHG EMP SAO JS TH BSE. Wrote the paper: JHG DQJ BSE.

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