SOX2 Plays a Critical Role in the Pituitary, Forebrain, and Eye during Human Embryonic Development

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Context: Heterozygous, *de novo* mutations in the transcription factor SOX2 are associated with bilateral anophthalmia or severe microphthalmia and hypopituitarism. Variable additional abnormalities include defects of the corpus callosum and hippocampus.

Objective: We have ascertained a further three patients with severe eye defects and pituitary abnormalities who were screened for mutations in *SOX2*. To provide further evidence of a direct role for *SOX2* in hypothalamo-pituitary development, we have studied the expression of the gene in human embryonic tissues.

Results: All three patients harbored heterozygous *SOX2* mutations: a deletion encompassing the entire gene, an intragenic deletion (c.70_89del), and a novel nonsense mutation (p.Q61X) within the DNA binding domain that results in impaired transactivation. We also show that human SOX2 can inhibit β-catenin-driven reporter gene expression *in vitro*, whereas mutant SOX2 proteins are unable to repress efficiently this activity. Furthermore, we show that *SOX2* is expressed throughout the human brain, including the developing hypothalamus, as well as Rathke's pouch, the developing anterior pituitary, and the eye.

Conclusions: Patients with *SOX2* mutations often manifest the unusual phenotype of hypogonadotropic hypogonadism, with sparing of other pituitary hormones despite anterior pituitary hypoplasia. *SOX2* expression patterns in human embryonic development support a direct involvement of the protein during development of tissues affected in these individuals. Given the critical role of Wnt-signaling in the development of most of these tissues, our data suggest that a failure to repress the Wnt-β-catenin pathway could be one of the underlying pathogenic mechanisms associated with loss-of-function mutations in *SOX2*. **(*J Clin Endocrinol Metab* 93: 1865–1873, 2008)**

SOX2 (OMIM 184429) belongs to the SOX family of transcription factors that contain a 79-amino acid high mobility group (HMG) box DNA-binding domain similar to that found in the sex-determining gene *SRY* (OMIM 480000) (1, 2). *SOX1* (OMIM 602148), *SOX2*, and *SOX3* (OMIM 313430) belong to the B1 subfamily and are expressed in various phases of embryonic development and cell differentiation, in which they play critical roles from the earliest stages of development (3, 4).

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Abbreviations: CS, Carnegie Stage; E, embryonic d; F, fetal stage; FT4, free T_4; HMG, high mobility group; MRI, magnetic resonance imaging; NR, normal range; SDS, SD score; TBST, Tris-buffered saline with Tween 20.

In the mouse, *Sox2* expression is first detected in cells at the morula stage at embryonic d (E) 2.5 and then in the inner cell mass of the blastocyst (E3.5) (5). After gastrulation, *Sox2* expression becomes largely restricted to presumptive anterior neuroectoderm, and by E9.5 is expressed throughout the central nervous system, sensory placodes, branchial arches, and gut endoderm, including the developing esophagus (1, 6, 7).

Sox2$^{-/-}$ null embryos die shortly after implantation; however, *Sox2* heterozygous mice appear relatively normal, but about one third show perinatal lethality, and others show a reduction in size and male fertility (5). We have recently demonstrated a role for *Sox2* in the development of the anterior pituitary gland in the mouse. Analysis of heterozygous mutant mice showed a variable hypopituitary phenotype affecting the size and shape of the pituitary gland, and a significant lowering of pituitary GH, LH, ACTH, and TSH contents in affected mice (8).

Heterozygous mutations within *SOX2* in humans have been associated with bilateral anophthalmia or severe microphthalmia, in addition to hypopituitarism characterized by anterior pituitary hypoplasia and gonadotropin deficiency (hypogonadotropic hypogonadism) with genital abnormalities in males (7–9). Additional forebrain defects may also include hypoplasia of the corpus callosum, hypothalamic hamartoma, and hippocampal malformation (8, 10), frequently associated with additional abnormalities, including esophageal atresia, sensorineural hearing loss, and learning difficulties.

We now report the endocrine phenotype in a further three patients with *de novo* heterozygous mutations in *SOX2*, including a novel mutation in the HMG box (p.Q61X), in addition to an individual with an interstitial deletion of chromosome 3q26-q27 encompassing the entire gene. We describe here for the first time the expression pattern of *SOX2* during human embryonic development using *in situ* hybridization and immunohistochemistry. In addition, because the disruption of Wnt signaling and the normal function of β-catenin have been associated with pituitary defects in mice (11, 12), and because SOX proteins have been reported to interact with β-catenin (13, 14), we have used an *in vitro* reporter system to show that several of the naturally occurring mutations identified in patients impair the ability of human SOX2 to antagonize the transcriptional activity of β-catenin.

Patients and Methods

Patients (Table 1)

Patient 1

Patient 1 has previously been reported to show an unusual karyotype consisting of a *de novo* translocation with an associated interstitial deletion of chromosome 3q at the break point: 46,XX,del (3) (q26.33q28)t (3, 7) (q28;q21.1) (patient 2 in Ref. 15). This female patient was born to nonconsanguineous parents at term by a normal vaginal delivery with a birth weight of 1.42 kg [−5.68 SD score (SDS)]. She had right anophthalmia and left microphthalmia, and mild pulmonary stenosis. Global developmental delay was noted at the age of 15 months. A right ocular prosthesis was inserted and a corneal graft performed in the left eye. Visual evoked responses and an electroretinogram revealed good retinal and optic nerve function. Audiometry revealed normal hearing.

TABLE 1. Summary of patient phenotypes

Patient no.	Mutation	Age (yr)	Height (SDS)	Peak GH (μg/liter)	IGF-I	Peak TSH (mU/liter)	Basal prolactin (μg/liter)	Peak (basal[a]) cortisol (μg/dl)	Peak LH (IU/liter)	Peak FSH (IU/liter)	Ocular phenotype	Additional features
1	*SOX2* deletion	1.5	−2.0	26.8	+1 SDS	23.5	12.1	24	3.5	7.8	Right anophthalmia, left microphthalmia	AP hypoplasia, thin corpus callosum, mild pulmonary stenosis, developmental delay
2	p.Q61X	19	−1.2	ND	264.6 ng/ml (215–885)[b]	ND	ND	7.3[a]	2.1	2.0	Bilateral anophthalmia	Developmental delay, absence of pubertal development with HH, MRI not performed
3	c.70del20	14.5	−1.31	14.1 (C), 10.2 (A)	312 ng/ml (97–699)[b]	12.3	24.6	ND	2.6	2.7	Bilateral anophthalmia	Absence of pubertal development with HH

A, Arginine; AP, anterior pituitary; C, clonidine; HH, hypogonadotropic hypogonadism; ND, not determined.

[a] Basal value.

[b] Normal range.

Magnetic resonance imaging (MRI) revealed a hypoplastic anterior pituitary, a normal infundibulum, an eutopic posterior pituitary, and a thin corpus callosum with prominent ventricles and some frontotemporal volume loss (Fig. 1). Subsequently, growth velocity was poor over the first 18 months (height 65.7 cm, −5 SDS; weight 5.54 kg, −4.36 SDS; height velocity −2 SDS between the ages of 0.5 and 1.55 yr). Investigations revealed a peak GH of 26.8 μg/liter (67.2 mU/liter), with a normal cortisol of 24 μg/dl (670 nmol/liter), normal prolactin [12.1 μg/liter (242 mU/liter)], and normal thyroid function [free T_4 (FT4) 1.5 ng/dl (19.4 pmol/liter); peak TSH to TRH 23.5 mU/liter]. Gonadotropin deficiency could not be excluded (peak LH to LHRH 3.5 IU/liter, peak FSH to LHRH at 60 min of 7.8 IU/liter at 20 months age). Subsequently, her height velocity ranged from −1.93 to −2.7 SDS but with an IGF-I and its binding protein-3 concentrations of 227 ng/ml (+1 SDS) and 3.30 mg/liter (0 SDS), respectively. The proband is now aged 6.5 yr, and no endocrine treatment is required at present.

Patient 2

This female patient was referred to the pediatric endocrine service aged nearly 19 yr due to a lack of pubertal development. She exhibited bilateral anophthalmia, but no symptoms of wider pituitary dysfunction. She had evidence of significant neurodevelopmental delay. She reported a normal sense of smell, and there was no family history of delayed puberty. She had Tanner stage V pubic hair, stage II axillary hair, mature nipple development but no significant underlying breast tissue. Her height was on the 9th centile, and the weight was between the 25th and 50th centiles. Investigations demonstrated a normal karyotype, early morning urinary concentrating capacity (838 mOsm/kg), thyroid function [FT4 1.3 ng/dl (16.8 pmol/liter), TSH 1.3 mU/liter], cortisol [7.3 μg/dl (201 nmol/liter); normal range (NR) 5–25 μg/dl (138–690 nmol/liter)], and IGF-I (264.6 ng/ml; NR 215–885 ng/ml) concentrations. Basal gonadotropins were undetectable (both < 1.0 U/liter), and in response to an LHRH stimulation test, LH increased to 2.1 U/liter and FSH to 2.0 U/liter. A pelvic ultrasound showed a 3-cm length uterus, but no ovaries were identified. These clinical, biochemical, and radiological findings were interpreted to be in keeping with hypogonadotropic hypogonadism. Her parents were reluctant for her to undergo intracranial imaging given the likely need for sedation or anesthesia.

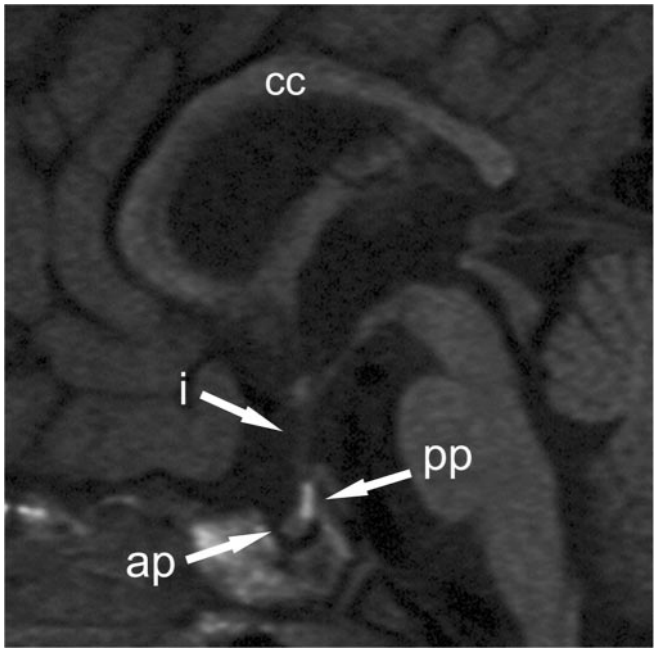


FIG. 1. Midline sagittal MRI section of patient 1 showing hypoplasia of the anterior pituitary (ap), normal infundibulum (i), with an eutopic posterior pituitary (pp) and a thin abnormal corpus callosum (cc).

Patient 3

Patient 3 is a 14.5-yr-old girl who is the second child of four offspring, born to unrelated parents at full term after a pregnancy complicated by uterine contractions in the fifth month that waned with tocolytic therapy that was maintained until delivery. The birth weight was 3.35 kg (−0.5 SDS) and birth length 50 cm (−0.38 SDS). She presented with bilateral anophthalmia. At 15 d of life, she underwent corneal transplantation, and brain MRI showed an arachnoid cyst in the suprasellar area, with right deflection of the pituitary pedunculus. She was referred to the endocrinologist at the age of 9.4 yr because of short stature (height 125.8 cm, −1.31 SDS; weight 24.2 kg, −1 SDS). Investigations performed at the age of 11.4 yr revealed normal thyroid function [FT4 1.01 ng/dl (13 pmol/liter), TSH 2.45 mU/liter], and a peak GH response to clonidine of 14.1 μg/liter (42.3 mU/liter) and to arginine stimulation of 10.2 μg/liter (30.6 mU/liter). The IGF-I concentration was within the NR (312 ng/ml; NR 97–699 ng/ml). Subsequent investigations at the age of 14.5 yr, at which stage she had no evidence of puberty (height −2.58 SDS, weight −0.34 SDS), revealed gonadotropin deficiency with low basal gonadotropin concentrations (LH undetectable, FSH 0.6 IU/liter) and poor responses to GnRH stimulation (peak LH 2.6 IU/liter; peak FSH 2.7 IU/liter). TRH testing performed at this time showed normal thyroid function with normal TSH (peak 12.3 mU/liter) and prolactin [peak 24.6 μg/liter (492 mU/liter)] responses, and a normal FT4 [1.3 ng/dl (16.8 pmol/liter); NR 0.8–1.8] concentration. At the last outpatient visit, no pubertal development had occurred, and estrogen replacement is currently being considered. There was no evidence of learning difficulties.

Mutation analysis

To detect chromosomal abnormalities, we used the Agilent 44k Human Genome CGH microarray (Agilent Technologies, Wokingham, UK). Genomic DNA from patient 1 and reference normal human male genomic DNA (Promega Corp., Madison, WI) were digested and labeled incorporating either 3 mM Cy5-deoxyuridine 5-triphosphate (test sample) or Cy3-deoxyuridine 5-triphosphate (male reference). Purified Cy5- and Cy3-labeled DNA was combined and mixed with human Cot-1 DNA (Invitrogen, Paisley, UK). Automated hybridization and washes were performed in an HS Pro 4800 Pro Hybridization Station (Tecan UK Ltd., Reading, UK). Slides were scanned using an Agilent microarray scanner (Agilent), and data analysis was performed using Agilent Analytics v3.3.27 software.

For intragenic mutations, the coding sequence of *SOX2* was amplified from genomic DNA and directly sequenced as described elsewhere (8). Sequences were compared with *SOX2* reference sequence (RefSeq NM_003106). Ethical committee approval was obtained from the Institute of Child Health/Great Ormond Street Hospital for Children Joint Research Ethics Committee. Informed written consent was obtained from the parents, and, where applicable, the patients before collection of samples and genomic analysis.

Plasmid constructs

PCR products comprising the entire *SOX2* coding region from patient 2 were generated and cloned into pcDNA3.1(+) (Invitrogen) and pCMV/SV-Flag, containing an in-frame N-terminal FLAG epitope. Constructs containing wild-type *SOX2*, in addition to the mutations c.60_61insG, c.387_388delC, and p.Q177X, have been previously described (8). A clone containing the full-length human β-catenin coding sequence (*CTNNB1*; IMAGE Consortium CloneID 6151332) (16) was obtained from Geneservice Ltd. (Cambridge, UK). To generate a constitutively active phosphorylation mutant of β-catenin, the mutation c.96C>A (p.S33Y) (17) was introduced using the QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA).

Luciferase assay experiments

SOX2 luciferase reporter assays were performed using Chinese hamster ovary cells as previously described (8). For the TOPFLASH assay (18), human embryonic kidney 293 cells were cotransfected with 20 ng

TOPFLASH reporter, 30 ng β-catenin expression construct, and increasing amounts of wild-type *SOX2* expression construct (1–70 ng). Luciferase activity was measured using a BMG FLUOstar Optima multiplate reader (BMG LABTECH Gmbh, Offenburg, Germany), and experiments were each repeated three times independently and performed in triplicate.

Cell localization studies

Chinese hamster ovary cells were transfected with 50 ng FLAG-tagged SOX2 expression construct (wild type or p.Q61X). After transfection, cells were washed, fixed, and immunostaining was performed as described elsewhere (8).

EMSA

SOX2 proteins were generated using the TNT Quick Coupled Transcription/Translation System (Promega). EMSAs were performed as described previously (8).

In situ hybridization

Analysis of human embryonic/fetal material was performed by the Medical Research Council-Wellcome Trust Human Developmental Biology Resource *in situ* hybridization service with full ethical approval from the Joint Great Ormond Street Hospital National Health Service Trust/Institute of Child Health Ethics Committee.

Human embryos/fetuses at selected stages were dissected and fixed in 4% paraformaldehyde, then dehydrated and embedded in paraffin wax. Sections of 7 μm were cut using a standard microtome and mounted on Superfrost Plus slides (BDH Laboratory Supplies, Poole, UK). *In situ* hybridization was performed essentially as described by Wilkinson (19) using digoxigenin incorporated riboprobes generated from a 697-bp fragment of the 3′ untranslated region of human *SOX2* and cloned into the pCR4-TOPO vector (Invitrogen). For antibody detection, slides were incubated with antidigoxigenin antibody conjugated with alkaline phosphatase (diluted 1:1000, containing 2% fetal calf serum). Expression patterns were visualized using the Nitro-Blue Tetrazolium Chloride/5-Bromo-4-Chloro-3′-Indolyphosphate p-Toluidine Salt system (Roche, Welwyn Garden City, UK). Sections were mounted in VectaMount (Vector Laboratories, Burlingame, CA) and analyzed using the Axioplan2 imaging system (Carl Zeiss, Inc., Thornwood, NY).

Immunohistochemistry

Human embryo and fetal sections were dewaxed and rehydrated. Antigen retrieval was performed by boiling for 10 min in 0.01 M citric acid buffer (pH 6.0). After cooling to room temperature, slides were transferred to Tris-buffered saline with Tween 20 (TBST) [0.5 M Tris Base, 9% NaCl (pH 7.6), 0.025% Triton] and blocked with 10% heat-inactivated rabbit serum (Sigma-Aldrich, St. Louis, MO) in TBST. Slides were incubated with goat anti-SOX2 antibody (1:500 dilution; Immune Systems Ltd., Paignton, UK) in TBST with 1% heat-inactivated rabbit serum, then washed and incubated with a polyclonal rabbit antigoat IgG biotinylated secondary antibody (1:100 dilution; Dako Corp., Carpinteria, CA). Biotin was detected using streptavidin-Alexa Fluor 555 conjugate in PBS (1:300 dilution; Molecular Probes, Inc., Eugene OR). Slides were mounted in Vectashield with DAPI (Vector Laboratories) and images captured on a Leica DC500 microscope using Fire Cam Software (Leica Microsystems GmbH, Wetzlar, Germany).

Results

Mutation analysis

Fluorescent *in situ* hybridization analysis of patient 1 showed that *SOX2* was deleted on the der(3) chromosome of the patient in all metaphase preparations examined (Fig. 2A). Subsequently, microarray comparative genomic hybridization analysis defined the extent of the deletion to approximately 7.8 Mb of chromosomal region 3q26.33-q27.3 (start: 181216930 bp; end: 189014508 bp) (Fig. 2B). The array CGH data showed no other significant copy number changes.

Two other individuals were found to harbor *de novo* heterozygous mutations in *SOX2*. In patient 2 we identified the novel transition c.181C>T (Fig. 2C), resulting in the nonsense mutation p.Q61X, which is predicted to result in a mutant protein truncated within the HMG domain. This mutation creates a BfaI restriction site that was used to genotype the parents of the affected individual, neither of whom carried the mutation (Fig. 2C). Patient 3 was found to harbor a deletion of 20 nucleotides upstream of the HMG domain (c.70_89del), which occurred *de novo* in this individual because it was not present in her unaffected parents (data not shown).

Molecular analysis of *SOX2* mutations

The single exon structure of *SOX2* implies that nonsense-mediated decay mechanisms are unlikely to result in degradation of the mutant transcript (20, 21). Therefore, we sought to confirm the functional consequence of the p.Q61X mutation *in vitro*. Luciferase reporter assays using a reporter construct previously shown to be activated by SOX2 *in vitro* (8) showed that the mutant construct failed to activate this reporter (Fig. 2D) as a result of the complete loss of DNA binding, compared with wild-type SOX2 (Fig. 2E). We also investigated the localization of the mutant protein within the cell. Whereas the majority of wild-type SOX2 was localized within the cell nucleus, the p.Q61X mutant protein showed predominantly cytoplasmic staining (Fig. 2F). The c.70_89del mutation has previously been identified in other unrelated individuals and shown to result in the loss of function of the predicted protein product (8, 22, 23).

Previous studies have demonstrated that murine Sox2 is capable of interfering with the activity of a β-catenin responsive promoter containing TCF/LEF binding sites mediated by the COOH terminal portion of Sox2 outside of the DNA binding HMG domain (13). To investigate the ability of human SOX2 to repress β-catenin-TCF-mediated transcription, we performed a TOPFLASH reporter assay (18). Cotransfection of the reporter with an expression construct containing human β-catenin leads to increased activation of the reporter. Cotransfection with increasing amounts of wild-type human SOX2 led to a dose-dependent reduction in β-catenin induced activation, whereas SOX2 alone had no effect on transcriptional activation of the reporter (Fig. 3A). To determine the effect of various naturally occurring truncating mutations in *SOX2*, we cotransfected SOX2 expression constructs containing the mutations c.60_61insG, p.Q61X, c.387_388delC, and p.Q177X (c.529C>T); the latter two maintain an intact HMG domain and retain the ability to bind DNA *in vitro* (8). Products from all four of these mutations failed to repress β-catenin-mediated transcription (Fig. 3B). Repeating the experiments using a constitutively active β-catenin mutant construct (17) showed identical results to wild-type β-catenin (data not shown).

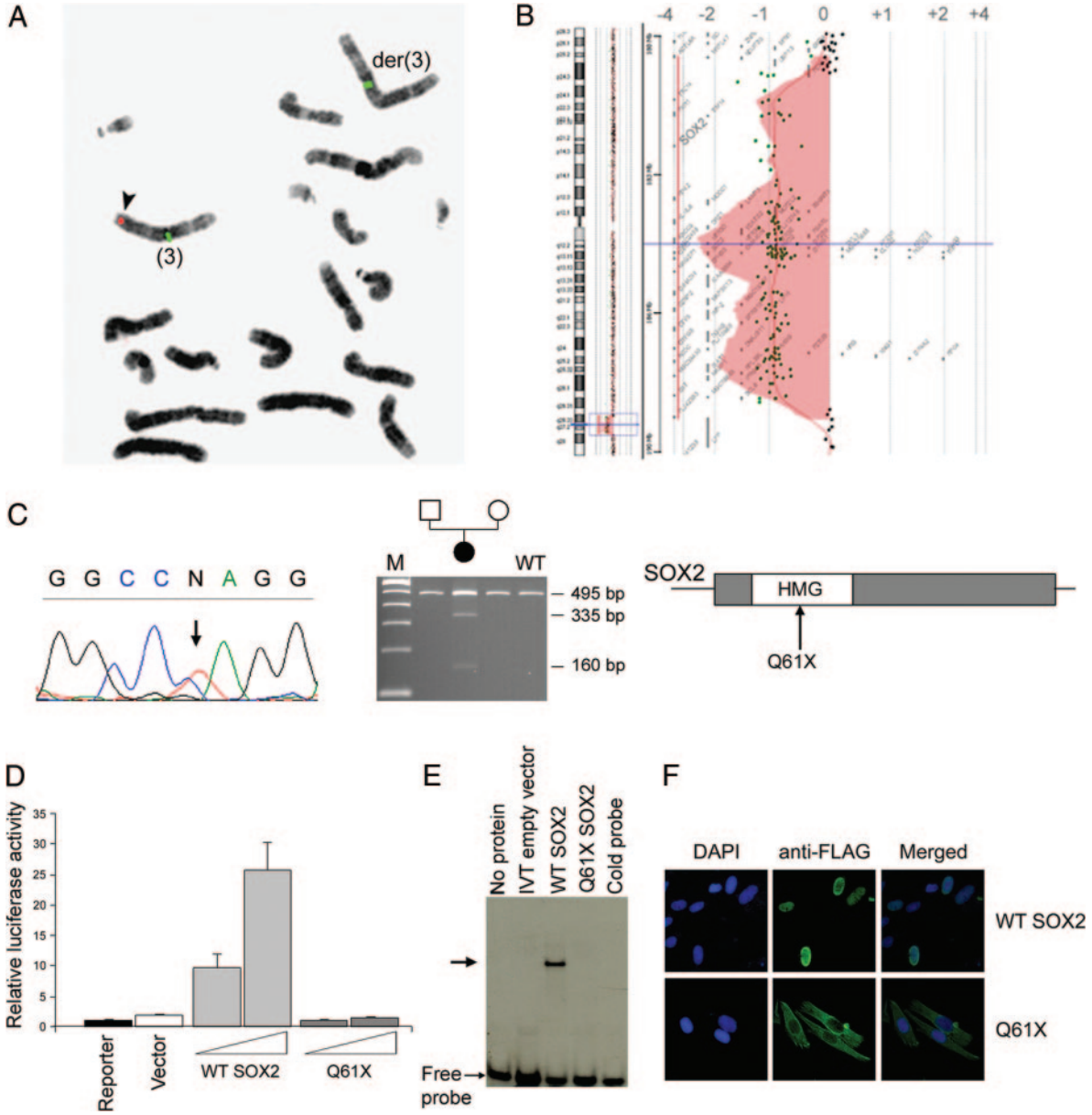


FIG. 2. Analysis of *SOX2* mutations. A, *SOX2* gene deletion in patient 1. Metaphase chromosomes were hybridized with BAC clone RP11–43F17 containing *SOX2* (*red signal*; *arrowhead*) and a chromosome 3 centromeric clone (*green signal*). The normal chromosome 3 shows both *red* and *green signals*. The der(3) shows only a *green signal* indicating that one copy of *SOX2* is deleted. B, Array-CGH profile from patient 1. The chromosome 3 ideogram (*left*) and enlargement of the deleted region (*right*) show the ratio of probes (each represented by a *single dot*) plotted as a function of chromosomal position; loss of copy number of a probe shifts the ratio to the left. C, Electropherogram showing the c.181C>T transition (p.Q61X) in patient 2 (*arrow*). BfaI digestion of PCR products showing that the heterozygous mutation occurred *de novo* in patient 2 (*filled circle*) and is not present in the unaffected parents or in a normal control individual [wild type (WT)]. M, 100-bp DNA ladder. D–F, Functional effects of the p.Q61X mutation. D, The p.Q61X mutation failed to activate the reporter showing similar levels of activation to empty expression vector compared with wild-type SOX2. E, EMSA of wild-type and p.Q61X SOX2 proteins shows that the mutant SOX2 is unable to bind DNA. F, The p.Q61X mutation results in impaired nuclear localization of the mutant protein compared with wild-type SOX2, which stains predominantly within the nucleus. DAPI, 4′,6-diamidino-2-phenylindole; IVT, *in vitro* translated empty expression vector.

Analysis of *SOX2* expression during human embryonic development

To understand the consequences of *SOX2* mutations directly involved in specific tissue abnormalities, it is important to establish its expression patterns during human development. To assess this, we analyzed available sections of normal human embryos from Carnegie Stage (CS) 14 through to fetal stage (F) 2 [~ 4.5- to 9-wk development (24)]. Expression of *SOX2* mRNA and protein was observed throughout the central nervous system at all stages investigated. Strong expression is observed in the forebrain at CS16, which is maintained throughout embryonic development (Fig. 4, A–E), with *SOX2* transcripts detected in the cortex and neuroepithelium of the lateral ventricles at Fs (data not shown). *SOX2* expression is observed within Rathke's pouch at CS16 (Fig. 4, A and B), and expression is maintained within the pouch and throughout anterior pituitary development, in addition to the overlying presumptive hypothalamus and generally within neural ectoderm. SOX2 transcripts and protein are also

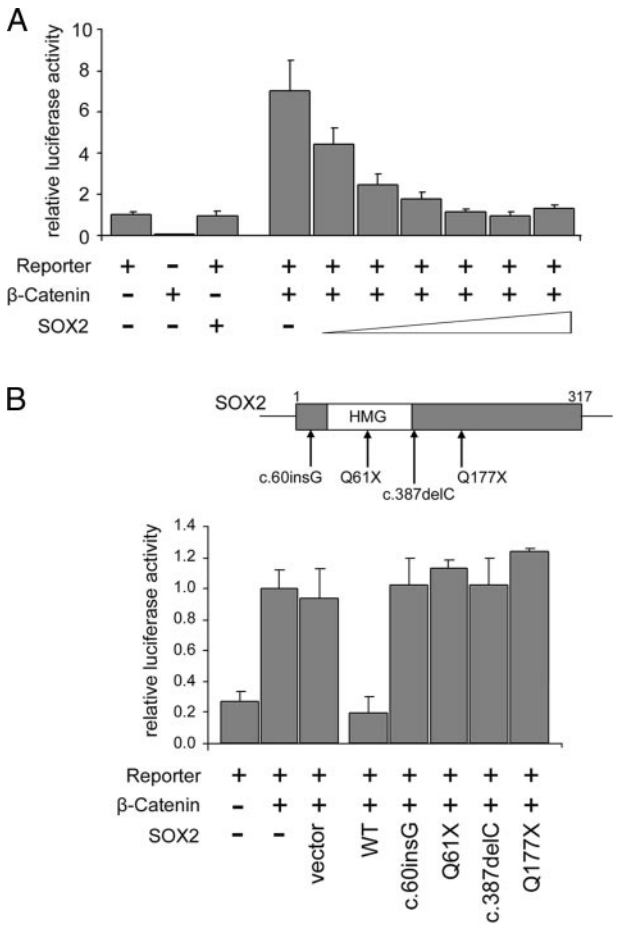


FIG. 3. SOX2 disrupts β-catenin-TCF/LEF mediated transcriptional activation. A, Human embryonic kidney 293 cells were cotransfected with the TCF/LEF reporter construct TOPFLASH with human SOX2 expression construct alone (70 ng) or human β-catenin expression construct with variable amounts of SOX2 (1–70 ng). Increasing amounts of SOX2 led to dose-dependent repression of β-catenin-mediated activation of the reporter. B, *SOX2* mutations disrupt the interaction with β-catenin. *SOX2* mutant constructs were tested and compared with wild-type (WT) SOX2 using 20 ng TOPFLASH, 30 ng β-catenin, and 20 ng *SOX2* expression construct. Truncating *SOX2* mutations fail to repress β-catenin-mediated activation, showing levels of activation comparable to cotransfection with β-catenin alone or empty expression vector. Schematic representation of the *SOX2* gene with approximate positions of the mutations is shown in the *top right*.

present within the cells lining the lumen of the maturing anterior gland during Fs (Fig. 4, C and D; data not shown). No expression was observed in the infundibulum or posterior pituitary.

SOX2 is expressed throughout the telencephalon, with strong expression in the region of the primordial hippocampus dentate gyrus (data not shown). The midbrain and hindbrain also stain uniformly for *SOX2*, including the developing cerebellum and in the isthmic area of the midbrain-hindbrain boundary at CS19 (Fig. 4E). In later stages of fetal development, *SOX2* is expressed abundantly in the thalamus, and specific populations of SOX2 positive cells are detected throughout the hypothalamus, which may represent neuronal and/or glial cell populations (Fig. 4, F–H). *SOX2* also shows significant expression along the entire length of the developing neural tube, in addition to the dorsal root ganglia in sections analyzed from CS14–CS20 (data not shown).

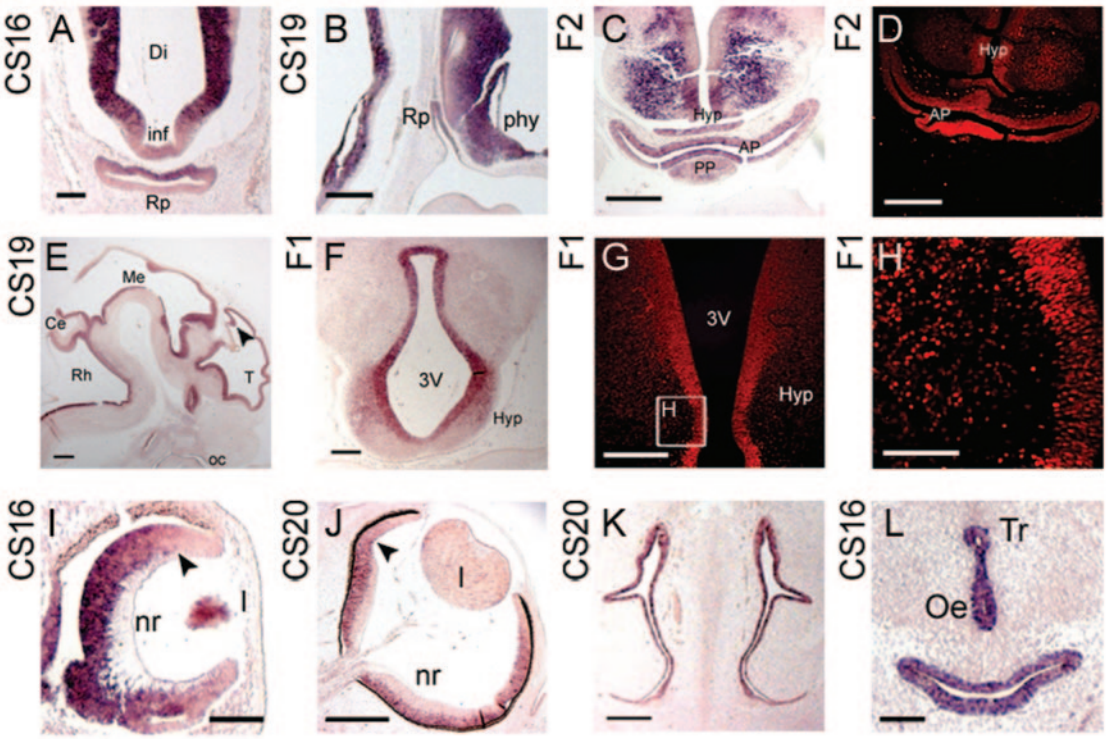
Expression of *SOX2* was observed during eye development at all stages investigated between CS16 and F2. At CS16, low levels of *SOX2* expression are detected in the developing lens vesicle (Fig. 4I) and proximally within the developing neural retina, with a boundary of expression at the distal region of the retina. By CS20, and during Fs thereafter, *SOX2* expression is limited to the inner layer of the neural retina, and is also present in the optic nerve (Fig. 4J). Expression within the lens appears to be maintained at low levels as the lens differentiates, becoming undetectable by *in situ* hybridization after F2 (~9-wk development; data not shown). *SOX2* is also expressed within the cells lining the nasal epithelium where SOX2 continues to be detected during fetal development, in addition to the developing laryngopharynx extending ventrally at the point of division of the trachea and esophagus, as well as in the embryonic lung (Fig. 4, K and L; data not shown).

In situ hybridization and immunohistochemistry failed to detect the presence of *SOX2* within the developing gonad from CS14 onwards. Using RT PCR analysis, we detected *SOX2* transcripts in total RNA extracted from the developing gonads of both males, from 7 wk (the earliest material available) through to 12 wk, and females at 10-wk development (data not shown). Strong expression was observed in the epithelium lining the lumen of the stomach and gut endoderm. No expression was observed by *in situ* hybridization in the heart, kidneys, liver, adrenal glands, or pancreas at any stage investigated (data not shown).

Discussion

Heterozygous, *de novo* mutations in *SOX2* were initially described in patients associated with severe bilateral eye malformations, most commonly anophthalmia (9, 25). We have recently reported the additional phenotype of hypopituitarism in six (of six) patients with mutations in *SOX2* with anterior pituitary hypoplasia and hypogonadotropic hypogonadism (8). Here, we report an additional two patients who manifest hypogonadotropic hypogonadism with lack of pubertal development. To date, all eight patients with *SOX2* mutations in our cohort in whom a confirmed diagnosis can be made (excluding patient 1 in this study) manifest hypogonadotropic hypogonadism. Sato *et al.* (26) also recently reported a female patient with unilateral anophthalmia and isolated hypogonadotropic hypogonadism, suggesting that all individuals harboring *SOX2* mutations are at high risk for gonadotropin deficiency, which has important implications for their clinical management. Patient 1 is as yet too young for a diagnosis of hypogonadotropic hypogonadism at this stage, however, several aspects of the phenotype exhibited by this patient are entirely consistent with phenotypical features observed in other patients with *SOX2* mutations (8). Continued follow-up of this and other individuals with *SOX2* mutations and timely diagnosis of sex steroid deficiency would lead to prompt treatment with the prevention of associated long-term morbidity.

Mice with a heterozygous disruption of *Sox2* also display endocrine deficits and hypoplasia of the anterior pituitary; moreover, *SOX2* is expressed in the developing hypothalamus and



FOG. 4. SOX2 expression pattern in human embryonic and fetal tissue (mRNA: A–C, E, F, and I–L; protein: D, G, and H). Transverse (A) and sagittal (B) sections show expression of *SOX2* within the developing Rathke's pouch (Rp) and overlying neural ectoderm at CS16 and CS19, respectively. C and D, Coronal sections of F2 tissue showing *SOX2* transcripts and protein continue to be expressed in the cells lining the lumen of the anterior pituitary (AP). Note the absence of expression in the infundibulum (inf) and posterior pituitary (PP). E, Transverse section showing *SOX2* expression in the forebrain, including the region of the presumptive hippocampus (*arrowhead*), and throughout the midbrain and hindbrain in addition to the cerebellum (Ce). F–H, Transverse sections of the forebrain in human fetal tissues at approximately 8-wk development showing expression of *SOX2* in the hypothalamus (Hyp). H, Magnified section corresponding to the boxed region in G. I and J, Transverse sections showing expression of *SOX2* in the lens (l) vesicle and developing neural retina (nr) at CS16, where expression is restricted to the proximal inner layer, with a boundary of expression in the distal region adjacent to the lens (*arrowhead*). J, Expression within the lens diminishes by CS20. *SOX2* is also expressed in the nasal epithelium (K) and in the tracheoesophageal tract (L), as shown in transverse section. *Scale bars*: 500 μm (E); 300 μm (B–D, F, G, J, and K); and 100 μm (A, H, I, and L). Di, Diencephalon; Me, mesencephalon; oc, oral cavity; Oe, developing esophagus; phy, presumptive hypothalamus; Rh, rhombencephalon; T, telencephalon; Tr, developing trachea; 3V, third ventricle.

Rathke's pouch in both mice and humans, suggesting a direct role for the gene in hypothalamo-pituitary development in both species. The phenotype observed in patients of anterior pituitary hypoplasia with isolated gonadotropin deficiency is unusual, particularly because other pituitary hormone axes appear to be clinically unaffected in the majority of patients, although a few patients do exhibit GH insufficiency. It is of interest to note that *SOX2* expression is not uniform in the developing hypothalamus in humans (Fig. 4, G and H), suggesting that haploinsufficiency for *SOX2* may affect only certain populations of glia, neuroendocrine neurons, their progenitors, or their afferent inputs. The report of a patient with isolated hypogonadotropic hypogonadism without pituitary hypoplasia on imaging (26) suggests that *SOX2* might be involved independently at multiple levels during the development of the hypothalamo-pituitary-gonadal axis. Because mice with haploinsufficiency of *Sox2* show more generalized pituitary deficits, the apparent selectivity for *SOX2* mutations impairing the gonadotrope axis in human subjects remains intriguing.

The ocular phenotype associated with *SOX2* mutations is fully penetrant in all patients described to date; however, this may be a result of ascertainment bias with patients selected for screening based on the manifestation of bilateral anophthalmia or microphthalmia. On the other hand, we have previously screened a cohort of 235 patients with varying degrees of hypopituitarism and eye defects, and only identified pathogenic mutations in those with bilateral anophthalmia/microphthalmia. More recently, we have not detected any coding sequence variations in *SOX2* in a small cohort of patients (n = 20) with isolated hypogonadotropic hypogonadism (data not shown). The manifestation of hypogonadotropic hypogonadism with unilateral eye disease and no other obvious neurological defects in the patient described by Sato *et al.* (26) suggests that in humans, the hypothalamo-pituitary-gonadal axis may be more sensitive to SOX2 levels, or timing of expression, than other tissues. Consistent with this, we note that mice do not display an eye phenotype unless the level of *Sox2* expression is reduced to levels less than 40% that of wild type (27), whereas heterozygous knockout mice do exhibit abnormal pituitary morphogenesis and endocrine deficits (8).

It is always difficult to assign quantitative loss of function to different mutations based on *in vitro* model assays on a single reporter target, but there is good reason to suppose that the effects we report on the β-catenin/Wnt signaling pathway could be relevant. Previous studies have shown that murine Sox2, as well as *Xenopus* XSox3, XSox17α, and XSox17β, are capable of

associating with β-catenin and repressing the activity of a β-catenin responsive reporter (13, 14, 28). This inhibitory activity is mediated by the COOH terminal domain and is independent of either the HMG domain or the ability to bind DNA. We have shown that human SOX2 protein is also capable of inhibiting β-catenin-mediated reporter gene activation, and that several naturally occurring *de novo* mutations, producing truncated mutant proteins of varying length, were unable to repress this activity *in vitro*. Both the c.387_388delC and p.Q177X mutations retain the ability to bind DNA (8) but show impaired repression of β-catenin. This suggests that the ability of SOX2 to repress transcription in this assay is not a result of competition for binding sites on the reporter construct.

Wnt-β-catenin signaling pathways provide key signals for forebrain and pituitary development, and members of the Tcf/Lef family of transcription factors show distinct patterns of expression in the developing anterior pituitary gland. The formation of a complex between β-catenin and Prop1 mediates the attenuation of expression of the transcriptional repressor *Hesx1*, and simultaneous activation of the pituitary specific transcription factor *Pou1f1* (*Pit1*) (11); both of these events are critical for the determination of specific hormone secreting cell types (29–31). Furthermore, premature activation of β-catenin signaling in the developing anterior pituitary in mice results in complete loss of the gland by E13.5 (11). *Hesx1* is an interesting target in this context because it is also implicated in Wnt signaling pathways during forebrain development, demonstrated by the abnormal domain of *Wnt1* expression and ectopic activation of Wnt/β-catenin targets in *Hesx1* mutant embryos (12). Furthermore, the phenotype associated with mutations in *HESX1* in humans is somewhat reminiscent of that in individuals with *SOX2* mutations, including ocular abnormalities, anterior pituitary hypoplasia, and midline forebrain abnormalities (8, 32–34). Disruption of these interactions with β-catenin and consequent effects on the regulation of β-catenin target genes may be one mechanism by which loss-of-function mutations in *SOX2* can result in such a pleiotropic phenotype.

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