社会へ向けての研究成果の発信
（サイエンススクール・公開市民講座・患者会での講演）

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研究要旨

本研究の最終目標のひとつは、先天性内分泌疾患患者のQOL改善である。この目的を達成する上で「研究成果の社会への還元」は必要不可欠である。このために、中学3年生を対象としたサイエンススクールを3回、一般市民を対象とした公開市民講座を1回、患者会での講演を2回おこない、国民と双方向コミュニケーションした。

A. 研究目的

研究班の研究成果を国民との科学・技術対話として双方向コミュニケーションする。

B. 研究方法

（1）サイエンススクール

中学校3年生を対象としたサイエンススクール（特別授業）を2012年2回（神奈川県、東京都）、2013年に1回（神奈川県）行った。

（2）公開市民講座

一般市民を対象とした公開市民講座を2012年3月10日（山梨県）に行った。

（3）患者会講演

先天性内分泌疾患の一つである骨形成不全症患者会（ネットワークOI）を対象とした講演（東京都）を2012年8月5日に行った。また成長ホルモン分泌不全性低身長患者会（ポプラの会）を対象とした講演（東京都）を2012年10月21日に行った。

C. 研究結果

（1）サイエンススクール

参加者は160～254名であった。

（2）公開市民講座

参加者は100名以上であった。

（3）患者会講演

参加者は以下の通りであった。

2012年8月5日　30名以上

2012年10月21日　10名

D. 考察

厚生労働省大臣官房厚生科学課による平成23年度「厚生労働科学研究費補助金公募要項」（難病・がん等の疾患分野の医療の実用化研究事業）には、「特に、1件当たり年間3千万円以上の公的研究費（競争的資金又はプロジェクト研究資金）の配分を受ける研究者等においては、国民との双方向コミュニケーション活動に積極的に取り組む」と記載されている。中学校での特別授業、地域の市民公開講座での研究成果の講演は、いずれもこの活動の一つの例として記載されており、上記の成果はこの活動に合致する。

E. 結論

研究班の研究成果を国民との科学・技術対話として双方向コミュニケーションすることにより研究成果が社会に還元された。また、中学校3年生を対象としたサイエンススクールは次世代の研究者養成の端緒（知的資産への間接的な社会的波及効果）ともなりうる。

F. 研究発表

1. 論文発表

該当なし

2. 学会発表

該当なし

G. 知的財産権の出願・登録状況

（予定を含む。）

1. 特許取得

該当なし

2. 実用新案登録

該当なし

3. その他

該当なし

III. 研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書籍名	出版社名	出版地	出版年	ページ
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安達昌功	Part 1 ホルモンの作用と病態：成長ホルモンと身長増加	有阪治	ビギナーのための小児内分泌診療ガイド	中山書店	東京	2014	8-11
安達昌功	Prt 2 さまざまな症状や検査異常への対応と診断･治療：低身長	有阪治	ビギナーのための小児内分泌診療ガイド	中山書店	東京	2014	80-85
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IV. 研究成果の刊行物・別冊

Identification and Functional Characterization of Two Novel *NPR2* Mutations in Japanese Patients With Short Stature

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Context: C-type natriuretic peptide-natriuretic peptide receptor B (NPR-B) signaling is critical for endochondral ossification, which is responsible for longitudinal growth in limbs and vertebrae. Biallelic *NPR2* mutations cause acromesomelic dysplasia, type Maroteaux, which is bone dysplasia characterized by severe short stature and short limbs. A monoallelic *NPR2* mutation has been suggested to mildly impair long bone growth.

Objective: The goal of this study was to identify and characterize *NPR2* mutations among Japanese patients with short stature.

Subjects and Methods: We enrolled 101 unrelated Japanese patients with short stature. *NPR2* and *NPPC* were sequenced, and the identified variants were characterized in vitro.

Results: In two subjects, we identified two novel heterozygous *NPR2* mutations (R110C and Q417E) causing a loss of C-type natriuretic peptide-dependent cGMP generation capacities and having dominant-negative effects. R110C was defective in trafficking from the endoplasmic reticulum to the Golgi apparatus. In contrast, Q417E showed clear cell surface expression.

Conclusions: We identified heterozygous *NPR2* mutations in 2% of Japanese patients with short stature. Our in vitro findings indicate that *NPR2* mutations have a dominant negative effect, and their dominant-negative mechanisms vary corresponding to the molecular pathogenesis of the mutations. **(*J Clin Endocrinol Metab* 99: E713–E718, 2014)**

C-type natriuretic peptide (CNP)-natriuretic peptide receptor B (NPR-B) signaling plays a critical role in endochondral ossification, which is responsible for longitudinal growth in limbs and vertebrae (1–3). *NPR2* encodes NPR-B, which transduces CNP signals. Knockout mice for *Nppc* (encoding CNP) or *Npr2* are severely dwarfed (4, 5). Biallelic loss-of-function *NPR2* mutations cause acromesomelic dysplasia, type Maroteaux (AMDM), which is characterized by severe short stature and short limbs (6–9). In contrast, gain-of-function *NPR2* mutations were identified in patients with tall stature and macrodactyly (10, 11). These studies showed that CNP-NPR-B signaling is essential in skeletal development in humans.

In the first report of biallelic *NPR2* mutations causing AMDM, height SD scores (SDSs) of the probands' parents who were heterozygous carriers were lower than the mean

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Abbreviations: AMDM, acromesomelic dysplasia, type Maroteaux; CNP, C-type natriuretic peptide; Endo H, endoglycosidase H; ER, endoplasmic reticulum; HA, hemagglutinin; ISS, idiopathic short stature; NPR-B, natriuretic peptide receptor B; PNGase F, peptide-N-glycosidase F; SDS SD score; WT, wild type.

height SDS of population-matched standards (7). In a large pedigree including an AMDM proband, who carried a homozygous mutation (p.I364fs), the mean height SDS of the heterozygous carriers was -1.8 ± 1.1, whereas that of the noncarriers was -0.4 ± 0.8 (12). Based on these two studies, it is presumed that heterozygous *NPR2* mutations can mildly impair long bone growth. Recently heterozygous *NPR2* mutations were detected in a Brazilian cohort with idiopathic short stature (ISS) (13).

In this study, we conducted genetic analyses of these two genes in 101 Japanese patients with short stature of unknown etiology and performed functional analyses to elucidate the precise molecular mechanisms of the identified mutations.

Subjects and Methods

Details of subjects and methods are described in the Supplemental Data, published on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org.

Subjects

We enrolled 101 unrelated Japanese patients with short stature who were recruited by the Japan Growth Genome Consortium, a research network of Japanese pediatric endocrinologists (14).

Sequencing

NPPC and *NPR2* were sequenced. Variations were filtered with reference to the dbSNP database and the 1000 Genomes Project database. Detected mutations were analyzed in 100 Japanese control individuals and the family members of mutation-carrying patients.

Functional characterization of mutant NPR-B

The hemagglutinin (HA)-tagged wild-type (WT) human NPR-B construct (HA-WT-NPR-B) has been described previously (8). All variants and myc-tagged WT NPR-B construct were generated. CNP-dependent cGMP response of transiently transfected COS7 cells were measured by a competitive enzyme immunoassay. Total protein expression of HA-NPR-B was determined by Western blotting. Deglycosylation experiments were performed, and the effect of brefeldin A was evaluated. The subcellular localization of HA-NPR-B was observed under a confocal microscope using immunofluorescence experiments. Coimmunoprecipitation experiments were performed after cotransfection of myc-WT-NPR-B and each HA-NPR-B construct.

Results

Characteristics of the subjects

The characteristics of the 101 subjects are shown in Supplemental Table 1.

Sequencing

We identified three novel *NPR2* variations (c.328C>T, p.R110C; c.559G>A, p.V187I; and c.1249C>G, p.Q417E) in three subjects and no novel *NPPC* sequence variation (Figure 1A). These three *NPR2* variations were absent in the 100 con-

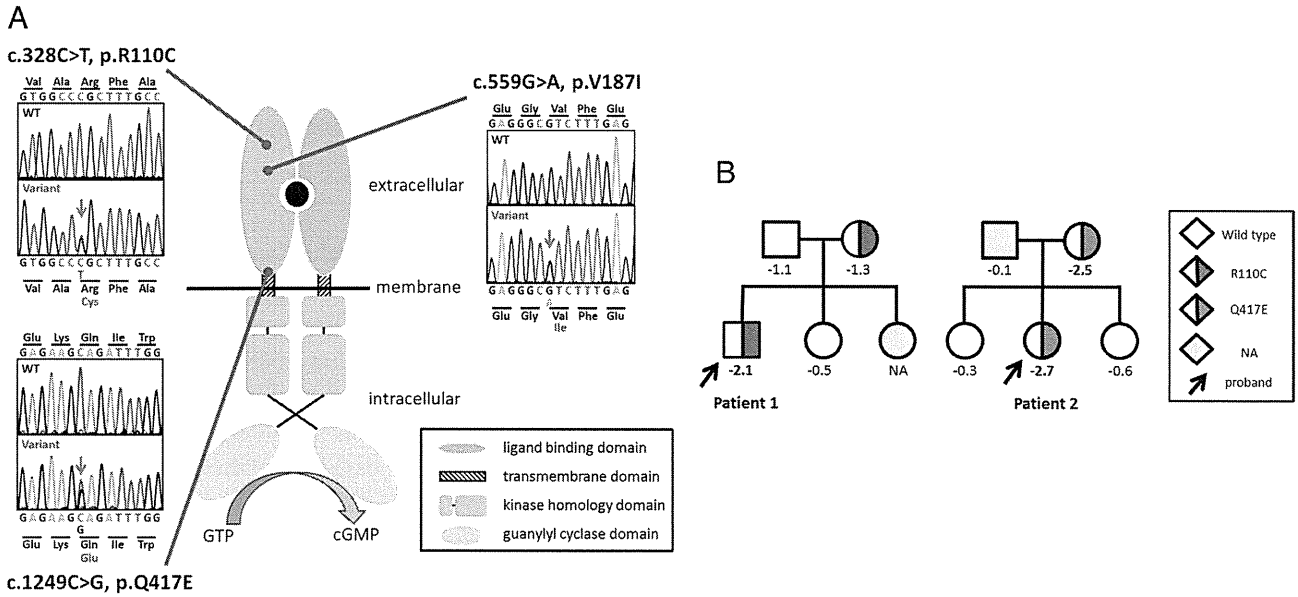

Figure 1. Identification of three *NPR2* sequence variations. A, A schematic diagram of NPR-B protein, which acts as a dimer and has an extracellular ligand binding domain, a transmembrane domain, a kinase homology domain, and a guanylyl cyclase domain. The blue closed circle is CNP. Three sequence variations are visualized by chromatograms. The three heterozygous substitution of cysteine in place of Arg110, isoleucine in place of Val187, and glutamic acid in place of Gln417 are indicated by arrows. The three sequence variations are located in the ligand binding domain. B, Pedigrees of patient 1 and patient 2 are shown. The values under the symbols show height SDS. NA, not available.

trol individuals. The three *NPR2* variants were located in the extracellular domain.

Functional characterization of NPR-B variants

To examine the pathogenicity of the three variations, we evaluated the CNP-dependent, cGMP-producing capacities. R110C showed a negligible cGMP response, and Q417E showed a markedly low response (Figure 2A). V187I had a comparable response with WT, indicating that the variant is functionally neutral.

We characterized HA-WT-NPR-B using Western blotting. HA-WT-NPR-B (total cell lysate, untreated) showed two bands (Figure 2B). Peptide-N-glycosidase F (PNGase F) treatment showed a single digested product band of smaller molecular size (Figure 2C), indicating that the two bands were derived from N-linked glycosylated protein. The lower band was sensitive to endoglycosidase H (Endo H), which digests the sites of N-glycosylation occurring in the endoplasmic reticulum (ER), whereas the upper band was digested by O-glycosidase, which digests the sites of O-glycosylation occurring in the Golgi apparatus. These results indicate that the lower band corresponded to N-glycosylated NPR-B produced in the ER, whereas the upper band corresponded to the mature NPR-B with N- and O-glycosylation produced in the Golgi apparatus. Treatment with brefeldin A, which inhibits protein transportation from the ER to the Golgi apparatus, resulted in the same immunoreactive pattern as that of O-glycosidase (Figure 2D). This result also indicates that the upper band was derived from the fully glycosylated NPR-B in the Golgi apparatus.

To clarify the mechanisms underlying the two loss-of-function mutants, we conducted a series of expression experiments in vitro. We performed Western blotting of the mutant HA-NPR-B. R110C showed only the lower band, whereas Q417E appeared as two bands (Figure 2E), indicating that R110C did not exist in the O-glycosylated form. We analyzed the subcellular localization of HA-NPR-B with an HA epitope in the extracellular domain. Under the nonpermeabilized condition, WT and Q417E displayed cell surface expression, whereas R110C did not (Figure 2F). Under the permeabilized condition, R110C was colocalized with an ER marker (Figure 2F).

We assessed possible dominant-negative effects of the two mutants. Coexpression of each mutant and WT led to a significant loss in the CNP-dependent cGMP response compared with that of the empty vector and WT, indicating a dominant-negative effect (Figure 2G).

To elucidate mechanisms causing the dominant-negative effects, we conducted further expression experiments. We performed Western blotting using cells coexpressing HA-WT-NPR-B and HA-R110C-NPR-B (Figure 2I). A decrease in the upper band (fully glycosylated receptor) and parallel an increase in the lower bands (N-glycosylated, not O-glycosylated) were observed in cells coexpressing the R110C mutant. To evaluate the interaction between WT and each mutant, we performed coimmunoprecipitation (Figure 2H). After precipitation of total cell lysates with an anti-HA antibody, immunoblotting of the precipitates with an antimyc antibody showed that both HA-R110C-NPRB and HA-Q417E-NPRB interacted with the myc-WT-NPR-B.

Family analyses and clinical phenotypes

The mutations (R110C and Q417E) were transmitted from their mothers (Figure 1B). Patient 1 showed normal serum IGF-I and normal GH responses by stimulation tests of arginine, L-DOPA, and growth hormone releasing hormone. After introduction of recombinant human GH treatment (50 μg/kg·d) for 2 years, the patient's height velocity slightly improved. However, the patient's adult height SDS was −2.8 (Supplemental Figure 1). Height SDS of the patient's mother was −1.3. Patient 2 also showed normal serum IGF-I level, and the patient's adult height SDS was −2.6. The patient's mother had short stature (height SDS, −2.5) (Supplemental Table 2).

Discussion

In the present study, we identified three *NPR2* variants (R110C, V187I, and Q417E) in a Japanese short stature cohort. The Arg110 residue is highly conserved among vertebrate species, whereas Val187 and Gln417 are conserved among terrestrial animals but not in fish (Supplemental Figure 2). Functional studies showed that R110C and Q417E lost the CNP-dependent cGMP generation capacities and had dominant-negative effects in different manners in vitro.

We identified heterozygous *NPR2* mutations in 2% (95% confidence interval 0%–7%) of Japanese patients with short stature. Assuming that the prevalence of AMDM is 1 in 2 000 000 by comparing the number of patients with AMDM with that with achondroplasia in the Little People of America, regardless of ethnicities, the frequency of heterozygous *NPR2* mutation carriers is calculated to be approximately 1 in 700 (12). Hypothesizing that the effect of a heterozygous *NPR2* mutation on height SDS is −1.8 according to the previous report, 2.6 in 100 subjects with short stature, which is defined as height SDS less than −2.0, are expected to be heterozygous for the *NPR2* mutation. This estimation is consistent with our observation. Recently *NPR2* mutations were identified in 6% of the Brazilian cohort with ISS (13). Further studies

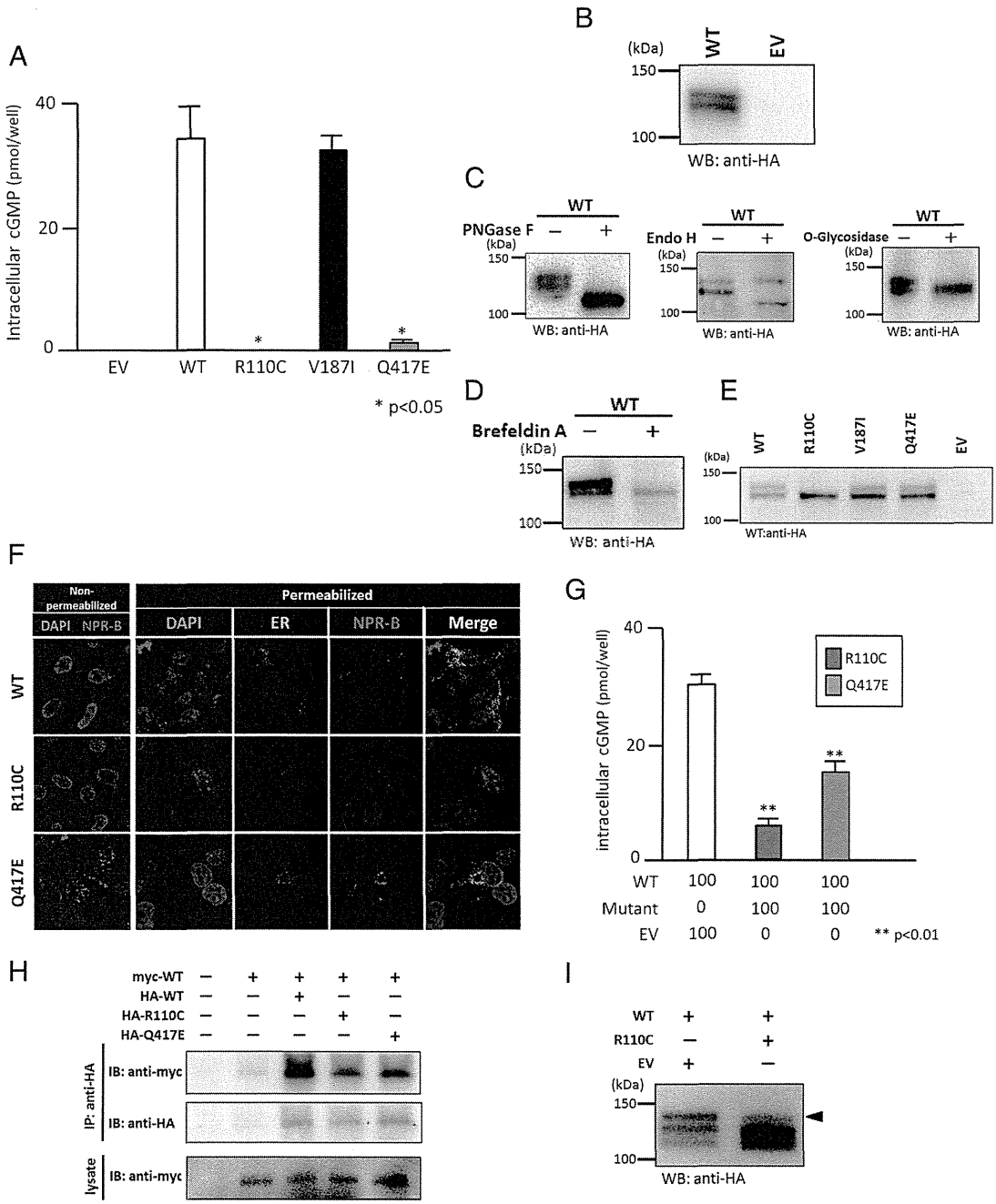

Figure 2. Functional characterization of the three NPR-B variants. A, CNP-stimulated cGMP accumulation in COS7 cells transiently transfected with empty vector (EV), wild-type (WT), and the three NPR-B variants (R110C, V187I, and Q417E) were evaluated. Data are presented as the mean ± SEM of triplicate samples and are representative of three independent experiments. B, Western blotting analysis of COS7 cells expressing HA-WT-NPR-B revealed two bands. C, Western blotting analysis of HA-WT-NPR-B, which was deglycosylated with PNGase F, Endo H, or O-glycosidase/neuraminidase is shown. Both bands were sensitive to PNGase F (left panel). The lower band was digested by Endo H (middle panel), whereas the upper band was sensitive to O-glycosidase/neuraminidase (right panel). D, Western blotting analysis of HA-WT-NPR-B under treatment with brefeldin A is shown. Only the lower band was detected. E, Western blotting analysis of HA-NPR-B (WT, R110C, and Q417E) shows that WT and Q417E revealed two bands, whereas R110C did as a single band. Migrations of molecular mass markers (in kilodaltons) are shown on the left of each panel in B–E. F, Subcellular localization analyses of HA-NPR-B proteins in COS7 cells under the nonpermeabilized or permeabilized condition are shown. WT (red) and Q417E (red) showed clear plasma membrane expression in nonpermeabilized cells, whereas R110C (red) colocalized with an ER (green) marker in permeabilized cells. G, CNP-stimulated cGMP accumulation in cells transfected with WT/R110C or WT/Q417E NPR-B is shown. H, Coimmunoprecipitation analysis of WT and the mutants is shown. Lysates from cells expressing myc-WT-NPR-B and HA-NPR-B (WT or mutants) were precipitated (IP) with an anti-HA antibody. The myc-WT-NPR-B alone and myc-WT-NPR-B being coexpressed with HA-WT-NPRB were used as negative and positive controls, respectively. Samples were assessed by Western blotting using anti-myc (top panel) and anti-HA antibodies (middle panel). As a control, the amount of myc-HA-NPR-B was verified in total cell lysates (bottom panel). The bands corresponding to HA-NPR-B (R110C and Q417E) were present in immunoprecipitates from cells coexpressing myc- and HA-WT-NPR-B (top panel). I, Western blotting analysis in cells coexpressing WT and R110C showed that the upper band (arrowhead) decreased and the lower bands increased.

are required to elucidate whether the frequencies of heterozygous mutation carriers in ISS differ, depending on ethnicities.

R110C showed defective trafficking from the ER to the Golgi apparatus. Subcellular localization studies have shown that 11 of 12 missense *NPR2* mutations, which were identified in AMDM patients, caused ER retention (15). Therefore, defective cellular trafficking from the ER to the plasma membrane is likely a major molecular mechanism of the missense *NPR2* mutations. Protein folding in the ER is monitored by ER quality control mechanisms, and misfolding proteins are retained and degraded in the ER by an ER-associated degradation pathway (16). We speculate that a change in conformation of the mutant NPR-B receptor promotes protein misfolding and defects in the normal intracellular trafficking from the ER to the Golgi apparatus.

In contrast to R110C, Q417E was expressed normally on the plasma membrane. We hypothesize that the pathogenesis of Q417E may be defective in ligand binding or receptor activation. Particularly, Gln417 is located in the junctional region between the ligand binding domain and the transmembrane domain. According to an investigation using an NPR-A crystal structure, counterclockwise rotation of juxtamembrane regions of a dimer is prerequisite for initiating transmembrane signaling by ligand binding (17). This rotation, which is transduced across the membrane, can reorient the intracellular domains and activate guanylyl cyclase. If the rotation of the NPR-A receptor is applied to the NPR-B receptor, a juxtamembrane region of NPR-B, including Gln417 might also play a critical role in initializing transmembrane signaling.

R110C and Q417E showed dominant-negative effects on the coexpressed WT receptor. Previously, four missense mutations (S76P, R263P, L658F, and R819C) were demonstrated to have dominant-negative effects (8, 13), but molecular mechanisms have not been elucidated. Our coexpression experiments indicated that R110C, which has defective trafficking from the ER to the Golgi apparatus, interacted with WT-NPR-B and relatively reduced the abundance of a fully glycosylated receptor. Some receptor mutants (eg, GnRH receptor, melanocortin 1 receptor, and α2-adrenergic receptor) were reported to have dominant-negative effects by heterodimerizing with and entrapping the WT receptor in the ER (18–20). A similar pathological mechanism can be true for the dominant-negative effect of R110C. Our coexpression experiments also showed that Q417E expressing on the plasma membrane interacted with WT-NPR-B. Q417E probably functions as a dominant-negative mutant, suppressing the activation and response of WT-NPR-B by forming an unproductive heterodimer, WT-NPR-B/Q417E.

In contrast, the I364fs mutant lacking the transmembrane domain was not thought to have a dominant-negative effect (ie, haploinsufficiency) (12). These findings necessitate the reinvestigation of other heterozygous *NPR2* mutations to discriminate whether the pathological effects (a dominant negative effect or haploinsufficiency) on WT-NPR-B in vitro significantly influence clinical phenotypes.

We provided observational data that were consistent with the hypothesis that a monoallelic *NPR2* mutation could cause short stature. However, we have not rigorously verified that hypothesis. It would be of interest to have genotype and phenotype information for the extra family member, as reported in the paper by Olney (12).

In summary, we identified two heterozygous loss-of-function *NPR2* mutations in a Japanese cohort with short stature. Both mutations had a dominant-negative effect, and their dominant-negative mechanisms varied corresponding to the molecular pathogenesis of *NPR2* mutations. Further studies involving other mutants should be conducted to clarify the pathological roles of different mutations in long bone growth.

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Novel Insights from Clinical Practice

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A Novel Mutation in SOX2 Causes Hypogonadotropic Hypogonadism with Mild Ocular Malformation

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Established Facts

- Heterozygous *SOX2* mutations have been reported to cause isolated hypogonadotropic hypogonadism (HH) in addition to ocular and brain abnormalities.
- The most common ocular phenotype associated with *SOX2* mutations is a severe bilateral eye defect such as anophthalmia or severe microphthalmia.

Novel Insights

- We report a novel missense *SOX2* (Y110C) mutation in an HH patient with mild ocular malformation, unilateral retinal detachment.
- The findings in this patient emphasize the importance of testing for *SOX2* mutations in HH individuals with mild ocular defects, such as retinal detachment, in the absence of anophthalmia or severe microphthalmia.
- We used a next-generation sequencing strategy to analyze 122 genes associated with congenital endocrine disorders. This approach is new in HH; it has never been reported to our knowledge.

Key Words
SOX2 · Hypogonadotropic hypogonadism · HMG domain · Targeted next-generation sequencing

Abstract
Background: Heterozygous *SOX2* mutations have been reported to cause isolated hypogonadotropic hypogonadism (HH) in addition to ocular and brain abnormalities. ***Objective:*** We report a novel missense *SOX2* (Y110C) mutation in an HH patient with mild ocular malformation. ***Patients:*** The 20-year-old male was referred because of typical signs of complete hypogonadism, with small intrascrotal testes (2 ml), no pubic hair (P1), and a micropenis. Hormone assays revealed very low plasma testosterone levels and very low levels of plasma gonadotropin. He was found to have retinal detachment in his right eye and surgery was performed at the age of 14 years. ***Results:*** Using a next-generation se-

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quencing strategy, we identified a novel heterozygous *SOX2* mutation, c.329A>G (p.Y110C). Y110C SOX2 had reduced transactivation and no dominant negative effect. Subcellular localization revealed no significant difference between wild-type and mutant SOX2. EMSA experiments showed that the Y110C SOX2 abrogated DNA-binding ability. ***Conclusion:*** The Y110C mutation affects a critical residue in the SOX2 protein. This study extends our understanding of the phenotypic features, molecular mechanism, and developmental course associated with mutations in *SOX2*. When multiple genes need to be analyzed for mutations simultaneously, targeted sequence analysis of interesting genomic regions is an attractive approach.

Introduction

Hypogonadotropic hypogonadism (HH) is a genetically heterogeneous condition, defined by absent or incomplete sexual maturation secondary to gonadotropin deficiency. Several genes have been linked to the pathogenesis of HH, including *KAL1*, *FGFR1*, *FGF8*, *PROK2*, *PROKR2*, *CHD7*, *GNRHR*, *GNRH1*, *KISS1R*, *KISS1*, *TAC3*, *TACR3*, and *SOX2* [1–8].

Heterozygous mutations in *SOX2* were first reported in patients with bilateral anophthalmia or severe microphthalmia who had additional abnormalities, including developmental delays, learning difficulties, esophageal atresia, and genital abnormalities [9–11]. Subsequently, *SOX2* mutations were also shown to be associated with anterior pituitary hypoplasia, HH, and variable growth hormone deficiency in association with other manifestations, including hippocampal abnormalities, defects of the corpus callosum, hypothalamic hamartoma, and sensorineural hearing loss [7, 8]. To date, more than 40 mutations in *SOX2* have been described [12]. The majority of these are truncating mutations such as nonsense or frameshift mutations, and only 10 missense mutations have been reported.

Here, we report an HH patient with mild ocular phenotypes carrying a novel missense mutation in *SOX2* (Y110C). Through molecular analyses, we showed that substitution of a conserved, critical amino acid near the DNA-binding high-mobility group (HMG) domain of SOX2 abrogated DNA-binding and pituitary gene *(HESX1)* activation. This study extends our understanding of the phenotypic features, molecular mechanism, and developmental course associated with mutations in the *SOX2* gene.

Table 1. Endocrinological findings (baseline) in the propositus

	20 years	Reference (adult)
IGF-1, ng/ml	307	male: 41–369
TSH, μU/ml	2.42	0.3–3.50
Free T4, ng/dl	1.15	1.09–2.55
Free T3, pg/ml	3.82	3.23–5.11
LH, mIU/ml	0.1	male: 2.2–8.4
FSH, mIU/ml	0.31	male: 1.8–12
Testosterone, ng/ml	0.38	male: 2.01–7.50

The conversion factors to the SI units are as follows: IGF-I 0.131 (nmol/l), TSH 1.0 (mIU/l), free T4 12.87 (pmol/l), free T3 1.54 (pmol/l), LH 1.0 (IU/l), FSH 1.0 (IU/l), and testosterone 0.035 (nmol/l).

Materials and Methods

Case Report

The propositus was a 23-year-old Japanese man born at 41 weeks of gestation after an uncomplicated pregnancy and delivery. The parents were nonconsanguineous and phenotypically normal. He had one younger sister who had no relevant clinical problems. His birth weight was 3,250 g (+0.6 SD), and length was 49.0 cm (0.0 SD). He had horizontal nystagmus and bilateral cryptorchidism, which were diagnosed in the first months of life. No anophthalmia or microphthalmia was recorded. His gross motor development was almost normal. At the age of 3 years, he presented with generalized seizures, which have been well controlled with sodium valproate. At the age of 14 years, he was found to have retinal detachment in his right eye and surgery was performed.

He was referred at the age of 20 years due to the typical signs of complete hypogonadism, with small intrascrotal testes (2 ml), no pubic hair (P1), and a micropenis. Hormone assays revealed very low plasma levels of testosterone and gonadotropin (table 1). Brain MRI showed a normal pituitary and olfactory bulb and no other abnormalities. He had a normal sense of smell. His karyotype was 46,XY. His height and weight were 174.4 cm (+0.7 SD) and 63.9 kg (+0.2 SD), respectively. The visual acuity of both his right and left eyes was 0.01 without glasses and 0.6 with glasses.

Mutation Screening

After obtaining informed consent, and with the approval of the Institutional Review Board of Keio University School of Medicine, genomic DNA was extracted from peripheral blood leucocytes of the propositus and his parents. We sequenced 13 genes implicated in HH, including *CHD7*, *FGFR1*, *FGF8*, *GNRH1*, *GNRHR*, *KAL1*, *KISS1*, *KISS1R*, *PROK2*, *PROKR2*, *TAC3*, *TACR3*, and *SOX2* using the MiSeq instrument (Illumina Inc., San Diego, Calif., USA) according to the SureSelect protocol (Agilent Technologies, Santa Clara, Calif., USA). In brief, 3 μg of genomic DNA were used for the SureSelect capture methods. Exons of 122 genes known to be associated with congenital endocrine disorders (including 13 HH-related genes) were identified in the University of California Santa

Cruz table browser (http://genome.ucsc.edu/). In total, we targeted 1,321 regions comprising 246,158 bp using SureSelect. DNA obtained from the SureSelect solution-based sequence capture was subjected to MiSeq sequencing according to the manufacturer's protocol. Base calling, read filtering, and demultiplexing were performed with the standard Illumina processing pipeline. We used BWA 0.6.1 and SAMtools 0.1.18 for alignment and variant detection against the human reference genome (NCBI build 37; hg19) with the default settings. Local realignment, quality score recalibration and variant calling were performed by GATK 2.3.9 with the default settings. We used ANNOVAR for annotation of called variants.

Crystal Structure Modeling

The crystal structure of the SOX2 HMG domain (protein data bank ID 1GT0; http://www.rcsb.org/pdb/) was used as a reference wild-type (WT) structure for modeling the structure of Y110C SOX2 using the PyMOL Molecular Graphics System (http://www.pymol.org).

Functional Studies

To generate SOX2 expression vectors, *SOX2* cDNA was cloned into pCMV-myc (Clontech, Palo Alto, Calif., USA). For subcellular localization analyses, we purchased a Halo-tagged clone vector (Kazusa DNA Research Institute, Chiba, Japan) containing human SOX2 cDNA. We introduced the Y110C mutation by site-directed mutagenesis using the PrimeSTAR Mutagenesis Basal Kit (TaKaRa, Otsu, Japan). A luciferase reporter vector was constructed by inserting the *HESX1* promoter sequence (–405 to +267 bp) into a pGL4.24 [luc2P/minP] vector (Promega, Madison, Wisc., USA). A transactivation assay was performed in HeLa cells using a dual-luciferase reporter assay system (Promega). For subcellular localization analyses, we visualized HeLa cells transfected with Halo-tagged SOX2 and TMRDirect™ ligands (Promega), according to the manufacturer's instructions. We photographed the cells using a Leica TCS-SP5 laser scanning confocal microscope (Leica, Exton, Pa., USA). The sequences of the biotin-labeled double-stranded oligonucleotide used as probe in the EMSA experiment was 5′-CAAACAAATAAACAATTAACTC -3′ [13]. Five micrograms of nuclear protein extraction was incubated at room temperature in a 20-μl binding reaction mixture containing a 20-fmol probe, 50 mM KCl, 5 mM $MgCl_2$, 2.5% glycerol, 0.05% NP-40, and 1 μg poly(dI-dC) for 20 min. For competition experiments, a large excess (200×) of unlabeled competitor oligonucleotides was included in the binding reactions. The protein-DNA complexes were subject to gel electrophoresis and transferred to a nylon membrane. The biotin-labeled probe was detected with the Lightshift Chemiluminescent EMSA Kit (Pierce).

Results

Mutation Screening

We identified a novel heterozygous *SOX2* mutation, c.329A>G (p.Y110C), the only gene among 13 HH-related genes where unknown variants were identified. We used Sanger sequencing of PCR products from genomic DNA to confirm the *SOX2* variant (fig. 1a). Y110 is immediately N-terminal to the DNA-binding HMG domain, which is highly conserved among SOX proteins and is critical for binding to both interacting proteins and target DNA sequences. Y110 is a highly evolutionarily conserved amino acid (fig. 1b), and this mutation was not detected in 150 healthy Japanese controls. No sequence variation was found in *CHD7*, *FGFR1*, *FGF8*, *GNRH1*, *GNRHR*, *KAL1*, *KISS1*, *KISS1R*, *PROK2*, *PROKR2*, *TAC3*, and *TACR3*. Parental analysis was refused.

Crystal Structural Modeling

Y110C SOX2 was predicted to lose a residue-DNA contact (fig. 1c).

Functional Studies

In Hella cells, WT SOX2 stimulated transcription of the *HESX1* reporter in a dose-dependent manner. Y110C SOX2 had reduced transactivation, and had no dominant negative effect (fig. 2a). Subcellular localization revealed no significant difference between WT and mutant SOX2 (fig. 2b), indicating that nuclear targeting was not affected by the mutation. WT SOX2 specifically bound to the DNA and this binding was competed by an excess (200×) of cold competitors. In contrast, Y110C SOX2 had abrogated DNA-binding ability (fig. 2c).

Discussion

We characterized a novel mutant (Y110C) of the SOX2 transcription factor that is associated with HH and a mild ocular phenotype. The Y110C SOX2 protein had abrogated DNA-binding affinity and decreased transcription activity compared to WT SOX2 with no dominant negative effect. The partial transcription activity suggests that the Y110C mutation is a hypomorphic mutation that retains residual activity. The most common ocular phenotype associated with *SOX2* mutations is a severe bilateral eye defect such as anophthalmia or severe microphthalmia. If an eye is present, it may be associated with ocular features, including sclerocornea. Our patient showed only unilateral retinal detachment. This mild phenotype was likely due to residual SOX2 activity. The findings in this patient emphasize the importance of testing for *SOX2* mutations in HH individuals with mild ocular defects, such as retinal detachment, in the absence of anophthalmia or severe microphthalmia.

To date, more than 40 mutations in *SOX2* have been described. Most of the mutations cause premature termination codons as a result of nonsense or frameshift muta-

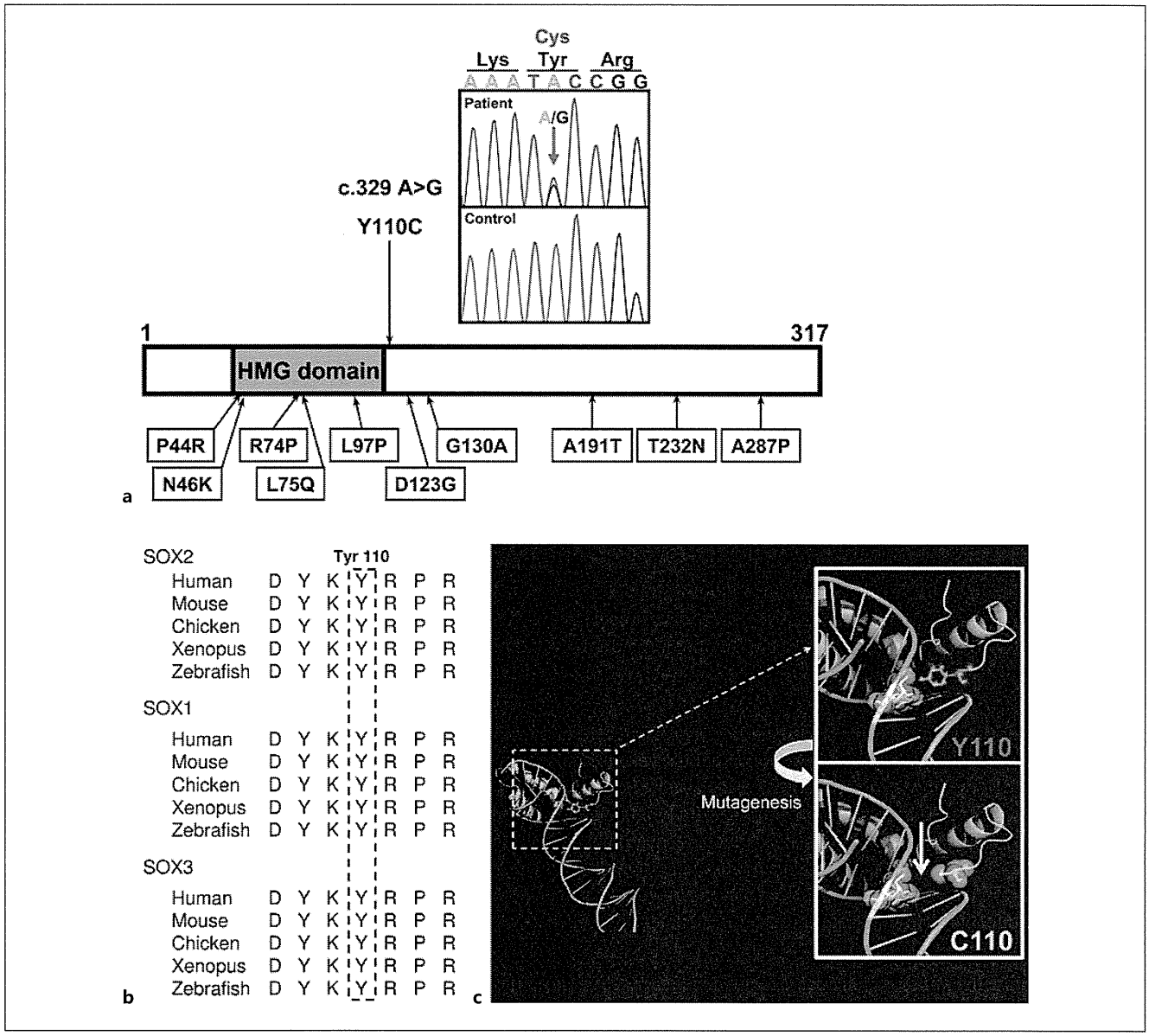

Fig. 1. Identification of sequence variation of *SOX2*. **a** Partial sequence of PCR product and schematic diagrams of the SOX2 protein. The chromatogram represents a heterozygous substitution of cysteine (TGC) in place of tyrosine (TAC) at codon 110. The arrow indicates the mutated nucleotide. Tyrosine 110 is located immediately 3′ of the HMG domain. The reported 10 missense mutations are summarized. **b** Homology study showed tyrosine at codon 110 is highly conserved through species in SOX2, SOX1, and SOX3. **c** Modeled structure of the Y110C in comparison with the WT structure (upper panels). Modeling of the mutant was performed using a built-in mutagenesis function of the PyMOL Molecular Graphics System. Crystal structural modeling showed Y110C SOX2 was predicted to lose a residue-DNA contact (yellow arrow).

tions; only 10 missense mutations have been reported. Among these 10 missense mutations, only 3 (R74P, L75Q, and L97P) have been confirmed as pathogenetic by functional assays [7, 10, 14]. All 3 mutations are located in the HMG domain and cause anophthalmia or severe microphthalmia. Therefore, Y110C is the only amino acid change located outside of the HMG domain that has been shown to be pathogenetic in functional assays.

Recently, Mihelec et al. [15] reported a 4-generation family with marked ocular phenotypic variability harbor-

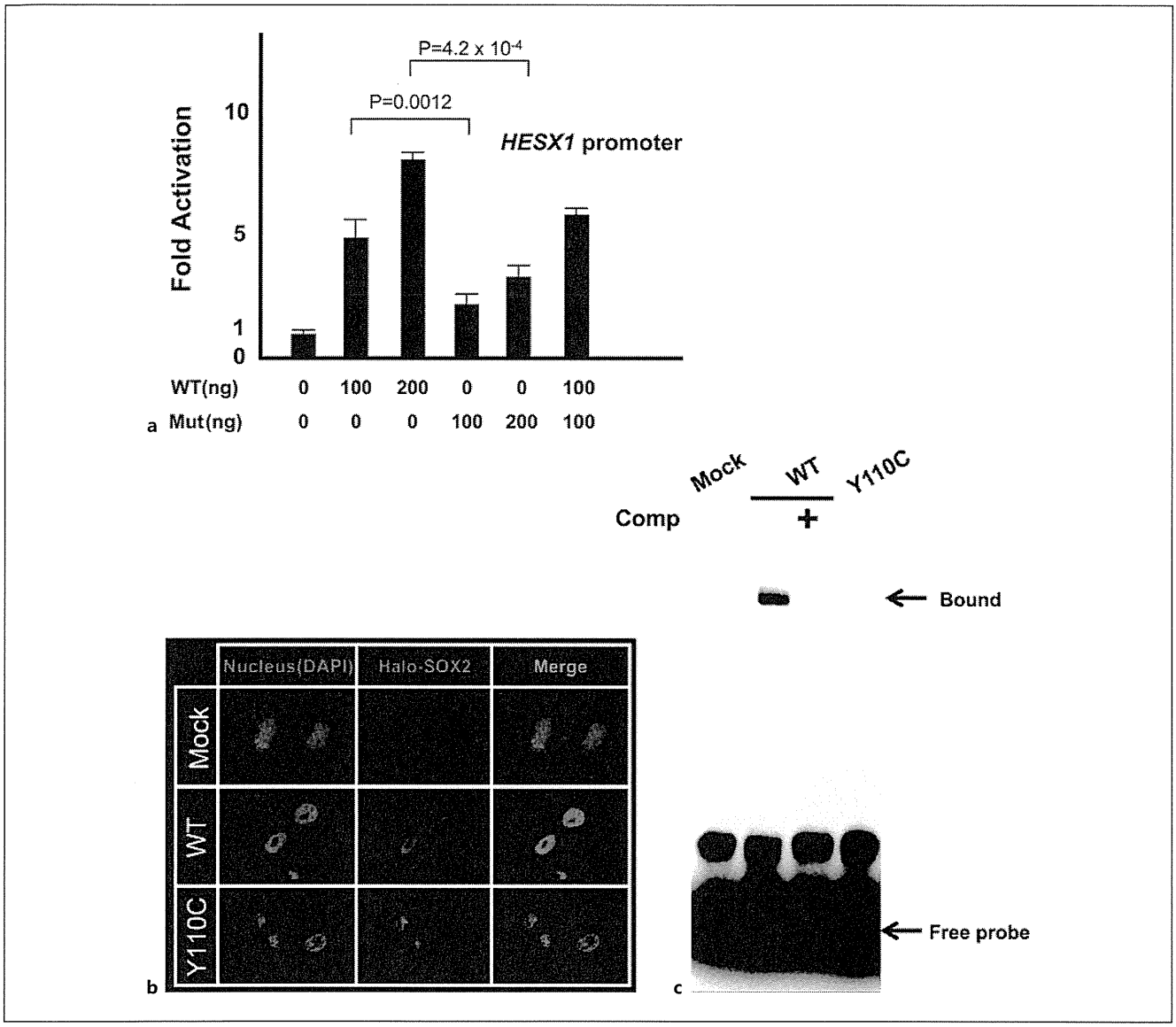

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Fig. 2. Functional characterization of Y110C SOX2. **a** Transactivation assays of Y110C SOX2 using *HESX1* reporter Hela cells were cotransfected with the pRL-CMV internal control vector, indicated amount (nanograms) of the effector plasmids, and the *HESX1* reporter. WT SOX2 stimulated transcription of the *HESX1* reporter in a dose-dependent manner. Y110C SOX2 exhibited reduced transactivation and had no dominant negative effect. The data are given as means ± SEM of at least 3 independent experiments performed in triplicate transfections. **b** Subcellular localization analysis. For subcellular localization analyses, we visualized and photographed Hela cells transfected with Halo-tagged SOX2 using a Leica TCS-SP5 laser scanning confocal microscope, after mounting the cells in a Vectashield-DAPI solution. The WT and Y110C SOX2 are localized to the nucleus. **c** EMSA experiments. WT SOX2 showed specific binding to the elements, which was competed by an excess amount of (200×) cold competitors. The Y110C SOX2 showed abrogated DNA-binding ability.

ing a D123G SOX2 mutation. These multigenerational patients suggested that there had been no fertility problems in the carriers of the D123G SOX2 mutation. D123G is located immediately C-terminal to the HMG domain, which was described as a partner-factor interaction region by Mihelec et al. [15]. SOX transcription factors exert tissue-specific effects in concert with tissue-specific partner factors. In the lens, SOX2 interacts with the lens-specific factor δEF3, and this interaction is dependent on the partner-factor interaction region [16]. Y110 is also