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Sox21 deletion in mice causes postnatal growth deficiency without physiological disruption of hypothalamic-pituitary endocrine axes

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ABSTRACT

The hypothalamic-pituitary axes are the coordinating centers for multiple endocrine gland functions and physiological processes. Defects in the hypothalamus or pituitary gland can cause reduced growth and severe short stature, affecting approximately 1 in 4000 children, and a large percentage of cases of pituitary hormone deficiencies do not have an identified genetic cause. SOX21 is a protein that regulates hair, neural, and trophoblast stem cell differentiation. Mice lacking *Sox21* have reduced growth, but the etiology of this growth defect has not been described. We studied the expression of *Sox21* in hypothalamic-pituitary development and examined multiple endocrine axes in these mice. We find no evidence of reduced intrauterine growth, food intake, or physical activity, but there is evidence for increased energy expenditure in mutants. In addition, despite changes in pituitary hormone expression, hypothalamic-pituitary axes appear to be functional. Therefore, *SOX21* variants may be a cause of non-endocrine short stature in humans.

1. Introduction

Children with heights more than 2 standard deviations (-2SD) below the mean are defined as being of clinically short stature, affecting those lower than the 3rd percentile for height. Pituitary growth hormone deficiency (GHD) leads to severe short stature and slow growth rates in children, occurring in approximately 1 in 4000 children (Bao et al., 1992; Lindsay et al., 1994; Tani, 1985; Vimpani et al., 1977), although up to 95% of cases of severe short stature cannot be attributed to endocrine issues (Lindsay et al., 1994). Congenital GHD and a lack of other pituitary hormones (hypopituitarism) is most commonly caused by mutations in the gene *PROP1* (Bottner et al., 2004; Pernasetti et al., 2000), with mutations in *POU1F1* (Turton et al., 2012) and other genes, including genes affecting hypothalamic development such as *OTX2*, accounting for only a small proportion of cases (Bancalari et al., 2012; Kelberman et al., 2009; Mortensen et al., 2015), leaving the majority of cases of hypopituitarism without a known cause.

The hypothalamus is an important regulator of the endocrine system, consolidating input from the cortex, autonomic system, end organs, and the environment. Hypothalamic neurons deliver neuropeptide hormone signals to the anterior pituitary gland, which in turn secretes polypeptide hormones that regulate the development and function of multiple target organs. Feedback loops between the hypothalamus, pituitary, and end organs form the different levels of the hypothalamic-pituitary axes. These axes regulate growth, lactation, reproduction, metabolism and stress response, in order to maintain homeostasis and respond to physiological demands.

SOX2 and SOX3 are important transcriptional regulators of the developing ventral diencephalon and hypothalamus (Jayakody et al., 2012; Rizzoti et al., 2004). The development and function of the pituitary gland also involves *Sry*-related HMG-box (SOX) proteins: SOX2 and SOX9 are both important transcription factors in the pituitary (Alatzoglou et al., 2009; Fauquier et al., 2008), and SOX2 is the primary marker for the postnatal pituitary stem cell population (Andoniadou et al., 2013; Rizzoti et al., 2013). SOX2, together with SOX1 and 3, make up the SoxB1 factors, while SOX21 and SOX14 are SoxB2 members (Cunningham et al., 2008; Sarkar and Hochedlinger, 2013). SoxB1 proteins usually act as transcriptional activators, while SoxB2 proteins are transcriptional repressors (Kamachi and Kondoh, 2013; Sandberg et al., 2005; Uchikawa et al., 1999). SOX21 regulates neuronal cell fate decisions (Sandberg et al., 2005; Matsuda et al., 2012; Whittington

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et al., 2015), counterbalancing the transcriptional activity of SOX2 (Sandberg et al., 2005). SOX21 also regulates trophoblast stem cell differentiation and placentation (Moretto Zita et al., 2015).

Sox21$^{-/-}$ mice are born normally but display cyclic alopecia because SOX21 plays a role in hair shaft cuticle differentiation in follicles of the skin (Kiso et al., 2009). At the same time, *Sox21*$^{-/-}$ mice were noted to be growth deficient without further physiological or endocrine investigations. We considered the possibility that SOX21 is involved in hypothalamic and/or pituitary development and function, and that *Sox21*$^{-/-}$ mutants may have reduced growth because of GHD or thyroid hormone deficiency. We have investigated the function of the hypothalamic-pituitary endocrine axes in these mice and present evidence for postnatal growth insufficiency of alternate origin.

2. Methods

2.1. Animals

The use of mice for this study was approved by the University of Michigan's Institutional Animal Care and Use Committee and the Unit for Laboratory Animal Medicine. Mice were housed under 12 h light/12 h dark cycles, and food and water were provided ad libitum. *Sox21*$^{-/-}$ mice were generated by replacing the coding region of the single exon with an enhanced green fluorescent protein (GFP) cassette and removing the floxed neomycin selection cassette with CAG-cre (Kiso et al., 2009). Mice were maintained on a C57BL/6 background. *Sox21*$^{+/-}$ males were bred to *Sox21*$^{+/-}$ females to generate *Sox21*$^{-/-}$ offspring. Males and females were both studied unless otherwise stated. In instances where *Sox21*$^{-/-}$ male and female mice show the same phenotype, only male mice are shown.

2.2. Histology

Tissues from embryonic day (e) 9.5 to postnatal (4-week-old) mice were dissected and fixed in 4% formaldehyde, processed for embedding, and sectioned at a thickness of 6–12 μm. Hematoxylin and eosin stainings were performed with a standard protocol as previously described (Mortensen et al., 2015; Jones et al., 2015) and examined under a light microscope.

2.3. Immunofluorescence

Immunofluorescence was performed on paraffin sections using a tyramide signal amplification system (PerkinElmer, Waltham, MA) as previously described (Mortensen et al., 2015; Gergics et al., 2015). The antibodies used in this study were: goat anti-GFP (Abcam Ab5450) at 1:1000; rabbit anti-rat GH (National Hormone and Peptide Program (NHPP), Torrance, CA) at 1:100; rabbit anti-human POMC (NHPP) at 1:150; goat anti-human SOX2 (Neuromics GT15098) at 1:100; goat anti-human SOX21 (R&D Systems AF3538) at 1:100; rabbit anti-mouse GHRH (gift from Frank Talamantes and Malcolm Low) at 1:1000; rabbit anti-mouse GNRH (ThermoFisher PA1-121) at 1:200.

2.4. In situ hybridization

In situ hybridization for *Trh* was performed on frozen brain sections using a 957 bp probe from Allen Brain Atlas probe (RP_050725_03_A12) with a non-radioactive protocol as previously described (Mortensen et al., 2015), and examined under a light microscope.

2.5. RNA and cDNA production

Pituitary glands and livers were dissected from 4-week-old *Sox2*$^{+/-}$ and *Sox21*$^{-/-}$ mice, and total mRNA was isolated using the RNAqueous 4PCR Isolation kit (Applied Biosystems, Life Technologies, Grand Island, NY) according to manufacturer's protocols. cDNA was produced using the Superscript II system (Invitrogen, Life Technologies) according to the manufacturer's protocols (Mortensen et al., 2015; Jones et al., 2015).

2.6. Quantitative PCR

Quantitative real time-PCR was performed on cDNA generated from pituitary and liver tissues from 4-week-old mice using intron-spanning Taqman Gene Expression Assays (Applied Biosciences) as previously described (Jones et al., 2015). The Taqman Assays used were: *Gh* mm00433590_g1; *Prl* mm00599949_m1; *Lhb* mm00656868_g1; *Tshb* mm00457190_m1; *Pomc* mm00435874_m1; *Igf1* mm00439559. Reactions were set up using Taqman Universal Master Mix (Applied Biosystems), and run on an Applied Biosystems 7500 Real Time-PCR system. Fold changes were calculated by normalizing to the mRNA expression of glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) using the Relative Expression Software Tool (REST) program (Pfaffl, 2001).

2.7. Thyroid hormone measurements

Serum total and free thyroxine (T4) were measured using enzyme immunoassay (EIA) test kits (07BC-1007 for total T4 and 07BC-1008 for free T4, MP Biomedicals, Solon, OH). 4-week-old male mice were sacrificed by carbon dioxide inhalation, and blood was collected by cardiac puncture. Sera were used directly for ELISA without dilution. Briefly, sera and standard solutions of known concentrations of T4 are pipetted into wells coated with anti-T4 antibody, and incubated with a solution containing horseradish peroxidase-labelled T4 for 60 min. The wells are washed with water, and a tetramethylbenzidine solution is added for 20 min to allow color development. The reaction is terminated with 1N HCl solution, and absorbance is measured at 450 nm on a SpectraMax 190 spectrophotometer (Molecular Devices).

2.8. Metabolic and calorimetric analyses

Oxygen consumption (VO_2), carbon dioxide production (VCO_2), spontaneous motor activity, and food intake were measured using the Comprehensive Laboratory Monitoring System (CLAMS, Columbus Instruments). Mice were individually placed into the sealed chambers with free access to food and water, and the study was carried out for 72 h under with 12 h light/12 h dark cycles. Food intake for each animal was monitored through a precision balance under the chamber. VO_2 and VCO_2 in each chamber were sampled sequentially for 5 s every 20 min and the motor activity was recorded every second. Total energy expenditure was calculated using the VO_2, VCO_2, and urinary nitrogen concentration (Simonson and DeFronzo, 1990).

2.9. Imaging and statistical analyses

Images were captured using a Leica Leitz DMB microscope and Leica DFC310 FX camera, or an Olympus FluoView 500 laser scanning confocal system. Images were analyzed and compiled using Adobe Photoshop CS6. All quantitative data show the mean ± standard error of the mean (SEM), and were analyzed by unpaired Student's *t*-test. In all cases, $^{*} = p < 0.05$; $^{**} = p < 0.01$; $^{***} = p < 0.001$.