

BRIEF REPORT

A WNT4 Mutation Associated with Müllerian-Duct Regression and Virilization in a 46,XX Woman

Anna Biason-Lauber, M.D., Daniel Konrad, M.D., Ph.D., Francesca Navratil, M.D., and Eugen J. Schoenle, M.D.

SUMMARY

WNT4, a secreted protein that suppresses male sexual differentiation, is thought to repress the biosynthesis of gonadal androgen in female mammals. An 18-year-old woman presented with primary amenorrhea and an absence of müllerian-derived structures, unilateral renal agenesis, and clinical signs of androgen excess — a phenotype resembling the Mayer–Rokitansky–Küster–Hauser syndrome and remarkably similar to that of female *Wnt4*-knockout mice. A genetic evaluation revealed a loss-of-function mutation in the *WNT4* gene. WNT4 appears to be important in the development and maintenance of the female phenotype in women, by means of the regulation of müllerian-duct formation and control of ovarian steroidogenesis.

From the Division of Pediatric Endocrinology and Diabetology, University Children's Hospital, Zurich, Switzerland. Address reprint requests to Dr. Biason-Lauber at University Children's Hospital, Steinwiesstr. 75, CH-8032 Zurich, Switzerland, or at anna.lauber@kispi.unizh.ch.

N Engl J Med 2004;351:792-8.

Copyright © 2004 Massachusetts Medical Society.

THE DIFFERENTIATION OF A TESTIS OR AN OVARY FROM THE BIPOTENTIAL gonad is a complex developmental process involving various genes and hormones.¹ Additional elements of the reproductive tract develop from an indeterminate stage through the differentiation of wolffian ducts (male reproductive tract anlage) and müllerian ducts (female reproductive tract anlage). Although factors involved in male sexual differentiation have been well studied, the pathways that regulate female sexual differentiation remain incompletely defined. To date, no genes have been shown to play a role in ovarian development equivalent to that played by the *SRY* or *SOX9* gene in testicular development. In mice, *Wnt4*, one of a few factors with a demonstrated function in the ovarian-determination pathway, has been found to be involved in sexual differentiation. WNT4, which suppresses male sexual differentiation,²⁻⁴ is a member of the WNT family of secreted molecules that function in a paracrine manner to effect a number of developmental changes. WNT proteins bind to members of the Frizzled family of cell-surface receptors and activate a cascade of intracellular signals leading to the transcriptional activation of target genes. Vainio et al.² studied a mouse model in which *Wnt4* is ablated⁵ and observed that, whereas both male and female *Wnt4*-knockout mice have similar defects in kidney development and adrenal function, gonadal development and steroidogenic function are affected exclusively in female *Wnt4*-knockout mice. *Wnt4*-knockout female mice are masculinized, as demonstrated by the absence of müllerian ducts and the presence of wolffian ducts, and express the steroidogenic enzymes 3β -hydroxysteroid dehydrogenase and 17α -hydroxylase, which are required for the production of testosterone and are normally suppressed in the female ovary.² *Wnt4* up-regulates *Dax1*, a gene known to antagonize the nuclear-receptor steroid factor 1, and thereby inhibits steroidogenic enzymes.³ In XX gonads, WNT4 suppresses

the migration of mesonephric endothelial and steroidogenic cells, preventing the formation of male-specific coelomic blood vessels and the production of steroids.⁴ Collectively, these data suggest that WNT4 normally functions to suppress the synthesis of gonadal androgen in females.

A previous search for clinically relevant WNT4 mutations in a large cohort of patients with abnormalities in sexual development was unsuccessful.⁶ We describe a woman without structures derived from müllerian ducts (uterine and fallopian tubes) who had unilateral renal agenesis and clinical signs of androgen excess. Her phenotype resembles that of patients with the Mayer-Rokitansky-Küster-Hauser syndrome^{7,8} and is also strikingly similar to that of *Wnt4*-knockout female mice. This constellation of findings prompted us to search for mutations in the *WNT4* gene in this patient.

CASE REPORT

A woman who was 18 years 7 months old was referred for evaluation of primary amenorrhea. Thelarche and adrenarche had occurred at 12 years of age, and both breast and pubic-hair development had progressed normally. Physical examination revealed a normal weight (47 kg; standard-deviation score, -0.9), height (158 cm; standard-deviation score, -1.1), body-mass index ([the weight in kilograms divided by the square of the height in meters] 18.8; standard-deviation score, -0.6), and blood pressure (110/65 mm Hg); acne (requiring antiandrogen therapy); pubic hair of the adult-female type (Tanner stage 5); and mature female breasts (Tanner stage 5). The clitoral size was normal, but the vaginal introitus was small and short (0.5 cm). Laboratory examination revealed elevated levels of androstenedione (25.4 nmol per liter; normal range, 2.8 to 8.0) and dehydroepiandrosterone sulfate (11.8 μ mol per liter; normal range, 2.2 to 9.2). Total and free testosterone levels were repeatedly slightly elevated (e.g., 4.6 nmol per liter [normal range, 0.3 to 3.4] and 19.9 pmol per liter [normal range, 2.4 to 12.4], respectively), whereas levels of luteinizing hormone (10 IU per liter), follicle-stimulating hormone (6.4 IU per liter), 17-hydroxyprogesterone (5.4 nmol per liter), progesterone (16.2 nmol per liter), and estradiol (179 pmol per liter) were normal. Chromosomal analysis of 20 cells showed a normal female 46,XX karyotype. *SRY* was absent in lymphocytes. Pelvic and abdominal magnetic resonance imaging revealed that the vagina and uterus

were absent, both ovaries were of normal size but ectopic (retroperitoneal, above the iliac crest), and the right kidney was aplastic, with compensatory hypertrophy of the left kidney.

METHODS

The study conformed to the guidelines of the institutional review board, and all determinations were conducted as part of diagnostic procedures approved by the institutional review board. Oral informed consent was obtained from all subjects.

MUTATIONAL ANALYSIS OF GENOMIC DNA

Genomic DNA was extracted from peripheral-blood leukocytes from the patient, her mother and sister, and 100 ethnically matched control subjects (200 alleles; 50 male and 50 female subjects), and all five exons of the *WNT4* gene were amplified by means of the polymerase chain reaction (PCR) with the use of a Qiagen DNA blood- and cell-culture kit. Sequences of the primers used to amplify the exons of *WNT4* (GenBank accession number, NT_004610) and the gene for hepatocyte nuclear factor 1 β (*TCF2*; GenBank accession numbers, U90279 and U90280-7) are available on request (Mycosynth). *SRY* amplification was conducted as described previously.⁹ Direct cycle sequencing of the PCR products was performed with the use of the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) and analyzed with the use of the ABI Prism 310 Genetic Analyzer (Applied Biosystems).

EXPRESSION STUDIES

To study the functional implications of the mutation we identified, we first performed reverse-transcriptase PCR on *WNT4* messenger RNA (mRNA) ectopically expressed in peripheral-blood leukocytes from control subjects.¹⁰ Primer sequences and PCR-amplification conditions used for wild-type *WNT4* complementary DNA (cDNA) are available on request. The wild-type cDNA was then subjected to PCR-mediated site-directed mutagenesis.¹¹ The sequence was verified as described above. Confluent cultures of a human ovarian adenocarcinoma cell line (OVCAR3, National Institutes of Health; American Type Culture Collection [ATCC], HTB-161), an adrenal adenocarcinoma cell line (H295R, National Cancer Institute; ATCC, CRL-2128), and renal 293T cells (ATCC, CRL-11268) were transfected with 10 μ g of wild-type *WNT4* DNA, 10 μ g

of mutant *WNT4* DNA, or a combination of the two (molar ratio, 1:1) in 60-mm plates with the use of the TransFast transfection system reagent (Promega) according to the manufacturer's instructions. The ovarian adenocarcinoma cells, which express steroid receptors¹² and steroidogenic enzymes,¹³ provide an in vitro model for evaluating the func-

tional effects of *WNT4*. The influence of wild-type and mutant *WNT4* on the expression of the steroidogenic enzymes 17 α -hydroxylase (GenBank accession number, NM_000102) and 3 β -hydroxysteroid dehydrogenase type 2 (GenBank accession number, 000198) and of the transcription factor hepatocyte nuclear factor 1 β (GenBank accession num-

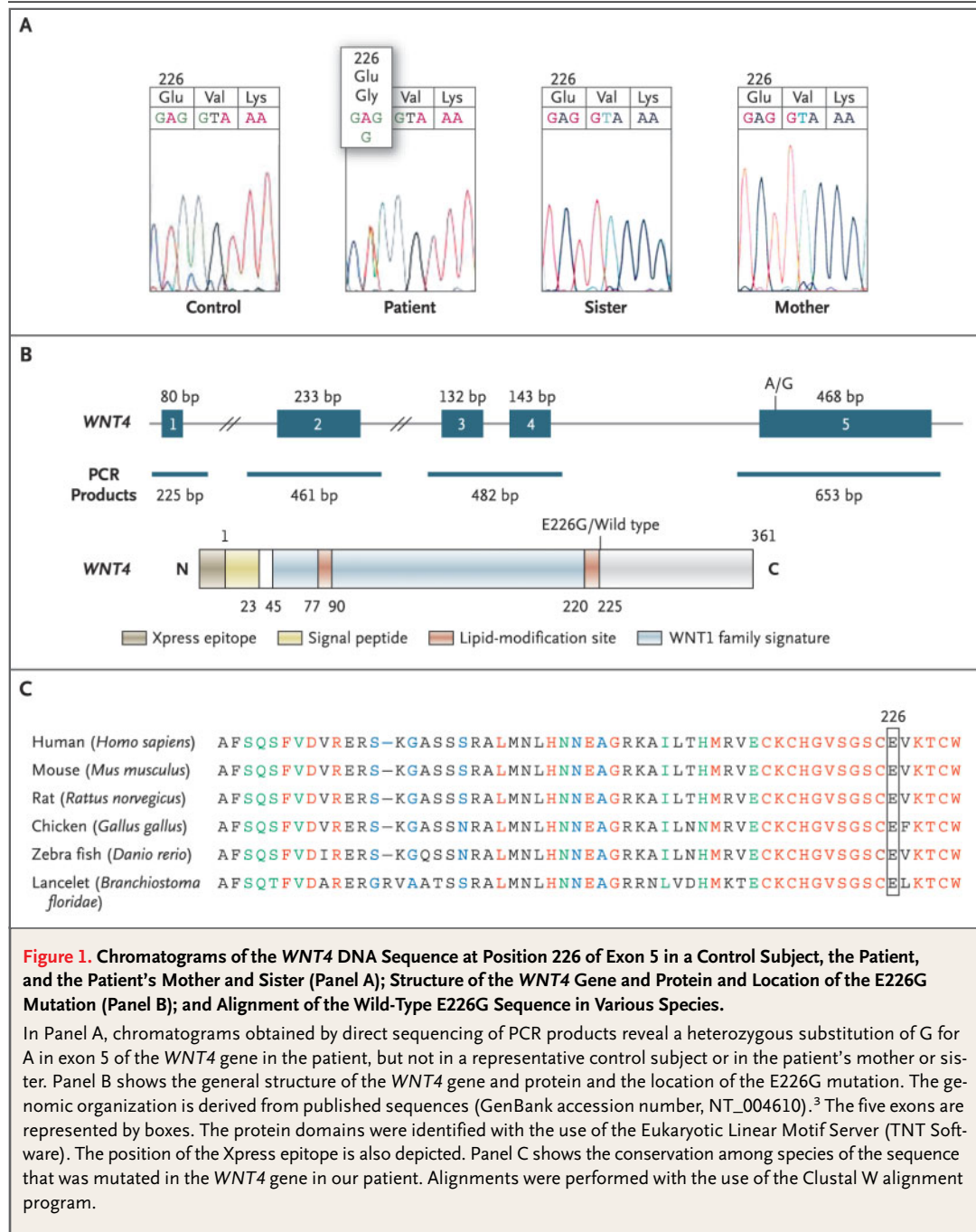
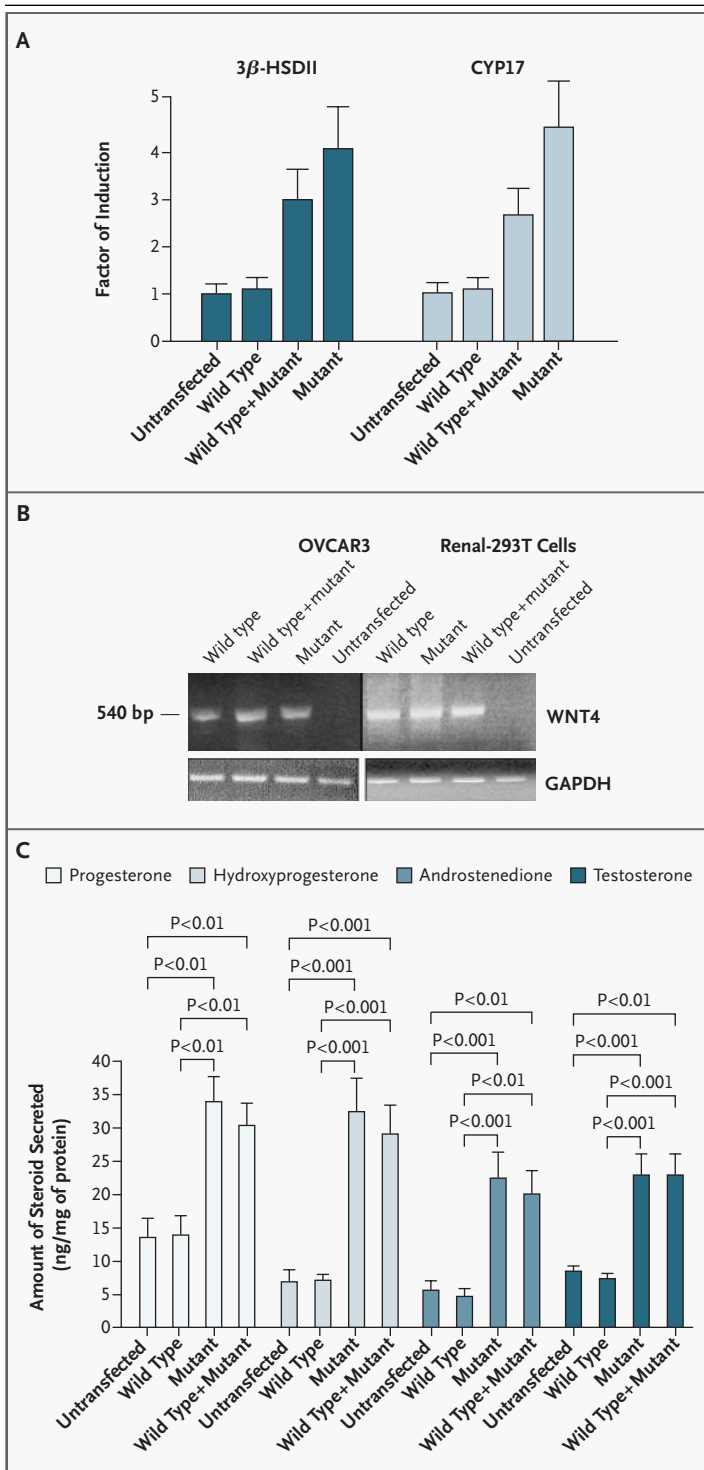


Figure 2. Dysregulation of the Signaling Pathway of the Mutant WNT4.

Panel A shows the quantification by real-time reverse-transcriptase–PCR of the expression of mRNA 3 β -hydroxysteroid dehydrogenase (3 β -HSDII) and 17 α -hydroxylase (CYP17) in an ovarian adenocarcinoma cell line (OVCAR3) in the presence and absence of transfection with mutant WNT4, wild-type WNT4 cDNA, or a 1:1 combination of the two. Results are expressed as the relative increase in transfected cells (by a factor of 1, 2, 3, 4, or 5), as compared with the untransfected cells. Panel B shows the results of reverse-transcriptase–PCR analysis of an ovarian adenocarcinoma cell line and renal 293T cells in the presence and absence of transfection with mutant WNT4, wild-type WNT4, or both with the use of primers specific for the subcloned 540-bp WNT4 reverse-transcriptase–PCR product alone. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a protein expressed in most tissues, was used as a control. As shown in Panel C, the mutant WNT4 failed to inhibit steroidogenesis, with a consequent increase in androgen production in ovarian cells. Levels of progesterone, 17-hydroxyprogesterone, androstenedione, and testosterone were measured in the medium of ovarian adenocarcinoma cells in the presence or absence of transfection with mutant WNT4, wild-type WNT4, or an equimolar ratio of the two. Values in Panels A and C are means \pm SD of three independent experiments. P values were obtained with the use of the paired t-test.



bers, U90279 and U90280-7) was studied by means of quantitative real-time PCR, performed with the use of an ABI 7000 Sequence Detection System (Applied Biosystems), and PCR products were quantified fluorometrically with the use of the SYBR Green Core Reagent kit. The reference mRNA cyclophilin was used to normalize the data. Western blot analysis was performed with the use of anti-Xpress (Invitrogen), an antibody that recognizes the epitope of the N-terminal Xpress peptide, according to the manufacturer's instructions. The activity of the steroidogenic enzymes was assessed by radioimmunoassays of progesterone (DPC Biermann) and 17-hydroxyprogesterone, androstenedione, and testosterone (CisBio International) in cell-culture medium after the addition of pregnenolone (100 ng per milliliter) as a precursor. Total protein content was assayed (BioRad protein assay).

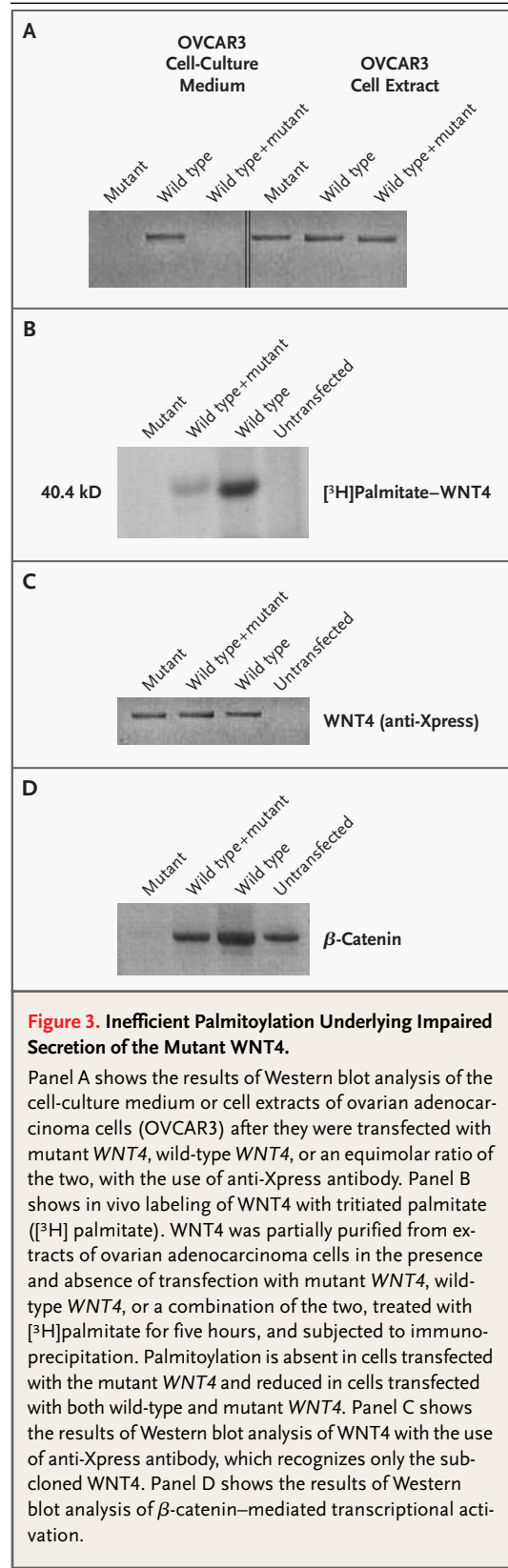
In vivo labeling of WNT4 with tritiated palmitate was performed as described previously,¹⁴ except that the assay was performed in cell extracts rather than in medium after immunoprecipitation of the protein¹³ with the use of anti-Xpress antibody. Western blotting was performed to deter-

mine the degree of β -catenin stabilization with the use of a commercially available antibody against β -catenin (U.S. Biological). All experiments were performed in triplicate.

RESULTS

Direct sequencing of PCR-amplified exonic fragments revealed a heterozygous substitution of guanine for adenine in exon 5 of the *WNT4* gene, leading to the Glu226Gly (E226G) missense mutation of the *WNT4* protein (Fig. 1A and 1B). Glutamate 226 is a *WNT4* residue conserved among human, mouse, rat, chicken, zebra fish, and lancelet species (Fig. 1C). The absence of the mutation in the DNA of the patient's mother and sister and 100 control subjects (200 alleles, data not shown) indicates that this substitution was either inherited from her father (whose DNA was not available for analysis) or a spontaneous event, and that it is not a common polymorphism. No mutation was found in the *TCF2* gene, which encodes the transcription factor hepatocyte nuclear factor 1 β that is crucial for the development of the kidneys and pancreas¹⁵ (data not shown). To determine the functional consequences of the rearrangement, we studied the expression of the steroidogenic enzymes 17 α -hydroxylase and 3 β -hydroxysteroid dehydrogenase type II and hepatocyte nuclear factor 1 β . The mutant *WNT4* was unable to suppress the expression of the steroidogenic enzymes in ovarian adenocarcinoma cells (Fig. 2A) and adrenal adenocarcinoma cells (data not shown). In renal cells, the mutant *WNT4* appeared to favor the expression of the short form of *TCF2*, which is devoid of the transactivation domain (data not shown). The effects were not due to differences in the expression of *WNT4* (Fig. 2B). The lack of inhibition of the expression of steroidogenic enzymes resulted in increased steroid production in ovarian adenocarcinoma cells transfected with the mutant *WNT4* (Fig. 3), as also appeared to be the case in the ovaries of our patient. The absence of the mutant *WNT4* in the medium of cultured ovarian cells (Fig. 3A) suggests that the mutant *WNT4* remains trapped within the cells.

Since proper processing of WNT proteins requires lipid-mediated modification,¹⁶ we evaluated the extent of the attachment of radiolabeled palmitate to wild-type and mutant *WNT4* in ovarian adenocarcinoma cells. The E226G mutant was inefficiently palmitoylated and, when transfected together with the wild type, partially prevented the palmitoylation of the transfected wild-type *WNT4* (Fig. 3B). The differences in lipid modification are not due to differences in the amount or stability of the protein (Fig. 3C). Evidence that the mutation did cause a defect in the *WNT4* signal-transduc-



tion pathway was provided by the failure to detect β -catenin (stabilized by the activation of the WNT signaling pathway) in ovarian adenocarcinoma cells transfected with the mutant *WNT4* (Fig. 3D).

DISCUSSION

The genetic factors controlling development in female mammals remain mysterious. With the exception of translocations in *SRY* and mutations in steroidogenic enzymes, the genetic mechanisms that cause a phenotypic XX male or XX virilization are poorly understood. We determined the biologic consequences of a mutation in *WNT4* and found that this gene appears to play a key role in human sexual differentiation.

The regression of the müllerian ducts in our patient suggests the occurrence in utero of ectopic ovarian expression of antimüllerian hormone, which is normally produced solely by testicular Sertoli cells at this stage of development. Furthermore, similar to *Wnt4*-knockout female mice, our patient had clinical and biochemical signs of ectopic activation of androgenesis. The androgen excess appears mild, since her external genitalia were not virilized, a feature that again resembles the phenotype of *Wnt4*-knockout female mice. We do not have direct proof that the mutation in *WNT4* led to morphologic masculinization of the ovaries or loss of oocytes in our patient, since we did not analyze ovarian tissue. Nevertheless, the inability of the mutant *WNT4* to inhibit the expression and activity of the steroidogenic enzymes in ovarian and adrenal cell lines provides good indirect evidence that the impairment of *WNT4* signaling may have caused the androgen excess. Unlike the female *Wnt4*-knockout mice,¹⁷ our patient did not have any defects in breast development, suggesting that this particular *WNT4* mutation does not affect mammary-gland growth in women.

The mutant Glu226 residue in *WNT4* in our patient (E226G) is conserved among species. However, other WNT proteins have different residues at that position, although never a glycine.

In searching for causes of the defective signaling, we found that the E226G mutant protein appears to be trapped inside the cell. The normal processing of WNT proteins is directly dependent on lipid modification. This apparently paradoxical phenomenon (cells have trouble releasing a molecule that is less hydrophobic than usual) is a very well known characteristic of WNT proteins and is probably due to misfolding of the protein during the formation of disulfide bonds between free cysteines.¹⁶ The E226G mutation prevents lipid modification and therefore represents a mechanism underlying the transduction defect. The mutated glutamate is not located in a consensus palmitoylation site (residues 77 through 90), and it is thus more likely that the mutation leads to misfolding, retention of the protein in the endoplasmic reticulum, and a subsequent lack of palmitoylation. The reasons for the surprisingly efficient secretion of wild-type *WNT4* are unclear, although we cannot exclude the possible involvement of the Xpress epitope. We obtained similar results in all functional assays when we evaluated the mutant and the wild-type *WNT4* together. This finding suggests that the mutant *WNT4* has dominant negative properties and provides a clear correlation between the genotype and the phenotype. However, the mechanism underlying such events remains unexplained, since WNT proteins do not seem to dimerize.¹⁴

The loss-of-function mutation we identified in the *WNT4* gene appears to cause developmental abnormalities in humans and indicates that *WNT4* is a major player in the development and maintenance of the female phenotype in women, by virtue of its ability to regulate the formation of müllerian ducts and control steroidogenesis in the ovary. Any generalizations regarding its role, however, must await the description and characterization of mutations in additional patients.

Supported by a grant (32-063629.00) from the Swiss National Science Foundation.

We are indebted to Dr. E. Liechti-Keusch for admitting the patient and for her collaboration.

REFERENCES

1. MacLaughlin DT, Donahoe PK. Sex determination and differentiation. *N Engl J Med* 2004;350:367-78.
2. Vainio S, Heikkilä M, Kispert A, Chin N, McMahon AP. Female development in mammals is regulated by *Wnt4* signalling. *Nature* 1999;397:405-9.
3. Jordan BK, Mohammed M, Ching ST, et al. Up-regulation of *WNT-4* signaling and dosage-sensitive sex reversal in humans. *Am J Hum Genet* 2001;68:1102-9.
4. Jeays-Ward K, Hoyle C, Brennan J, et al. Endothelial and steroidogenic cell migration are regulated by *WNT4* in the developing mammalian gonad. *Development* 2003;130:3663-70.
5. Stark K, Vainio S, Vassileva G, McMahon AP. Epithelial transformation of metanephric mesenchyme in the developing kidney regulated by *Wnt-4*. *Nature* 1994;372:679-83.
6. Domenice S, Correa RV, Costa EMF, et

- al. Mutations in the SRY, DAX1, SF1 and WNT4 genes in Brazilian sex-reversed patients. *Braz J Med Biol Res* 2004;37:145-50.
7. Bryan AL, Nigro JA, Counseller VS. One-hundred cases of congenital absence of the vagina. *Surg Gynecol Obstet* 1949;88:79-86.
8. Opitz JM. Vaginal atresia (von Mayer-Rokitansky-Kuster or MRK anomaly) in hereditary renal adysplasia (HRA). *Am J Med Genet* 1987;26:873-6.
9. Cui K, Warnes GM, Jeffrey R, Matthews CD. Sex determination of preimplantation embryos by human testis-determining-gene amplification. *Lancet* 1994;343:79-82.
10. Biason-Lauber A, Lang-Muritano M, Vaccaro T, Schoenle EJ. Loss of kinase activity in a patient with Wolcott-Rallison syndrome caused by a novel mutation in the *EIF2AK3* gene. *Diabetes* 2002;51:2301-5.
11. Rosa S, Biason-Lauber A, Mongan NP, Navratil F, Schoenle EJ. Complete androgen insensitivity syndrome caused by a novel mutation in the ligand-binding domain of the androgen receptor: functional characterization. *J Clin Endocrinol Metab* 2002;87:4378-82.
12. Hamilton TC, Young RC, McKoy WM, et al. Characterization of a human ovarian carcinoma cell line (NIH:OVCAR3) with androgen and estrogen receptors. *Cancer Res* 1983;43:5379-89.
13. Biason-Lauber A, Zachmann M, Schönle EJ. Effect of leptin on CYP17 enzymatic activities in human adrenal cells: new insight in the onset of adrenarche. *Endocrinology* 2000;141:1446-54.
14. Willert K, Brown JD, Danenberg E, et al. Wnt proteins are lipid-modified and can act as stem cell growth factors. *Nature* 2003;423:448-52.
15. Lindner TH, Njolstad PR, Horikawa Y, Bostad L, Bell GI, Sovik O. A novel syndrome of diabetes mellitus, renal dysfunction and genital malformation associated with a partial deletion of the pseudo-POU domain of hepatocyte nuclear factor-1 β . *Hum Mol Genet* 1999;8:2001-8.
16. Nusse R. Wnts and Hedgehogs: lipid-modified proteins and similarities in signaling mechanisms at the cell surface. *Development* 2003;130:5297-305.
17. Brisken C, Heineman A, Chavarria T, et al. Essential function of Wnt-4 in mammary gland development downstream of progesterone signaling. *Genes Dev* 2000;14:650-4.

Copyright © 2004 Massachusetts Medical Society.

VIEW CURRENT JOB POSTINGS AT THE NEJM CAREERCENTER

Visit our online CareerCenter for physicians at www.nejmjobs.org to see the expanded features and services available. Physicians can conduct a quick search of the public data base by specialty and view hundreds of current openings that are updated daily online at the CareerCenter.