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Production and Application of Polyclonal Antibody to Human Thyroid Transcription Factor 2 Reveals Thyroid Transcription Factor 2 Protein Expression in Adult Thyroid and Hair Follicles and Prepubertal Testis

Melwyn Sequeira,[1,2] Farakid Al-Khafaji,[1] SooMi Park,[3] Mark D. Lewis,[1] Malcolm H. Wheeler,[2] V. Krishna K. Chatterjee,[3] Bharat Jasani, and Marian Ludgate[1]

Germline mutations in thyroid transcription factor 2 (TTF2) cause thyroid agenesis, spiky hair, and cleft palate, indicating thyroidal and extrathyroidal expression. We sought to investigate this by producing and applying an antibody to human TTF2. The coding region of human TTF2 was cloned into a bacterial expression vector, production of the soluble TTF2 protein optimized, and pure TTF2 obtained by nickel chromatography. Rabbits were immunized and the resulting TTF2 polyclonal titrated on formalin-fixed, paraffin-embedded sections of thyroid. The optimized protocol was applied to a range of tissues. Nine milligrams of TTF2 protein was obtained per liter of culture and a high-titer antibody produced. This displayed specific staining of thyroid follicular cell nuclei/cytoplasm and not of the interstitium, connective tissue, smooth muscle, or endothelium. No staining was obtained with the preimmune serum in the same conditions, or with the majority of other tissues tested with the TTF2 polyclonal. The exceptions were testis and skin, in which nuclear TTF2 immunoreactivity was present in the seminiferous tubules and cells in the follicular outer root sheath, respectively. In conclusion, we have produced a polyclonal antibody for human TTF2 and demonstrated immunoreactivity for this transcription factor in adult human thyroid and hair follicles and prepubertal testis.

Introduction

Thyroid transcription factor 2 (TTF2) is a member of the forkhead/winged helix family of transcription factors, present in the developing thyroid. TTF2 is a helix-turn-helix DNA binding protein, which functions as a transcriptional activator in adult thyroid (1,2). Tissue-specific expression of thyroglobulin (Tg) and thyroid peroxidase (TPO) is regulated at the transcriptional level and both genes have binding sites for TTF2, which binds the DNA as a dimer and is regulated by the redox state (3,4). There is some controversy as to the precise role of TTF2 in the adult thyroid because, despite indirect evidence that it is a transcriptional activator (2,5), more recently it has also been found to function as a repressor (6). TTF2 is the main mediator of thyrotropin (TSH) and insulin regulation of TPO gene expression (7).

TTF2-null mice display features of either thyroid agenesis or maldescent (8). In humans, germline mutations of the TTF2 gene result in thyroid agenesis, cleft palate, choanal atresia, and spiky hair (9,10). This suggests that, in common with other thyroid transcription factors (11,12), TTF2 may be expressed in tissues other than the thyroid. Furthermore, northern analysis of a range of human tissues using a probe specific for the 3′ untranslated region (UTR) of TTF2 revealed a major transcript of 5.3 kb in the thyroid and a second transcript of 3.2 kb that was also present in the testis (9).

In a previous study (13), we investigated TTF2 gene expression in a range of benign and malignant thyroid lesions. In normal thyroid tissues TTF2 transcript levels are low; 18 of 36 were weakly positive and 18 of 36 negative. TTF2 transcripts were detected in 8 of 8 Graves' disease (GD); 3 of 7 Hashimoto's disease; 2 of 2 follicular hyperplasia; 15 of 21 follicular adenoma; 11 of 13 multinodular goiters, and 0 of 1 hyalinizing trabecular adenoma. In the malignant thyroid lesions, TTF2 transcripts were detected in 8 of 18 follicular cancers; 0 of 2 anaplastic carcinomas, and 11 of 17 papillary cancers. Confirmation that the transcripts are translated into the TTF2 protein in these thyroids and lesions requires evaluation of the tissue sections by immunohistochemistry (IHC), but antibodies to human TTF2 are lacking. Antibodies we

Departments of [1]Medicine (EMD Section), [2]Surgery and Pathology, University of Wales College of Medicine, Cardiff, U.K.
[3]Department of Medicine, Addenbrooke's Hospital, Cambridge, U.K.

raised to synthetic TTF2 peptides yielded disappointing results in IHC. In a recent study a polyclonal antibody to rat TTF2 was described (14) and applied to mouse embryo sections. It was not reported whether the antibody cross-reacts with the human protein.

The aim of this work was to produce a TTF2 fusion protein, use it to raise an antibody to human TTF2, suitable for immunohistochemical analysis of archival formalin-fixed, paraffin-embedded tissue sections, and apply it to investigate the extrathyroidal expression of TTF2 protein.

Materials and Methods

Subcloning human TTF2 into a procaryotic expression vector

The entire coding region of human TTF2 (accession number 51294, National Center for Biotechnology Information [NCBI]), was excised from pGEM III and subcloned into *Bam*H1 and *Hind*III restriction sites, in the poly linker of pTrcHis (InVitrogen, Carlsbad, CA), using standard ligation protocols (15). The vector uses the *trc* promoter (regulated by the lac operator) for high-level, inducible expression of recombinant proteins, which are tagged with six histidine residues for nickel column purification. The resulting pTrcHis-TTF2 construct was confirmed by complete sequencing of the TTF2 coding region and 200 base pairs of flanking vector sequence. Plasmid DNA was amplified using a Qiagen (Valencia, CA) maxiprep kit according to the manufacturer's instructions and used to transform the *Escherichia coli* Topp 10 strain.

Optimization of soluble TTF2 protein production

In pilot studies, 50 ml of LB with ampicillin was inoculated with 0.5 mL of an overnight culture of Topp 10 transformed with the pTrcHis-TTF2 construct and incubated in an orbital shaker at 230 rpm at 30°C or 37°C, until the optical density $(OD)_{600}$ reached 0.3. Expression of recombinant protein was induced by the addition of isopropylthiogalactopyranoside (IPTG) to a final concentration of 0.1 or 1 mM. One-milliliter aliquots of the culture were taken at hourly intervals for up to 4 hours after induction with IPTG, centrifuged to separate the pellet from the supernatant and the pellet resuspended in 100 μL of phosphate-buffered saline (PBS) and either treated immediately or kept on ice overnight. The samples were put through three cycles of freeze/thawing and the pellet (insoluble protein) and supernatant (soluble protein) analyzed separately. The samples were resuspended in 100 μL of sodium dodecyl sulfate-polyacrylamide gel electrophoreses (SDS-PAGE) loading buffer (16), boiled for 5 minutes, and 20 μL of each of the insoluble and soluble protein fractions were analyzed by 10% PAGE. A number of variables were evaluated for optimal protein production including incubation time after induction, immediate or delayed processing, IPTG concentration, and incubation temperature. The gels were stained in Coomassie blue solution, destained, and dried.

Large-scale production of TTF2 and nickel column purification

The cells from a 1 L optimized culture of Topp 10-TTF2 in LB medium containing 100 μg/mL ampicillin, were harvested by centrifugation, 5000 rpm for 5 minutes, 4°C, and resuspended in 10 mL of native binding buffer (20 mM phosphate, 500 mM NaCl, pH 7.8). Lysozyme was added to the lysate to a final concentration of 100 μg/mL and incubated on ice for 15 minutes. The suspension was kept on ice, and sonicated at medium intensity with three 10-second bursts and then submitted to three rounds of freeze/thawing. Insoluble debris was removed by centrifugation at 3000*g* for 15 minutes, at 4°C. The lysate was filtered through a 0.45-μm filter and then applied to the equilibrated nickel (ProBond, Invitrogen, Carlsbad, CA) column in two 5-mL aliquots. The polyhistidine tagged proteins were allowed to bind to the column by gently rocking the column with the cell lysate for 15 minutes at 4°C. The column was washed three times with 4 mL of native binding buffer (pH 7.8) followed by three washes with 4 mL of native wash buffer (pH 6.0). The protein was eluted by applying four 2.5-mL aliquots of increasing concentrations of imidazole (50–500 mM). The eluate was collected in 1-mL aliquots and quantified using 60% trichloroacetic acid precipitation with bovine serum albumin (BSA) as the control standard. The aliquots with the highest protein concentrations were pooled together.

Western blotting

Equal quantities (volume) of the cell lysate, the flow through and the purified protein were separated by 10% PAGE and analyzed both by Coomassie blue staining and Western blotting. The blot was incubated in a 1:1000 dilution of a rabbit antibody raised against a synthetic peptide of TTF2 (GVPGEATGRGAGGRRRKRPLQ–C). The blots were then incubated with a second antibody (anti-rabbit IgG-HRP conjugate, 1:5000 at room temperature for 1 hour) and detected using a chemiluminescent system (ECL Plus, Amersham Pharmacia Biotech, Uppsala, Sweden), films were exposed for 2 minutes. The membrane was stripped and reprobed with a 1:1000 dilution of the preimmune serum of the same rabbit used to generate the peptide antibody.

Immunization protocol

Prior to immunization the protein was dialyzed extensively against PBS to remove the imidazole. Two rabbits were immunized subcutaneously in several sites, with 300 μg purified and dialyzed TTF2 fusion protein, initially in complete Freund's adjuvant and subsequently boosted at approximately 3-week intervals with 300 μg TTF2 in incomplete Freund's adjuvant.

ELISA screening

Anti-TTF2 antibody activity in rabbit serum was determined by enzyme-linked immunosorbent assay (ELISA). Polypropylene 96-well microplates were coated with 100 μL of a 10 μg/mL dilution of the purified TTF2 fusion protein in coating buffer (15 mM carbonate, 35 mM bicarbonate, pH 9.6) overnight at 4°C. The plates were washed with ELISA wash buffer (10 mM phosphate, 150 mM NaCl, 0.1% Tween, 5% BSA, pH 7.4) and incubated with the immune sera from various bleeds at concentrations of 1:500, 1:1000, and 1:5000 for 2 hours. The plates were washed three times and incubated for 30 minutes in a 1:2000 dilution of anti-rabbit horseradish peroxidase conjugate solution containing a 1:50 con-

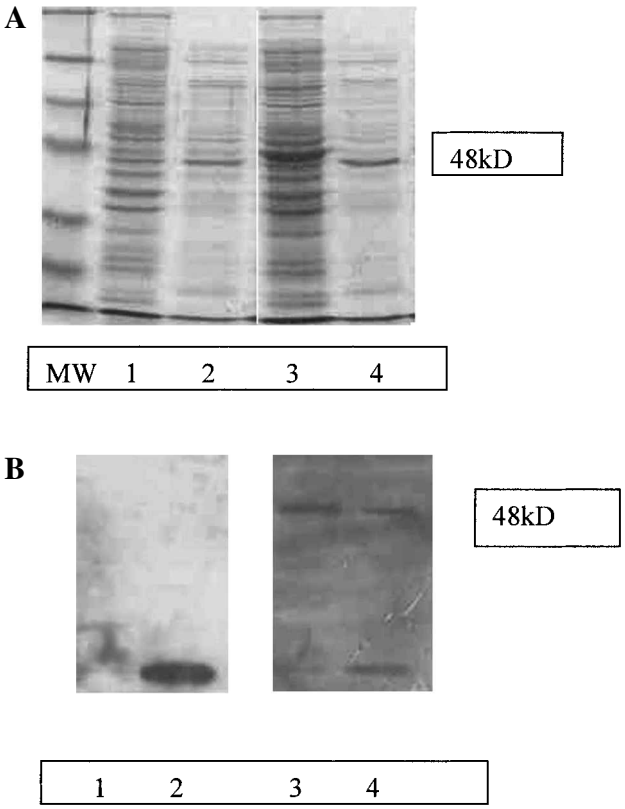

FIG. 1. **A:** Coomassie blue stain of bacterial lysate separated on 10% polyacrylamide gel. MW, molecular weight markers (Biolabs); Lanes 1 and 2, insoluble pellet and soluble fraction prior to induction with 0.1 mM IPTG (isopropylthiogalactopyranoside); Lanes 3 and 4, insoluble pellet and soluble fraction 3 hours postinduction with 0.1 mM IPTG. (Note the increased intensity of the band at 48 kd). **B:** Western blot of nickel column purified protein lysate using preimmune serum (left) and TTF2 peptide antibody (right) confirming the identity of the 48-kd band. Lanes 1 and 3, protein produced at 30°C; lanes 2 and 4, protein produced at 37°C.

centration of sheep serum. After washing, the antibody was detected by incubating in substrate solution (i.e., ABTS (Azinobisethylbenzthiazoline sulfonic acid, Sigma Chemical Co, St. Louis, MO) in citrate phosphate buffer for 30 minutes). The OD at A_{410} was recorded at 5-minute intervals for half an hour. Negative controls included preimmune serum at 1:250 concentration in addition to uncoated wells.

Immunohistochemistry

Paraffin-embedded human thyroid sections and sections of the testis, skin, appendix, lymph node, uterus, fallopian tube, liver, prostate, and gallbladder were retrieved from the archives of the Pathology Department at UWCM. The polyclonal TTF2 antibody was initially applied to sections of thyroid from a patient with GD using the streptavidin-biotin complex immunoperoxidase procedure, with and without antigen retrieval. In brief, the sections were deparaffinized, then incubated in methanol (98.4%) and hydrogen peroxide (1.6%) to block endogenous peroxidase activity and if antigen retrieval was to be performed, microwaved at full power for 25 minutes in 1 mM ethylenediaminetetraacetic acid (EDTA) solution. Endogenous biotin activity was blocked using a commercially available avidin/biotin blocking kit (Vector Labs, Burlingame, CA) by incubating the slides for 15 minutes each in 2–3 drops per slide of avidin followed by biotin. The slides were thoroughly washed in PBS between each step and prior to application of the primary antibody, overnight at 4°C. Multi-Link biotinylated anti-IgG (BioGenex, San Ramon, CA) and streptavidin peroxidase label (BioGenex) were utilized at a 1:40 dilution, each for 30 minutes at room temperature. Sections were washed with PBS between each step of the procedure. The chromogenic substrate utilized was diaminobenzidine tetrahydrochloride (DAB) made up in PBS with two drops of hydrogen peroxide. The sections were counterstained with Harris' hematoxylin.

Optimization of TTF2 antibody staining was performed using doubling dilutions of the antibody from 1:500 to 1:8000. Optimum dilution was set at the point where specific staining of thyroid follicular cell nuclei/cytoplasm was visible and there was little or no staining of the interstitium, the connective tissue, smooth muscle, or endothelium.

In later experiments, the sections were also incubated in 5% milk in PBS for 30 minutes prior to incubation in the primary antibody (the rest of the procedure being as detailed above) in order to further reduce any background staining.

Results

Generation of TTF2 fusion protein and polyclonal antibody

Optimal expression of the polyhistidine tagged TTF2 fusion protein in Topp 10 was achieved after 4 hours induction with IPTG. The majority of the protein was in the insoluble (pellet) form. Incubation at 30°C, produced a marginal improvement compared to 37°C but there was no difference between induction with 0.1 or 1 mM of IPTG. However, in subsequent experiments we found that the solubility of the TTF2 protein was improved by maintaining the crude cell lysate on ice overnight prior to the freeze/thaw cycles as shown in Figure 1A in which the TTF2 protein at 48 kD is clearly visible.

After nickel purification of the cell lysate, the 48-kD pro-

TABLE 1. OPTICAL DENSITY READINGS AT 450 nm OF PREIMMUNE AND IMMUNE SERA FROM RABBITS IMMUNIZED WITH THE PURIFIED TTF2 FUSION PROTEIN

	Rabbit A	*Rabbit B*
Preimmune 1:250	0.165	0.140
Immune-I 1:500	2.68	2.65
Immune-I 1:1000	2.61	2.49
Immune-I 1:5000	1.9	0.96
Immune-II 1:500	2.85	2.72
Immune-II 1:1000	2.64	2.39
Immune-II 1:5000	1.85	1.00

Immune-I, serum obtained after three immunizations; immune-II, serum obtained after four immunizations.

Optical density readings are the mean of at least duplicates which agree to within 10%.

TTF2, thyroid transcription factor 2.

tein was the major component. Its identity as TTF2 was confirmed in a Western blot using an antibody to a TTF2 peptide (Fig. 1B), which revealed bands at 48 kd and 25 kd. Immunoreactivity to the lower molecular weight protein was also present in the preimmune serum from the rabbit used to raise the TTF2 peptide antibody.

We were able to produce approximately 9 mg of purified TTF2 per liter of culture medium. The purified TTF2 fusion protein was used for the immunization of two rabbits and generated two high-titer antibodies, as summarized in Table 1.

Immunohistochemistry with the TTF2 polyclonal

The optimum protocol to produce specific staining of Graves' thyroid follicular cell nuclei/cytoplasm was preincubation of the sections with 5% milk in PBS for 30 minutes before incubation in 1:500 diluted TTF2 antibody, without antigen retrieval. This resulted in little or no staining of the interstitium, connective tissue, smooth muscle or endothelium as shown in Figure 2B. No staining was obtained with the preimmune serum in the same conditions in the Graves' thyroid (Fig. 2A). In the normal thyroid, approximately one third of the follicles displayed TTF2 staining, predominantly in the cytoplasm, although there were a few ($< 5\%$) positively staining nuclei (data not shown). In contrast, in the GD thyroid, the majority ($> 90\%$) of the thyrocytes displayed nuclear and cytoplasmic staining with only a minority being negative.

No staining was obtained with appendix, lymph node, uterus, fallopian tube, liver, prostate, or gallbladder tested with the TTF2 polyclonal (data not shown). The specificity of the TTF2 antibody was further confirmed using Chinese hamster ovary (CHO) cells stably transfected with full-length human TTF2 that displayed TTF2 immnoreactivity, in contrast to nontransfected cells that did not (data not shown).

TTF2 staining was detected in the epidermis of the skin but was most prominent in the hair follicles, as shown in Figure 2D. The immunoreactivity was detected in 50%–60% of nuclei in the epithelial cells of the outer root sheath. In con-

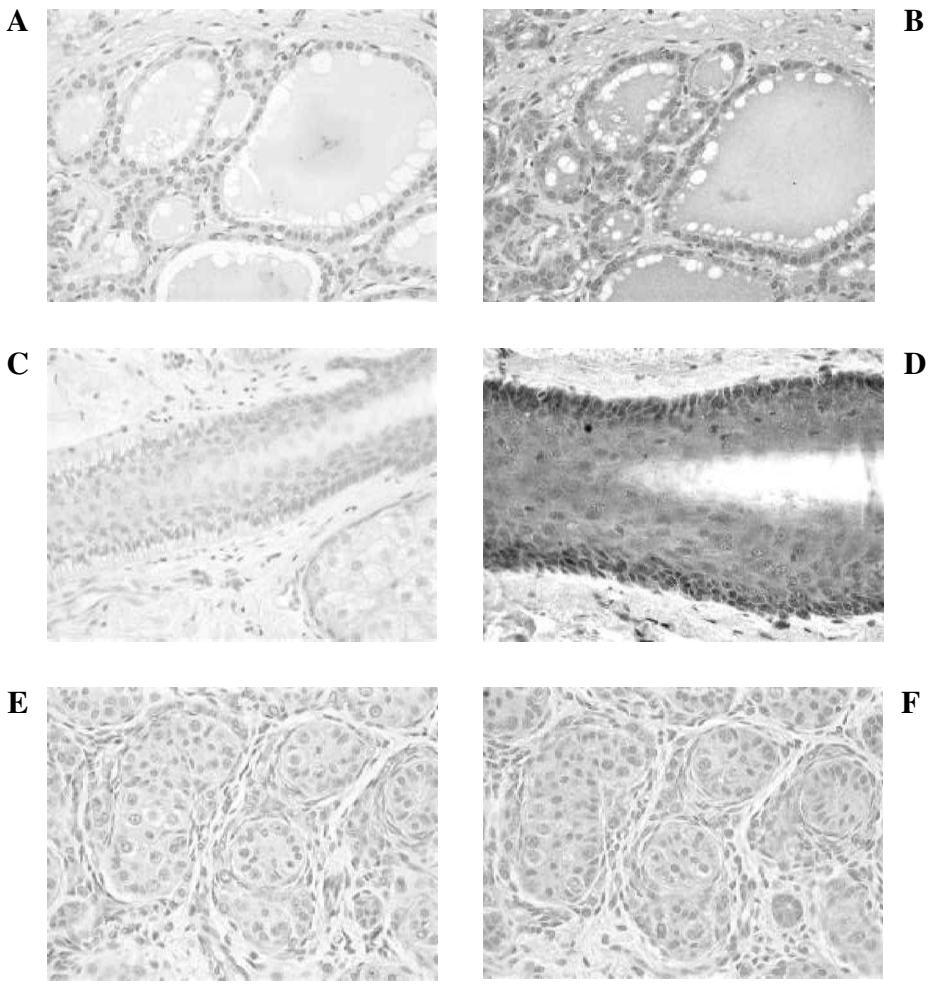

FIG. 2. Paraffin sections showing immunohistochemical staining. **A:** Graves' thyroid with preimmune serum. **B:** Graves' thyroid stained with the thyroid transcription factor 2 (TTF2) polyclonal, note the predominantly nuclear with some cytoplasmic staining in the majority of thyroid follicular cells. **C:** Section of skin with pre-immune serum. **D:** Section of skin stained with TTF2 polyclonal showing cytoplasmic and nuclear staining of cells in the outer root sheath of the hair follicle. **E:** Prepubertal testis with preimmune serum. **F:** Section of prepubertal testis showing TTF2 immunoreactivity predominantly in the cytoplasm and with some nuclear staining in the seminiferous tubules. Magnification, 40×.

trast, TTF2 staining was absent from the more differentiated cells in the suprabasal layer.

In sections of prepubertal testis, TTF2 immunoreactivity was present in the exocrine cells of the seminiferous tubules but absent from the germinal cell layer, as shown in Figure 2F. No staining was obtained with the preimmune serum in skin or testis, as shown in Figure 2C and 2E, respectively.

Discussion

TTF2 is a forkhead transcription factor encoded by a single exon. It differs from other members of the family, many of which are encoded by two exons, with the forkhead domain located in the first (17), but may have a more complex genomic organization (e.g., FKHL16, which spans 10 exons on human chromosome 12; 18). The absence of introns, makes transcript-based detection methods for TTF2 prone to false-positives because, for example, reverse transcription-polymerase chain reactions (RT-PCR) reactions could amplify contaminating genomic DNA and produce an amplicon of the expected size. *In situ* hybridization provides one solution but to confirm the presence of TTF2 protein requires IHC, which in turn depends on a suitable antibody.

Previous attempts to generate an antibody to a synthetic peptide of TTF2 yielded disappointing results. We opted to express the entire TTF2 coding region in bacteria, but experienced considerable difficulty in obtaining the protein in soluble form. This was slightly surprising, because TTF2 lacks membrane-spanning regions, which are known to hamper production in bacteria. However, TTF2 has a polyalanine tract, which is highly hydrophobic and probably contributed to the difficulties we encountered. This region is interesting, because it can be associated with transcriptional repression (19). A recent study has found variations in the number of alanine repeats in thyroid dysgenesis patients, although subsequent *in vitro* expression revealed that the transcriptional activity of the polymorphic TTF2 variants was equivalent to the wild-type (20).

We have produced an antibody to TTF2 and applied it to sections of various human tissues. There are relatively few immunohistochemical studies examining the expression of forkhead transcription factors in adult human tissues. Previous reports have investigated the expression of these proteins in developing mouse (21) and chick embryos (22) and provide evidence that some members of the family (e.g. *genesis* [23]), are restricted to embryonic stem cells or their malignant equivalent.

The expression and distribution of TTF2 during mouse embryonic development have recently been reported (14). The authors raised a polyclonal antibody to rat TTF2 and demonstrated TTF2 protein in structures where it has been inferred but not previously described, including the tongue, palate, choanae, and whiskers.

The results we have obtained in human thyroid for TTF2 protein expression, mirror our previously reported transcript findings, with normal thyroids expressing less TTF2 than GD thyroids (13). Increased expression of thyroid specific proteins is a common feature of GD thyroids and probably reflects the hyperactivity of the gland as reported by ourselves for the thyrotropin receptor (24) and for the sodium/iodide symporter and thyroid peroxidase by others (25).

We have demonstrated for the first time, the presence of TTF2 protein in the prepubertal testis (inferred from our previous Northern blot analysis) and defined its location as being the exocrine seminiferous tubules. The possible function of TTF2 in the testis is not clear but it may effect spermatogenesis. The reproductive potential of human subjects with TTF2 mutations is as yet unknown. Another forkhead protein, HFH-4, has been linked to the regulation and maintenance of the ciliated cell phenotype in epithelial cells and hence to cell motility (26). TTF2-null mice have either thyroid agenesis or maldescent, suggesting that it may control migration of the gland. The recent cloning of a TTF2 homologue, AmphiFoxE4, identified in amphioxus supports this hypothesis (27).

The spiky-hair phenotype of patients harboring germline mutations in TTF2 indicate that the protein has a role and is expressed in the hair follicle, at least during embryogenesis and this has been confirmed in the mouse (14). Our studies demonstrate for the first time that human adult hair follicles also express TTF2 protein and that it is located in the same region of the follicle as in the embryo, the outer root sheath, whose precise role in hair development is not known. Future studies will investigate whether TTF2 is present at all stages of the hair follicle life cycle, anagen, telogen, and catagen.

In conclusion, we have developed an antibody to TTF2, which has enabled us to demonstrate the presence of this transcription factor in adult human thyroid and in several extrathyroidal tissues, confirming our results obtained at the transcript level (9,13). Future studies will apply the antibody to investigate TTF2 protein expression in a range of benign and malignant thyroid lesions to determine whether it is affected by and/or contributes to malignant transformation.

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Address reprint requests to:
Marian Ludgate
EMD Section
Department of Medicine
University of Wales College of Medicine
Heath Park
Cardiff CF14 4XN
United Kingdom

E-mail: ludgate@cf.ac.uk